Category: Regular Articles
Section: Blood Coagulation

Fibrinopeptide A release is necessary for effective B:b interactions in polymerization of variant fibrinogens with impaired A:a interactions

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Short running title: FpA release is necessary for B:b interactions

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Word count: manuscript, 3711(excluding table, figure legends and references) ; summary, 230

Summary

Fibrin polymerization is mediated by interactions between knobs ‘A’ and ‘B’ exposed by thrombin cleavage, and holes ‘a’ and ‘b’. We demonstrated markedly delayed thrombin-catalyzed fibrin polymerization, through B:b interactions alone, of recombinant γD364H-fibrinogen with impaired hole ‘a’. To determine whether recombinant variant fibrinogens with no release of fibrinopeptide A (FpA) polymerize similarly to γD364H-fibrinogen, we examined two variant fibrinogens with substitutions altering knob ‘A’, Aα17A- and Aα17C-fibrinogen. We examined thrombin- or batroxobin-catalyzed fibrinopeptide release by HPLC, fibrin clot formation by turbidity and fibrin clot structure by scanning electron microscopy (SEM) and compared the results of the variants with those for γD364H-fibrinogen. Thrombin-catalyzed FpA release of Aα17A-fibrinogen was substantially delayed and none observed for Aα17C-fibrinogen; fibrinopeptide B (FpB) release was delayed for all variants. All variant fibrinogens showed substantially impaired thrombin-catalyzed polymerization; for Aα17A-fibrinogen it was delayed less, and for Aα17C more than for γD364H-fibrinogen. No variants polymerized with batroxobin, which exposed only knob ‘A’. The inhibition of variant fibrinogens’ polymerization was dose-dependent on the concentration of either GPRP or GHRP, and both peptides that block holes ‘b’. SEM showed that the variant clots from Aα17A- and γD364H-fibrinogen had uniform, ordered fibers, thicker than normal, whereas Aα17C-fibrinogen formed less

organized clots with shorter, thinner, and tapered ends. These results demonstrate that FpA release *per se* is necessary for effective B:b interactions during polymerization of variant fibrinogens with impaired A:a interactions.

Keywords: fibrin polymerization, B:b interaction, fibrinopeptide A release, knob 'A', hole 'a'

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 regions (central E region and two distal D regions) connected by linear segments called coiled-coil connectors. The E region contains the N-termini of all chains. The distal D regions contain the two nodules consisting of C-termini of the Bβ and γ chains: denoted as β- and γ-module, respectively. The C-termini of the Aα chains (αC regions) extend briefly through the D regions and fold back into the coiled-coil connectors; thereafter, the structure of this region 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, and converting fibrinogen to fibrin monomers (1). Fibrin monomers polymerize spontaneously

through a two-step process. In the first step, the release of FpA exposes a new N-terminal segment, knob 'A' that binds in hole 'a' in the γ-module of another fibrin molecule. These A:a interactions mediate the formation of half-staggered, double-stranded protofibrils (1). In the second step, these protofibrils grow in length and thrombin cleaves FpB, which exposes a new N-terminal segment, knob 'B', and dissociates the αC regions from the central E region. The protofibrils aggregate laterally to make thicker fibers and coalesce to form a complex, branching fibrin clot network.

Lateral aggregation is achieved by multiple interactions, including an interaction between knob 'B' and hole 'b' in the β-module of another fibrin molecule (2), intermolecular interactions between αC regions of different fibrin molecules (3), and interactions between two β-modules and two γ-modules of different protofibrils (4). It is not clear which interaction promotes this aggregation but the release of FpB enhances the extent of lateral aggregation (5). Thus, the exposure of knob 'B' and subsequent B:b interactions may contribute to this phase of polymerization. When FpA is cleaved alone by snake venom, batroxobin, normal looking fibers are still produced, although the fibers are thinner (6), suggesting that B:b interactions are not essential for lateral aggregation. Although the existence of B:b interactions and their importance for lateral aggregation is controversial, recently Doolittle et al. have demonstrated a novel function of B:b interactions—tethered knob 'B' become inserted in hole 'b', locking the β-modules in a conformation that allows

access to tissue type plasminogen activator (tPA) -plasminogen-binding sites, resulting in resistance to plasmin destruction of fibrin clots (7,8).

In our previous work, we demonstrated that recombinant variant fibrinogens modified at residue γD364 (located at the base of hole 'a') show markedly delayed thrombin-catalyzed fibrin polymerization through B:b interactions alone and form an organized clot with thicker fibers (9). We investigated whether recombinant variant fibrinogens with impaired A:a interactions, caused by the lack of FpA release, polymerize similarly to γD364H-fibrinogen. Here, we describe the analysis of thrombin-catalyzed fibrin polymerization of Aα17A- and Aα17C-fibrinogen. Our data indicate that thrombin-catalyzed polymerization of these variant fibrinogens is also contingent upon B:b knob-hole interactions. These interactions are enhanced with FpA release; that is, FpA release *per se* and not the exposure of knob 'A' is necessary for effective B:b interactions during polymerization.

Methods

Preparation of recombinant variant fibrinogens.

The fibrinogen Aα chain expression vector pMLP-Aα was altered by oligonucleotide-directed mutagenesis using the QuikChange II Site-Directed Mutagenic Kit (Stratagene, La Jolla, CA, USA) and the following primer pairs (the altered base is underlined); 5′-GGAGGCGTGCGTTGCCCAAGGGTTGTGG and

5′-CCACAACCCTTGGGCAACGCACGCCTCC for Aα17C, and

5′-GGAGGCGTGCGTGCCCCAAGGGTTGTGG and

5′-CCACAACCCTTGGGGCACGCACGCCTCC for Aα17A, according to the instruction manuals. The resultant expression vectors Aα17C and Aα17A were transfected into Chinese hamster ovary (CHO) cells that expressed normal human fibrinogen Bβ and γ chains (Bβγ-CHO cells), and the stable transfectants were selected as previously described (10). CHO cell lines producing γD364H- and wild-type Aα17G-fibrinogen were used (10). 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) (10). 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) (10). The fibrinogen concentration was determined from $\Delta A_{280\text{-}320}$, assuming that a 1 mg/ml solution has Δ absorbance of 1.51. The purity and characterization of the proteins was determined by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) under reducing conditions (10% polyacrylamide gel).

Kinetics of FpA and FpB release by thrombin or batroxobin.

Fibrinopeptide release was examined as previously described (10), with minor modifications. Briefly, fibrinogen (0.2 mg/ml) in HBS was added to human α-thrombin (0.02 U/ml) (Enzyme Research Laboratories, South Bend, IN; 3265 U/mg) or batroxobin (0.02 U/ml) (snake venom from *Bothrops atrox*, Pentapharm Ltd., Basel, Switzerland; 100 BU/mg) and incubated at ambient temperature for various incubation periods, with subsequent boiling for 3 min. After centrifugation, the supernatants were analyzed in duplicate by high-performance liquid chromatography (HPLC) using a Cosmocil 5C18P column (Nacalai Tesque, Inc, Kyoto, Japan; 4.5 × 150 mm). To calculate the percentage of fibrinopeptide release, the amount of FpA or FpB released from wild-type fibrinogen after 2 h of incubation with 2.4 U/ml (120-fold concentration) of thrombin or batroxobin at 37 °C was taken as 100%.

Thrombin or batroxobin-catalyzed fibrin polymerization.

Polymerization at ambient temperature was monitored at 350 nm using a UV-140-02 spectrophotometer (Shimadzu Corp., Tokyo, Japan). Briefly, fibrinogen (0.36 mg/ml) in HBS supplemented with 1 mM $CaCl_2$ was mixed with human α-thrombin (0.04 U/ml) or batroxobin (0.04 U/ml). The reactions were performed in triplicate and lag time, which reflects the rate of protofibril formation and maximum slope, which reflects the lateral aggregation rate were obtained from the turbidity curves, as described elsewhere (10).

Clottability of the fibrinogens was determined in triplicate, after mixing thrombin (0.04 U/ml) and fibrinogen (0.36 mg/ml) in HBS containing 1 mM $CaCl_2$ and incubating for 24 h at 37°C and fibrin clot or fibrin aggregates were removed by centrifugation at 13000 × *g* for 15 min. Fibrin that was not incorporated into the pellet was determined from the A_{280} of the supernatant, and clottability was calculated as (A_{280} at zero time −A_{280} of the supernatant) ÷ (A_{280} at zero time) × 100%. No correction was made for absorbance from the added thrombin.

Inhibition of thrombin-catalyzed fibrin 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 (purity >95%; MP Biomedicals Inc., Solon OH), was determined by adding each peptide to the fibrinogen solution prior to mixing with thrombin. Each reaction was performed in triplicate in the absence or presence of GPRP (0.03-0.5 mM), GHRP (0.03-0.5 mM) or GGG (0.5 mM).

Scanning electron microscopy.

Samples for scanning electron microscopy (wild-type, γD364H-, and Aα17A-fibrinogen) were prepared as described elsewhere (11). Briefly, fibrin clots were formed from fibrinogen (0.36 mg/ml) and α-thrombin (0.04 U/ml), incubated at 37 °C for 24 h. The fixed, stained, and mounted clots were osmium plasma-coated at 5 nm thickness in Neo-AN

(Meiwafosis Co. Ltd., Tokyo, Japan), and finally viewed on a JSM-6510LV (Japan Electron Optics Laboratory Co. Ltd). Images were taken at 3,000× or 20,000× magnification with 10.0 kV accelerating voltage. The fiber diameters were measured using a vernier caliper on printed photographs taken at a magnification of 20,000×. On the other hand, clots made from Aα17C-fibrinogen were packed by centrifugation (15,000 rpm for 10 min) followed by sample preparation as described above. Because the Aα17C-fibrinogen did not produce a solid clot to proceed but rather appeared like a suspension of more transparent and fragile precipitate.

Statistical analysis.

The statistical significance of differences between wild-type and variant fibrinogen was determined using unpaired *t*-tests. A difference was considered significant when $p < 0.05$.

Results

Synthesis and characterization of recombinant fibrinogens.

We synthesized three variant fibrinogens with single amino acid substitutions, γD364H-, Aα17A-, and Aα17C-fibrinogen, as described in the Methods section. SDS-PAGE run under reducing conditions (Figure 1) showed the usual pattern of three bands corresponding to the Aα, Bβ and γ chains. Aα chain of Aα17A-fibrinogen showed slightly faster electric

mobility than other fibrinogens. Each recombinant fibrinogen, including the wild-type, was pure, not degraded, and suitable for functional analyses.

Fibrinopeptide release.

We monitored thrombin- and batroxobin-catalyzed fibrinopeptide release by HPLC as described in the Methods section; the data are shown in Figure 2. The rate of thrombin-catalyzed FpA release from the γD364H-fibrinogen did not differ from that from the wild-type fibrinogen. Low concentrations of thrombin (0.02 U/ml) did not release any FpA from Aα17C-fibrinogen within 120 min, but there was a slight FpA release from Aα17A-fibrinogen. Namely, 3.7% and15.9% of FpA was released at 10 min (versus 36.1 % for wild-type) and 120 min (versus 90.9 % for wild-type), respectively (Figure 2A). FpB release levels from Aα17A-, Aα17C-, and γD364H-fibrinogens were all diminished in comparison with the wild-type (Figure 2B). The delayed releases of FpB were consistent with previous work that suggests FpB release occurs more rapidly from protofibrils (12). Namely, these three fibrinogens indicated markedly impaired thrombin-catalyzed fibrin polymerization, as shown in Figure 3C. Batroxobin-catalyzed FpA release was slower than thrombin-catalyzed, using equivalent units as defined by the vendors. The rate of batroxobin-catalyzed FpA release from the γD364H variant was the same as that from the wild-type fibrinogen. Interestingly, at a low concentration of batroxobin (0.02 U/ml) there

was a slight release of FpA from Aα17C at 120 min (9.2% versus 79.3 % for the wild-type), whereas FpA release from Aα17A was equivalent to the release in the presence of thrombin (Figure 2C). Importantly, no FpB release was observed from any of the fibrinogens even at a 120-fold higher concentration of batroxobin (data not shown).

Polymerization.

Polymerization of wild-type and variant fibrinogens was measured by turbidity, using 0.04 or 4.0 U/ml thrombin or batroxobin. Representative curves are shown in Figure 3 and averaged parameters are shown in Table 1. Polymerization of Aα17A-, Aα17C-, and γD364H-fibrinogens using 0.04 U/ml thrombin was markedly lower than the wild-type (Figure 3A, 3C and Table 1). When 4.0 U/ml thrombin was used, all three variant fibrinogens polymerized faster than using 0.04 U/ml, namely, the lag periods were shorter, but still markedly lower than that of the wild-type (Figure 3B, 3D and Table 1). Polymerization of wild-type fibrinogen using batroxobin was observed similarly as thrombin-catalyzation (Figure 3E, 3F and Table 1). In contrast, we saw no change in turbidity up to 24 h for any of the variants using a high concentration of batroxobin (Table 1 and figure not shown).

To examine these marked differences in thrombin-catalyzed polymerization further, we measured clottability after 24 h. Typically, clottability was 95.8 ± 2.0% for wild-type fibrinogen. For Aα17A-, Aα17C-, and γD364H-fibrinogens, the corresponding values were

65.0 ± 0.3% (p <0.001), 60.3 ± 6.6% (p <0.001), and 65.3 ± 2.0% (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, for Aα17A and γD364H variants the images showed organized clots with uniform fibers but appeared different from wild-type clots, with thicker fibers and larger pores. Measurements of the fiber diameters showed that the variant fibers were thicker: 196 ± 47 nm for Aα17A (n = 50, p <0.0001), and 339 ± 109 nm for γD364H (n = 50, p <0.0001), compared with 146 ± 31 nm for wild-type (n = 50) fibrinogen. On the other hand, Aα17C-fibrinogen made only fragile clots; the clots packed by centrifugation underwent sample preparation as described above. Surprisingly, Aα17C clots were less organized, with aberrant fibers which were shorter and thinner than normal clots, and had tapered ends (fiber diameter; 80 ± 28 nm, n = 50, p <0.0001). There is a possibility that the Aα17C-fibrin clot structure was modified by centrifugation, in particular, the pore size was reduced, and thus we did not compare its structure to others; however, we think that the characteristics of fibrin fibers were retained.

Inhibition of thrombin-catalyzed fibrin polymerization by GPRP or GHRP.

To examine the participation of B:b and/or B:a interactions in the polymerization of variant fibrinogens, we assessed polymerization in the presence of peptides that mimic knobs 'A' and 'B', GPRP and GHRP, respectively. We followed the changes in turbidity with time (curves

not shown) and determined the percentage of absorbance (absorbance with peptide/absorbance without peptide) at 30 min for wild-type fibrinogen, and at 10 h for Aα17A and γD364H (Figure 5). Since the turbidity of the Aα17C polymerization mixture increased after 5 h and absorbance at10 h was very low (Figure 3C), the inhibition of polymerization was assessed by examining fibrin clottability after 24 h incubation. In the presence of GPRP, polymerization was inhibited dose-dependently and to the same extent for all four fibrinogens (wild-type and variant); 0.5 mM peptide was sufficient to inhibit polymerization completely (Figure 5). In contrast, the addition of 0.5 mM GHRP to wild-type fibrinogen led to only 10% decrease in the final absorbance (Figure 5A), whereas the same GHRP concentration completely (Aα17C- and γD364H-fibrinogen) or almost completely (90%, Aα17A-fibrinogen) inhibited polymerization of the variant fibrinogens (Figure 5B, 5C, and 5D). This dose-dependent inhibition by GPRP or GHRP indicates that the variant fibrinogens did not all polymerize when the peptide was bound in hole 'b'. Using GGG as a control peptide, we saw no inhibition of polymerization under the same conditions.

Discussion

Our previous study with γD364A-, γD364V-, and γD364H-fibrinogens showed delayed thrombin-catalyzed fibrin polymerization, but with well-organized clot formation (9).

Furthermore, our data demonstrated that B:b interactions are necessary for fibrin fiber formation but A:b interactions are not, when A:a interactions are compromised because these variant fibrinogens showed no polymerization by catalyzation with batroxobin, which releases FpA but not FpB (9). In our experiments presented herein, we synthesized Aα17A- and Aα17C-fibrinogens with impaired A:a interactions. Comparison of the polymerization of these fibrinogens with the thrombin-catalyzed polymerization of γD364H-fibrinogens provides valuable insight into the functional basis of the altered function. Our new data indicate that FpA release plays a crucial role in effective B:b interactions during the polymerization of variant fibrinogens with impaired A:a interactions.

Our studies showed that thrombin polymerizes Aα17C- and γD364H-fibrinogen through B:b interactions alone. No FpA was released from Aα17C-fibrinogen by thrombin, and hole 'a' in γD364H-fibrinogen was almost completely dysfunctional (13); however, there is a possibility that B:a interaction functions in aberrant clot formation from Aα17C-fibrinogen. Two analyses have been reported that reject this potential. First, GPRP, which mimics knob 'A', was shown to bind to fibrinogen and inhibit thrombin-catalyzed fibrin polymerization. In contrast, GHRP, which mimics knob 'B', binds to fibrinogen but does not inhibit thrombin-catalyzed fibrin polymerization, namely, knob 'B' does not bind to hole 'a' (14). Second, Litvinov et al. used laser tweezers-based force spectroscopy and measured rupture forces of the interactions of the desAB fibrin and desAB N-terminal disulfide knot with

fibrinogen and fragment D, and demonstrated that not only B:a interactions but also A:b and B:b interactions were absent in fibrin polymerization (15). Furthermore, batroxobin-catalyzed fibrinopeptide release using 0.02 U/ml showed a slight release of FpA from Aα17C-fibrinogen at 120 min, but batroxobin-catalyzed fibrin polymerization was still not observed after 24h. These results indicate that the variant knob 'A', CPRV, which is exposed on Aα17C-desA fibrin monomer, did not bind to hole 'a', whereas Aα17A-fibrinogen polymerized through not only B:b interactions but also through weakened A:a interactions. We observed that a small amount of FpA was released from Aα17A-fibrinogen at low concentrations of thrombin (0.04 U/ml), and the variant knob 'A', APRV, which is exposed on Aα17A-desA fibrin monomer, bound to hole 'a' to some extent and participated in the interaction between variant knob 'A' and normal hole 'a'. Concerning the weakened interactions between variant knob 'A' and hole 'a', Wada et al. have already observed that synthetic peptides APRV and VPRV partially inhibit the polymerization of fibrin monomer made from thrombin and heterozygous dysfibrinogen with AαG17V (Fibrinogen Bremen) (16). These results support our observation that the weak interactions between variant knob 'A' and hole 'a' mediate thrombin-catalyzed polymerization of Aα17A-fibrinogen.

Furthermore, a 100-fold increase in the concentration of thrombin (4.0 U/ml) accelerated the release of FpA from Aα17C- and γD364H-fibrinogen and the release of FpB from all three variant fibrinogens; the lag period was shorter, the slope of polymerization curve steeper, and

the final absorbance of turbidity lower than those in the lower thrombin concentration (0.04 U/ml). These phenomena have also been observed on wild-type fibrinogen; increasing thrombin forms clots with thinner and more fiber bundles, and show a lower final absorbance of turbidity curve (17). Inhibition of thrombin-catalyzed polymerization by a peptide that mimics knob 'B', GHRP, also indicated that B:b interactions were involved in the polymerization of not only γ364H-fibrinogen but also Aα17A- and Aα17C-fibrinogens. Further, the knob 'A' mimic, GPRP, also inhibited polymerization. Previous experiments have shown that in the presence of mM calcium, GPRP binds not only to hole 'a' but also hole 'b' (18). In addition, X-ray analysis of fragment D isolated from γ364A showed that GPRP bound both holes 'a' and 'b' (13). Thus, the observed inhibition was likely a result of GPRP binding to hole 'b'.

Although no naturally occurring AαG17 residue variants have been reported except for AαG17V (16), homophenotypic AαR16 variant fibrinogens have been reported: Metz (19), Frankfurt XIII (20), and Kingsport (21). Fibrinogen Metz is an AαR16C homozygote (19), Frankfurt XIII is a compound heterozygote of AαR16C with an unknown deficiency mutation (20), and Kingsport is a compound heterozygote of AαR16H and *FGA* 11kb deletion (21). Interestingly, it has been reported that no variant FpA is released from Aα16C-fibrinogen (19, 20) and it is only partially released from Aα16H-fibrinogen (21). Moreover, Aα16C-fibrinogen does not polymerize within 4 hours (19, 20) and Aα16H-fibrinogen

polymerizes after a 90-minute lag period (21). These characteristics demonstrate that recombinant Aα17C-fibrinogen is similar to Aα16C-fibrinogen and recombinant Aα17A-fibrinogen resembles Aα16H-fibrinogen. Surprisingly, treatment with thrombin at 14 °C results in a clot formed from Aα16C (Metz)-fibrinogen; this clot is more transparent, less viscous, and more easily disrupted than the wild-type clot; scanning electron microscopy shows thin, twisting, and highly branched fibrils (22). These clot and fiber characteristics are also similar to those of the clots made with recombinant Aα17C-fibrinogen. Fibrin fibers formed from Aα16H-fibrinogen are thinner than wild-type fibers (21) and markedly different from those made with Aα17A-fibrinogen. We found that the fibrin fibers made from γD364H-fibrinogen through B:b interactions alone were much thicker than those produced with Aα17A-fibrinogen. These results led us to believe that A:a interactions cause the formation of solid and thin fibers.

We expected thrombin-catalyzed Aα17C-fibrinogen polymerization to be similar to the polymerization of γD364H-fibrinogen through B:b interactions alone. Since Aα17C-fibrinogen does not release FpA and no knob 'A' is exposed, the effects of A:a interactions are similar to the interactions of γD364H-fibrinogen. Surprisingly, Aα17C-desB fibrin monomers were less clottable and the final clot structure was less ordered than that in the case of γD364H-fibrinogen, although both variant fibrinogens can polymerize through B:b interactions alone. Our interpretation of the polymerization of

γD364H-fibrinogen is as follows: after FpA release, a strong negative charge is diminished and reduces repulsive electrostatic effects among the fibrin monomer molecules, the tertiary structure of N-termini of fibrinogen molecules changes, FpB is released slowly, interacts with knob 'B' and hole 'b', and forms double-stranded protofibrils. However, the presence of FpA in Aα17C-fibrinogen does not diminish the strong negative charge and also does not change the tertiary structure of N-termini of fibrinogen molecules, and thus disturbs the B:b interactions and the subsequent formation of protofibrils. Moreover, there is a possibility that the sulfhydryl groups in substituted Cys residue bind to Cys, homocysteine , and/or glutathione etc., and resulting in disturbing the B:b interactions and the subsequent formation of protofibrils. Our conclusion is that FpA release *per se* is necessary for effective B:b interactions in the polymerization of variant fibrinogens with impaired A:a interactions.

Contribution of authors

N. Okumura: designed the study. K. Soya, N. Okumura, F. Terasawa: performed the experiments. K. Soya, N. Okumura: designed the figures. K. Soya, N. Okumura: wrote the manuscript.

Disclosure of Conflicts of Interest

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

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

fibrinogen	enzyme	0.04U/ml		4.0 U/ml	
		lag (min)	slope ($x10^{-5}$/s)	lag (min)	slope ($x10^{-5}$/s)
wild-type	thrombin	2.6 ± 0.1	325 ± 3	< 0.2	> 1090
Aα17A	thrombin	105 ± 21	2.1 ± 0.2	23 ± 3	4.2 ± 0.4
Aα17C	thrombin	320 ± 28	0.3 ± 0.1	137 ± 6	0.4 ± 0.1
γD364H	thrombin	198 ± 11	4.6 ± 0.7	84 ± 7	5.6 ± 0.6
wild-type	batroxobin	6.2 ± 03	135 ± 9	< 0.2	> 1530
Aα17A	batroxobin	np	np	np	np
Aα17C	batroxobin	np	np	np	np
γD364H	batroxobin	np	np	np	np

np: not polymerized

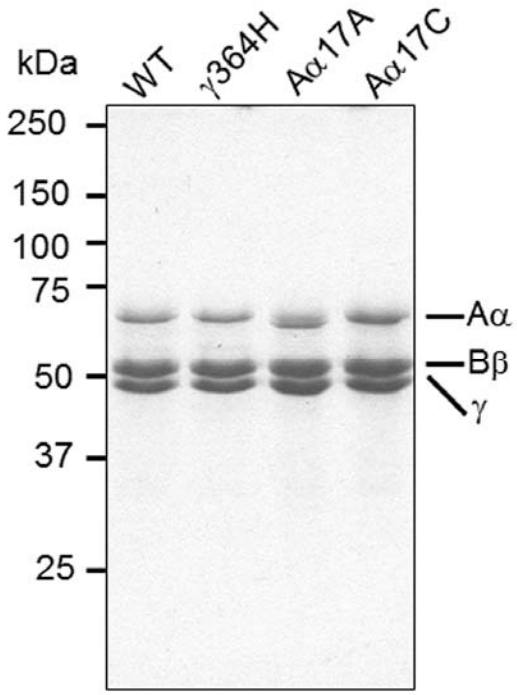

Figure 1. SDS-PAGE analysis of purified fibrinogens. One microgram of purified fibrinogen was resolved on 10% acrylamide gel under reducing conditions and stained using Coomassie Brilliant Blue R-250.

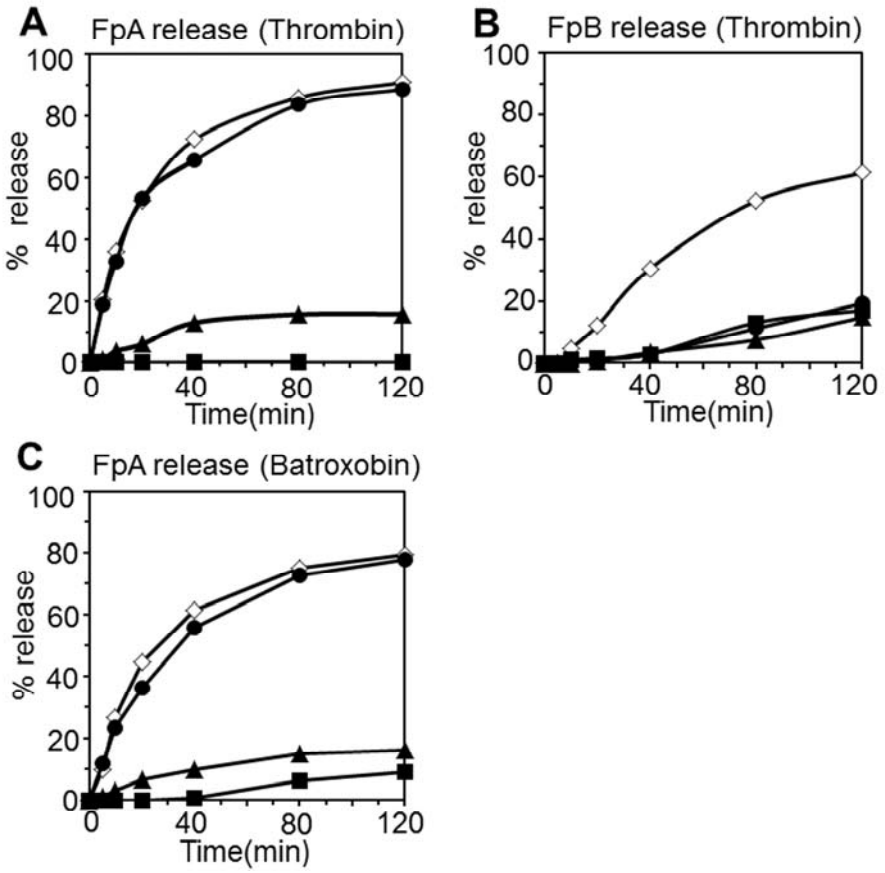

Figure 2. Thrombin- and batroxobin-catalyzed fibrinopeptide release. Mean fibrinopeptide release curves from duplicate HPLC assay are shown in Panel A: FpA and Panel B: FpB with thrombin (0.02 U/ml) catalysis, Panel C: FpA with batroxobin (0.02 U/ml) catalysis. Reactions were run with wild-type fibrinogens (◇), γD364H- (●), Aα17A- (▲), and Aα17C- (■) fibrinogens (0.20 mg/ml) at ambient temperature in HBS with 1 mM $CaCl_2$. Percentage of released FpA and FpB was calculated as described in Materials and Methods. No FpB was released from any of the fibrinogens after the addition of batroxobin (0.02 or 2.4 U/ml) (data not shown).

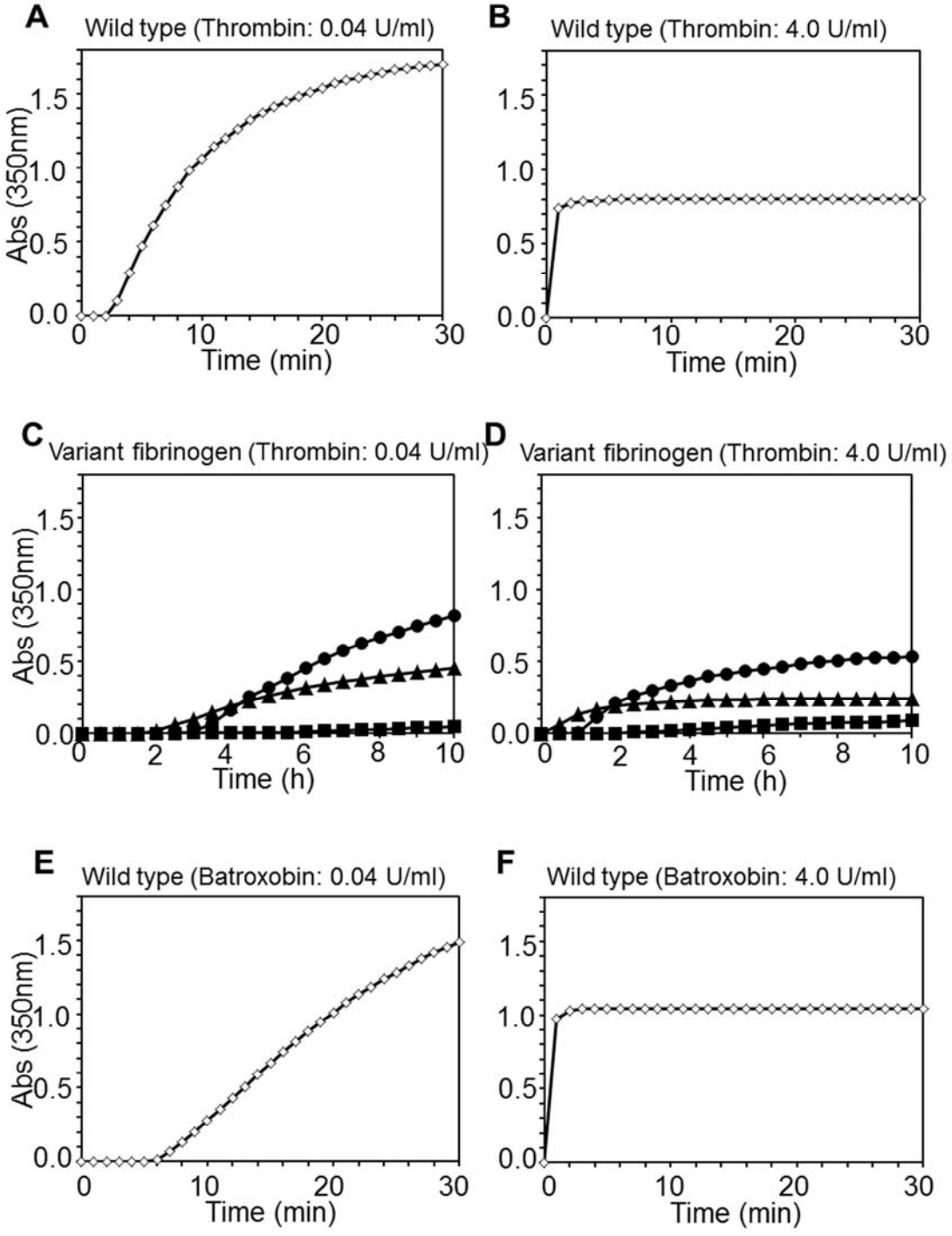
A
Wild type (Thrombin: 0.04 U/ml)
B
Wild type (Thrombin: 4.0 U/ml)
C
Variant fibrinogen (Thrombin: 0.04 U/ml)
D
Variant fibrinogen (Thrombin: 4.0 U/ml)
E
Wild type (Batroxobin: 0.04 U/ml)
F
Wild type (Batroxobin: 4.0 U/ml)
Abs (350nm)
Time (min)
Time (h)
0.0
0.5
1.0
1.5
0
2
4
6
8
10
20
30

Figure 3. Thrombin- or batroxobin-catalyzed polymerization. Polymerization of fibrinogens (0.36 mg/ml) was initiated with thrombin or batroxobin. Panel A: Wild-type fibrinogen with thrombin (0.04 U/ml), Panel B: Wild-type fibrinogen with thrombin (4.0 U/ml), Panel C: Variant fibrinogens with thrombin (0.04 U/ml), Panel D: Variant fibrinogens with thrombin (4.0 U/ml), Panel E: Wild-type fibrinogen with batroxobin (0.04 U/ml), Panel F: Wild-type fibrinogen with batroxobin (4.0 U/ml) in the presence of 1 mM $CaCl_2$. Representative turbidity curves from triplicate experiment are shown for wild-type fibrinogen (◇, Panels A, B, E, and F), γD364H- (●), Aα17A- (▲), and Aα17C- (■) fibrinogens (Panels C and D). Note that the time scale is in minutes for panels A, B, E, and F and in hours for panels C and D. No polymerization was observed for any of the variant fibrinogens after the addition of batroxobin (0.04 or 4.0 U/ml) (data not shown).

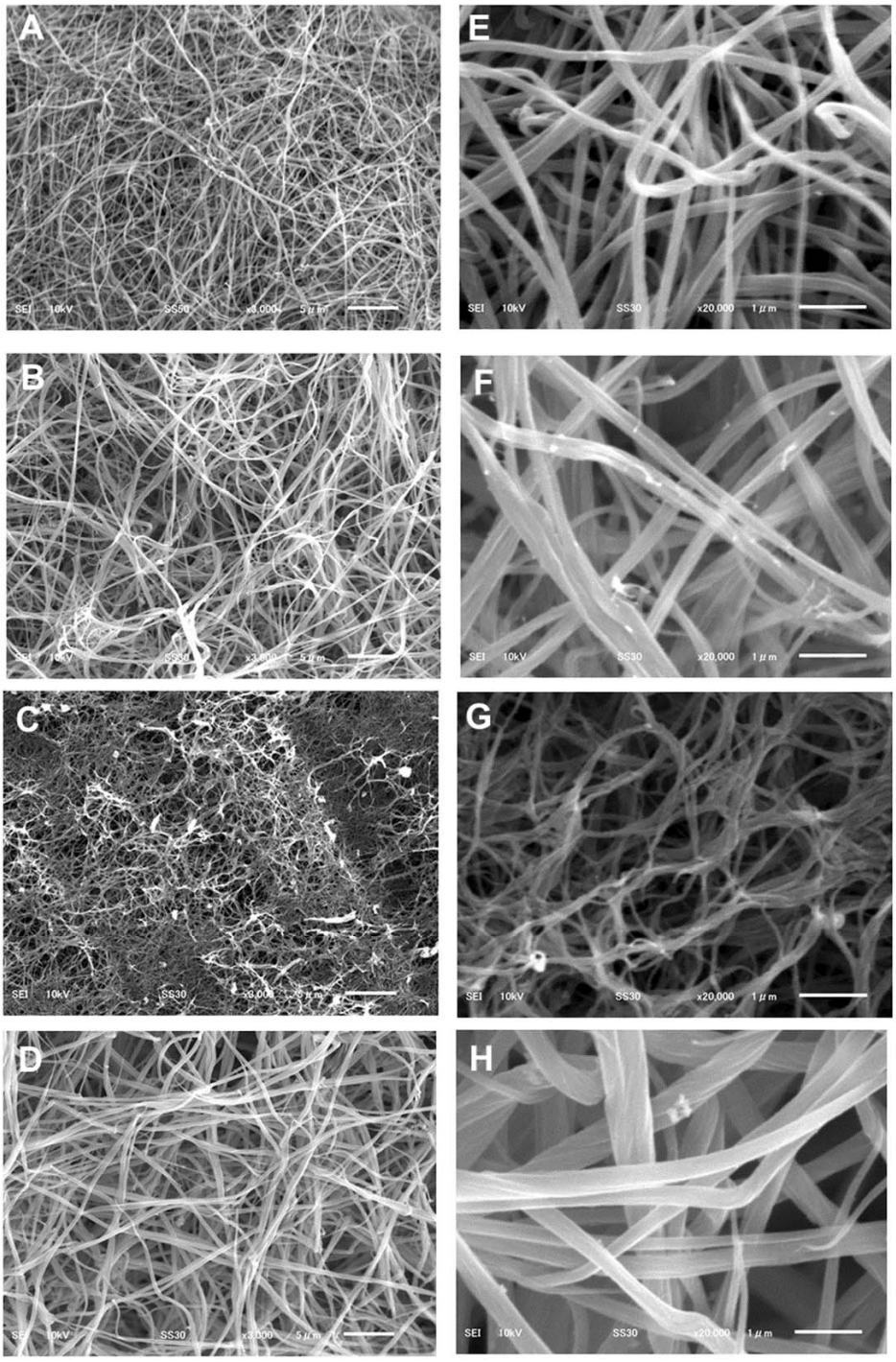
A
E
B
F
C
G
D
H

Figure 4. Scanning electron microscopy of fibrin clots. Fibrinogens (0.36 mg/ml) were incubated with thrombin (0.04 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 were made for wild-type (A, E), Aα17A- (B, F), Aα17C- (C, G), and γD364H- (D, H) fibrinogens. White bar represents 10 μm in A–D and 1.0 μm in E–H.

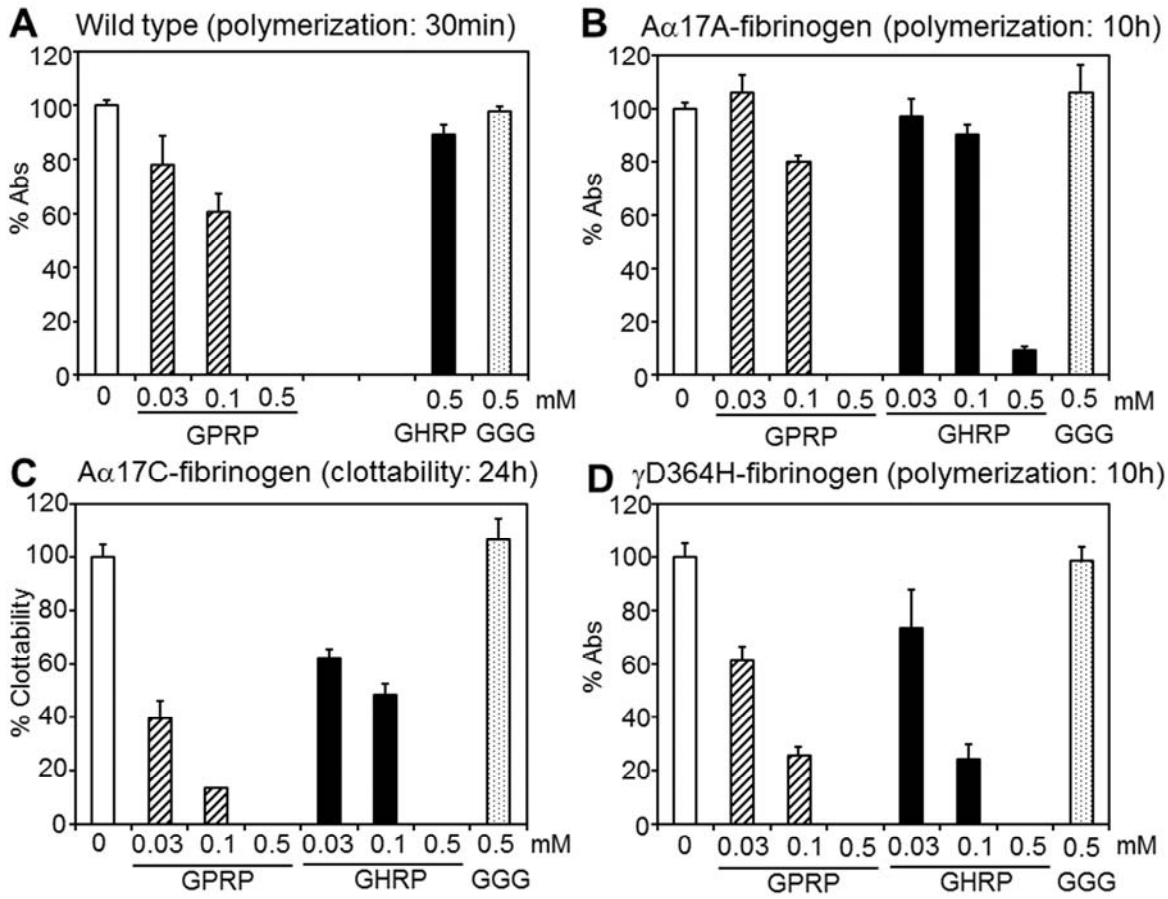
A
Wild type (polymerization: 30min)
% Abs
120
100
80
60
40
20
0
0
0.03
0.1
0.5
0.5
0.5
mM
GPRP
GHRP
GGG
B
Aα17A-fibrinogen (polymerization: 10h)
% Abs
0
0.03
0.1
0.5
0.03
0.1
0.5
0.5
mM
GPRP
GHRP
GGG
C
Aα17C-fibrinogen (clottability: 24h)
% Clottability
0
0.03
0.1
0.5
0.03
0.1
0.5
0.5
mM
GPRP
GHRP
GGG
D
γD364H-fibrinogen (polymerization: 10h)
% Abs
0
0.03
0.1
0.5
0.03
0.1
0.5
0.5
mM
GPRP
GHRP
GGG

Figure 5. Inhibition of fibrin polymerization by GPRP or GHRP. Thrombin-catalyzed polymerization of fibrinogen (0.36 mg/ml = 1.06 μM) was performed with 0.04 U/ml of thrombin without or with GPRP (0.03–0.5 mM), GHRP (0.03–0.5 mM), or GGG (0.5 mM), as described in Materials and Methods. The percent absorbance = [absorbance with peptide/absorbance without peptide] × 100% was determined at 30 min for wild-type fibrinogen (A) and 10 h for Aα17A- (B) and γD364H- (D) fibrinogens. The percent clottability = [clottability with peptide/clottability without peptide] × 100% was determined after 24 h for Aα17C-fibrinogen (C). The mean values and standard deviations from triplicate experiments are shown. Open bars: without peptide, diagonally striped bars: with GPRP, black bars: with GHRP, and dotted bars: with GGG. In the presence of 0.5 mM GPRP, the polymerization or clottability of all fibrinogens was completely inhibited, but in the presence of 0.5 mM GHRP, polymerization of γD364H-fibrinogen and clottability of Aα17C-fibrinogen were completely inhibited.