Heterozygous variant fibrinogen γA289V (Kanazawa III) was confirmed as hypodysfibrinogenemia by plasma and recombinant fibrinogens

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Short running Title; **γA289V fibrinogen showed hypodysfibrinogenemia**
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Manuscript: 3935 (total word count)

Abstract

Introduction: Congenital fibrinogen disorders are classified as afibrinogenemia, hypofibrinogenemia, dysfibrinogenemia, and hypodysfibrinogenemia. However, difficulties are associated with discriminating between dysfibrinogenemia, hypofibrinogenemia, and hypodysfibrinogenemia using routine analyses. We previously reported a heterozygous variant fibrinogen (γA289V; Kanazawa III) as hypodysfibrinogenemia; however, the same variant had previously been described as hypofibrinogenemia. To clarify the production of γA289V fibrinogen, we expressed recombinant γA289V (r-γA289V) fibrinogen and compared it with wild-type (WT) and adjacent recombinant variant fibrinogens.

Methods: Target mutations were introduced into a fibrinogen γ-chain expression vector by site-directed mutagenesis, and the vector was then transfected into Chinese hamster ovary cells to produce recombinant fibrinogen. Fibrinogen was purified from the plasma of the proposita, and culture media and fibrinogen functions were analyzed using fibrin polymerization, plasmin protection, and FXIIIa-catalyzed fibrinogen cross-linking.

Results: The fibrinogen concentration ratio of the culture media to cell lysates was markedly lower for r-γA289V fibrinogen than for WT. Because the secretion of recombinant γF290L (r-γF290L) fibrinogen was similar to WT, we compared r-γF290L

fibrinogen functions with WT. The fibrin polymerization of Kanazawa III plasma (K-III) fibrinogen was significantly weaker than normal plasma fibrinogen. Moreover, K-III fibrinogen showed a markedly reduced "D:D" interaction. However, all functions of r-γF290L fibrinogen were similar to WT. An *in silico* analysis confirmed the above results.

Conclusion: The present results demonstrated that γA289 is crucial for the γ-module structure, and the γA289V substitution markedly reduced fibrinogen secretion. Moreover, K-III fibrinogen showed markedly reduced fibrin polymerization and "D:D" interactions. γA289V fibrinogen was confirmed as hypodysfibrinogenemia.

Introduction

Fibrinogen is a 340-kDa plasma glycoprotein composed of two sets of three different polypeptide chains (Aα, Bβ, and γ),[1] which are encoded by *FGA*, *FGB*, and *FGG*, respectively. Each chain is synthesized, assembled into a three-chain monomer (Aα-Bβ-γ), and held together with each N-terminal portion into a six-chain dimer (Aα-Bβ-γ)$_2$,[2] which is then secreted into the circulation. Fibrinogen is a fibrous protein with a symmetrical trinodular structure consisting of a central E region and two distal D regions through a link with triple helix coiled-coil connectors. The globular D regions are composed of independently folding C-terminal regions of the Bβ- and γ-chains and these are called the β- and γ-modules, respectively.[3] The fibrinogen γ-module contains many functional sites and/or structures for fibrin polymerization, hole 'a', the "D:D" interface, γ-γ cross-linking, and high-affinity Ca^{2+}-binding sites.[4]

In the final step of the blood coagulation cascade, soluble fibrinogen is converted into fibrin fibers that form blood clots under physiological conditions to prevent bleeding, but form a thrombus in the pathogenesis of ischemic disorders. At the initiation of fibrin formation, thrombin releases fibrinopeptides A and B from the N-termini of the fibrinogen Aα- and Bβ-chains, respectively, and converts fibrinogen into fibrin monomers.[5] Fibrin monomers polymerize spontaneously through a two-step process. In

the first step, so-called "A-a" knob-hole interactions (knob 'A', which is the N-terminal

sequence of the α-chain; Gly-Pro-Arg-, and hole 'a', which is in the γ-module of another

fibrin molecule) and the so-called "D:D" interaction, which abuts the γ-chain of two

adjacent molecules, form double-stranded protofibrils.[5,6] In the second step, these

protofibrils grow in length and the so-called "B-b" knob-hole interaction (knob 'B',

which is the N-terminal sequence of the β-chain; Gly-His-Arg-, and hole 'b', which is in

the β-module of another fibrin molecule) promotes the lateral aggregation of protofibrils,[7]

resulting in the formation of fibrin clots consisting of a multi-stranded and branched fiber

network.[8]

Up to 400 congenital fibrinogen disorders (CFDs) have been listed on the GFHT

homepage;[9] however, these include heterogeneous groups with broad molecular

abnormalities and clinical features. CFDs are classified according to functional and

antigenic fibrinogen levels as afibrinogenemia (complete absence of fibrinogen) and

hypofibrinogenemia (proportional reduction of functional and antigenic levels) of

quantitative disorders and dysfibrinogenemia (reduction of functional level and normal

antigenic level) and hypodysfibrinogenemia (disproportional reduction of functional and

antigenic levels) of qualitative disorders.[10] We reported previously a heterozygous variant

fibrinogen (γA289V; Kanazawa III) as hypodysfibrinogenemia;[11] however, Dear et al.

described the same heterozygous variant (Dorfen) as hypofibrinogenemia.[12] γA289 is located at the "D:D" interface, which plays a central role in the initial alignment of fibrin monomers into protofibrils.[13]

To compare and clarify the synthesis and secretion of variant fibrinogens between γG287R and γG292V in the "D:D" interface region of the γ-module, we produced six recombinant variant fibrinogens and compared the function of γA289V plasma fibrinogen with adjacent γF290L recombinant fibrinogen.

Materials and Methods

The present study was approved by the Ethics Review Board of Shinshu University School of Medicine (approved number 603). After informed consent had been obtained from the proposita, blood samples were collected for biochemical and genetic analyses.

Coagulation tests and patient

Prothrombin time (PT), activated partial thromboplastin time (APTT), and fibrinogen concentrations, which were assessed using the thrombin time method, were measured with the automated analyzer, Coapresta 2000 (Sekisui Medical Co., Tokyo, Japan), and immunological fibrinogen concentrations were measured using a latex photometric

immunoassay with anti-fibrinogen antibody-coated latex particles (Mitsubishi Chemical Medicine Co., Tokyo, Japan).[14]

As reported previously,[11] the proposita of Kanazawa III (γA289V) was a 25-year-old Japanese woman with abnormal uterine bleeding after normal spontaneous vaginal delivery and genital bulging around the repaired perineal incision site. The PT and APTT were 14.5 and 33.5 seconds (normal range: 10.8–13.2 and 23.0–38.0 seconds, respectively). Functional and immunological fibrinogen concentrations were 0.38 g/L and 0.50 g/L, respectively (both normal ranges: 1.80–3.50 g/L). Her mother had the same heterozygous mutation and her fibrinogen concentrations were 0.75 g/L (functional) and 0.87 g/L (immunological).

Establishment of variant fibrinogen-producing Chinese hamster ovary (CHO) cells

Target mutations were introduced into the fibrinogen γ-chain expression vector pMLP-γ [15] using a QuikChange II Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA).[14] Mutated plasmids were co-transfected with the histidinol selection plasmid into CHO cells that expressed normal human fibrinogen Aα- and Bβ-chains, and colonies were selected on histidinol (Aldrich Chem., Milwaukee, WI, USA).[15]

Immunoblotting analysis and enzyme-linked immunosorbent assay (ELISA)

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting analysis were performed as described previously,[15] and a mouse anti-human fibrinogen γ-chain monoclonal antibody (2G10; Accurate Chemical and Scientific, Westbury, NY, USA) was used to detect the γ-chain. Culture media and cell lysates were prepared and fibrinogen concentrations were measured by ELISA.[15]

Purification of fibrinogen from plasma and serum-free media

The purification of fibrinogen from the proposita was performed by immunoaffinity chromatography using an anti-IF-1 monoclonal antibody (LSI Medience, Tokyo, Japan)-conjugated to a Sepharose 4B column and purified fibrinogen concentrations were measured as described previously.[16] The γF290L fibrinogen-producing CHO cell line was cultured on a large scale, and fibrinogen was purified from the pooled serum-free medium by ammonium sulfate precipitation and immunoaffinity chromatography, as described previously.[16]

Thrombin- or batroxobin-catalyzed fibrin polymerization

The turbidity curves of fibrin polymerization were recorded at 350 nm using a UV-

1280 (Shimadzu, Tokyo, Japan). Human α-thrombin (Enzyme Research Laboratories, South Bend, Ma, USA)- and batroxobin (snake venom from *Bothrops atrox*; Pentapharm Ltd., Basel, Switzerland)-catalyzed fibrin polymerization were performed, as described previously.[16] Three parameters: lag time, maximum slope (Max-slope), and absorbance change at 30-minute (ΔAbs), were obtained from the turbidity curves, as described previously.[14] Reactions were performed in triplicate for each sample.

Protection assay of plasmin digestion for fibrinogen

Fibrinogen (0.30 mg/mL) in 20 mM N-[2-hydroxyethyl] piperazine-N'-[2-ethansulfonic acid] pH 7.4, 0.12 M NaCl (HBS) buffer containing 1 or 5 mM $CaCl_2$, 1 or 5 mM GPRP peptides (synthetic peptides Gly-Pro-Arg-Pro acetate salt, purity >97%; Sigma-Aldrich), or 5 mM ethylene diamine tetraacetic acid (EDTA) was incubated with plasmin (0.18 U/mL; Chromogenix AB, Molngal, Sweden) at 37°C for 2 hours. The reactions were stopped by adding non-reducing sample buffer followed by boiling for 5 minutes. Plasmin digests were analyzed on 10% SDS-PAGE gels and stained with Coomassie brilliant blue (CBB).[14]

Factor (F) XIIIa-catalyzed cross-linking for fibrinogen

FXIII (Enzyme Research Laboratories) was activated with human α-thrombin at 37°C for 1-hour in HBS buffer with $CaCl_2$, then fibrinogen (0.47 mg/mL) was incubated at 37°C with FXIIIa (66.5 U/mL) in the presence of hirudin (3.3 U/mL; Sigma-Aldrich) as a thrombin inhibitor.[14] The reactions were stopped by the addition of reducing sample buffer followed by boiling for 5 minutes. Samples were separated using 8% SDS-PAGE gels and stained with CBB.[14]

In silico *molecular analysis*

The human fibrinogen crystal structure was obtained from the protein data bank[17] (PDB ID: 3GHG). Each substitution was analyzed using the Mutagenesis Wizard of PyMOL.[18] We selected each rotamer with the least steric clashes of available rotamers. All figures with molecular modeling were prepared with PyMOL.

Statistical analysis

The significance of differences in fibrinogen production and in three parameters of fibrin polymerization were assessed using Dunnett's test and Student's t-test, respectively. A difference was considered to be significant when the *p*-value was <0.05.

Results

Synthesis and secretion of variant fibrinogen in CHO cells

Immunoblotting analysis demonstrated that γ-chains were synthesized and fibrinogens were assembled in all variant cell lines (Figure 1A, B, C). The fibrinogen concentrations (μg/mL) of wild-type (WT) (mean ± S.D.) were 0.75 ± 0.30 in culture media and 0.89 ± 0.48 in cell lysates, resulting in a ratio of culture media to cell lysates (M/C ratio) of 0.88 ± 0.17. The culture medium concentrations of γF290L (3.06 ± 1.33) and γD291Y (1.83 ± 0.51) were significantly higher than that of WT. On the other hand, the cell lysate concentrations of γD288Y (5.49 ± 1.64), γA289V (6.38 ± 2.60), and γG292V (3.70 ± 0.95) were significantly higher than that of WT. The M/C ratios of γD288Y (0.04 ± 0.01), γA289V (0.07 ± 0.02), and γG292V (0.35 ± 0.12) were significantly lower than that of WT, whereas that of γD291Y (1.98 ± 0.52) was significantly higher (Figure 1D, E, F).

Fig.1->

Thrombin- or batroxobin-catalyzed fibrin polymerization

Turbidity curves were obtained as shown in Figure 2, and three parameters were calculated and are shown in Table 1. For fibrinogen from the plasma of the patient heterozygous for Kanazawa III (K-III), fibrin polymerization by thrombin and batroxobin

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were both significantly reduced. Recombinant γF290L fibrinogen also showed a slightly

significantly reduced polymerization compared with recombinant WT fibrinogen.

Fig.2 and Table 1->

Protection assay of plasmin digestion for fibrinogen

The plasmin protection assay was performed to assess the impaired function of hole

'a' and Ca^{2+}-binding sites (data not shown). In the presence of 5 mM EDTA, K-III

fibrinogen showed the same pattern with normal plasma fibrinogen. However, in the

presence of $CaCl_2$ or GPRP peptides, degradation products of K-III fibrinogen slightly

increased more than those of normal plasma fibrinogen, indicating a slight impairment of

the protection of plasmin digestion. On the contrary, recombinant γF290L fibrinogen was

similar to recombinant WT fibrinogen in any conditions.

FXIIIa-catalyzed cross-linking for fibrinogen

To confirm the difference in "D:D" interactions, FXIIIa-catalyzed cross-linking for

fibrinogen was performed. As shown in Figure 3A and 3C, the γ-γ dimer and α-polymer

bands of normal plasma and recombinant WT fibrinogen appeared after a 30-minute

reaction and increased in a time-dependent manner. In contrast, the Aα- and γ-chains

decreased over time. For K-III fibrinogen, increases in the γ-γ dimer and α-polymer bands

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were markedly slower than that of normal plasma fibrinogen (Figure 3B). However, for recombinant γF290L fibrinogen, increases in these bands were slightly slower than that for recombinant WT fibrinogen (Figure 3D).

Fig.3->

In silico *molecular analysis*

To assess steric clashes by each amino acid substitution in the γ-module, an *in silico* molecular analysis was performed using the human fibrinogen crystal structure. The γA289 residue was located inside of the molecule, whereas other residues were exposed to the molecular surface (Figure 4B). The substitutions of γD288Y, γA289V, and γG292V collided significantly more than other substitutions (Figure 4C-H). The substitution of γF290L collided the least of all substitutions (Figure 4F). Thus, the substitutions of γD288Y, γA289V, and γG292V induced significant changes in the structure of the γ-module. In contrast, the substitutions of γG287R, γF290L, and γD291Y did not appear to affect this structure.

Fig.4->

Discussion

We previously identified and reported two heterozygous hypodysfibrinogenemic patients (Kanazawa III: γA289V), based on the weak dissociation between functional and

antigenic fibrinogen level (ratio: 0.76 [proposita] and 0.86 [her mother]).[11] To clarify the

synthesis and secretion of γA289V, we established γA289V fibrinogen-producing CHO

cell lines and subjected them to ELISA and immunoblotting analysis. Recombinant

γA289V fibrinogen was synthesized and accumulated inside cells, whereas its secretion

and M/C ratio were markedly lower than WT fibrinogen. These results indicated that

patients with γA289V manifest the phenotype of hypofibrinogenemia or

hypodysfibrinogenemia.

Moreover, fibrinogen purified from the K-III patient (heterozygous γA289V) showed

significantly reduced, especially longer lag time in thrombin- and batroxobin-catalyzed

fibrin polymerization, demonstrating the presence of variant fibrinogen in the patient's

plasma. Based on these results, we concluded that the K-III patient manifested

hypodysfibrinogenemia with markedly reduced secretion of variant fibrinogen. Our

results suggested that the K-III patient bleeding tendency was caused by the extremely

reduced level of fibrinogen plus aberrant function of variant fibrinogen. Dear et al.

showed the lack of a variant γ-chain in the plasma of a heterozygous γA289V (Dorfen)

patient using mass spectrometry.[12] We attributed the discrepancy between the two

families to the fact that the amount of γA289V fibrinogen in plasma was too low and it

was inadequate for the mass analysis sensitivity. In addition, because the novel

heterozygous mutation, γA289D, was recently reported as dysfibrinogenemia,[19] the

γA289 residue is important for fibrinogen function. The heterozygous γG292V

fibrinogens, Baltimore I and St. Gallen I, have been reported as dysfibrinogenemia and

hypodysfibrinogenemia, respectively.[20,21] Because our results indicated that the M/C ratio

of recombinant γG292V fibrinogen was lower than that of recombinant WT fibrinogen,

we speculated that the γG292V patient manifested hypodysfibrinogenemia with the

reduced secretion of variant fibrinogen. In conclusion, three out of six recombinant

variant fibrinogens between γG287R and γG292V in the γ-module showed markedly

reduced secretion. These results suggested that this region is crucial for fibrinogen

secretion, but not synthesis.

The secretion of recombinant γA289V fibrinogen was markedly reduced and not

purified from culture media; therefore, we compared functional defects in purified K-III

fibrinogen to recombinant γF290L fibrinogen. In contrast to our expectations, the

thrombin- and batroxobin-catalyzed fibrin polymerization of recombinant γF290L

fibrinogen was only slightly reduced, whereas that of purified K-III fibrinogen was

markedly reduced. Moreover, the "D:D" interaction of K-III fibrinogen was affected more

severely than that of recombinant γF290L fibrinogen. These results indicated that the

defect in polymerization was due to the "D:D" interface being affected and not the

function of hole 'a' or the high-affinity Ca^{2+}-binding site.

The differences in secretion and function among the six substitutions of fibrinogen were confirmed by *in silico* analysis. The substitutions of γD288Y, γA289V, and γG292V were estimated as prominent steric hindrance, resulting in markedly reduced secretion of fibrinogen into the media. In contrast, the substitutions of γG287R and γD291Y were estimated as causing less collision than the above three and did not influence the secretion of fibrinogen into the media. Moreover, the substitution of γF290L was estimated as having the smallest collision, leading to a similar M/C ratio to WT and slightly reduced fibrin polymerization than WT.

It is difficult to classify the heterozygous dysfibrinogenemia, hypodysfibrinogenemia, and hypofibrinogenemia based on functional and immunological fibrinogen levels and their ratio, because the ratio was influenced by assay reproducibility and the reference material. Casini et al. showed that the cut-off (ratio < 0.7) is not sufficient to diagnose hypodysfibrinogenemia and reported that expression studies of recombinant protein are important to determine the hypofibrinogenemia, and functional analysis of most dysfibrinogenemic variants show the impairment of polymerization, calcium-binding and/or hypofibrinolysis.[22] Thus, it is important to determine the phenotype of variant fibrinogen using an expression experiment and thrombin-catalyzed fibrin polymerization

of purified plasma fibrinogen.

In conclusion, the present study demonstrated that fibrinogen γ-chain residues in the "D:D" interface, γA289, were crucial for the construction of a three-dimensional structure, and substitution resulted in markedly reduced fibrinogen secretion. Moreover, purified K-III fibrinogen showed markedly reduced "D:D" interactions and fibrin polymerization. These results demonstrated that this patient with fibrinogen γA289V substitution was classified as having hypodysfibrinogenemia, and the structure of the γ-module around γA289 is important for fibrinogen secretion and protofibril formation.

Authorship

Takahiro Kaido and Masahiro Yoda performed the research, analyzed the data, and wrote the manuscript. Tomu Kamijo, Chiaki Taira, Yumiko Higuchi, and Nobuo Okumura designed the research and discussed the data. N. Okumura and T. Kamijo reviewed the manuscript.

Conflict of interest

Competing interest: the authors have no competing interests.

Acknowledgments

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Table 1

|	Thrombin-catalyzed fibrin polymerization			Batroxobin-catalyzed fibrin polymerization		
	Lag time (min)	Max-slope ($\times10^{-3}$/sec)	ΔAbs	Lag time (min)	Max-slope ($\times10^{-3}$/sec)	ΔAbs
Purified plasma fibrinogen						
Normal plasma	2.3 ± 0.1	1.13 ± 0.08	0.254 ± 0.015	6.8 ± 0.2	0.72 ± 0.10	0.375 ± 0.007
	***	***	**	*	n.s.	*
Kanazawa III	3.4 ± 0.1	0.46 ± 0.03	0.149 ± 0.015	7.3 ± 0.2	0.55 ± 0.06	0.353 ± 0.006
Purified recombinant fibrinogen						
Wild-type	1.9 ± 0.2	1.55 ± 0.25	0.307 ± 0.012	6.0 ± 0.4	0.52 ± 0.03	0.424 ± 0.017
	**	*	n.s.	**	**	**
γF290L	2.8 ± 0.2	1.01 ± 0.06	0.271 ± 0.024	7.4 ± 0.3	0.42 ± 0.01	0.336 ± 0.010

Statistical analysis of each parameter between control fibrinogens and variant fibrinogens was performed using Student's t-test.

n.s.: Not significant, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$

Figure legends

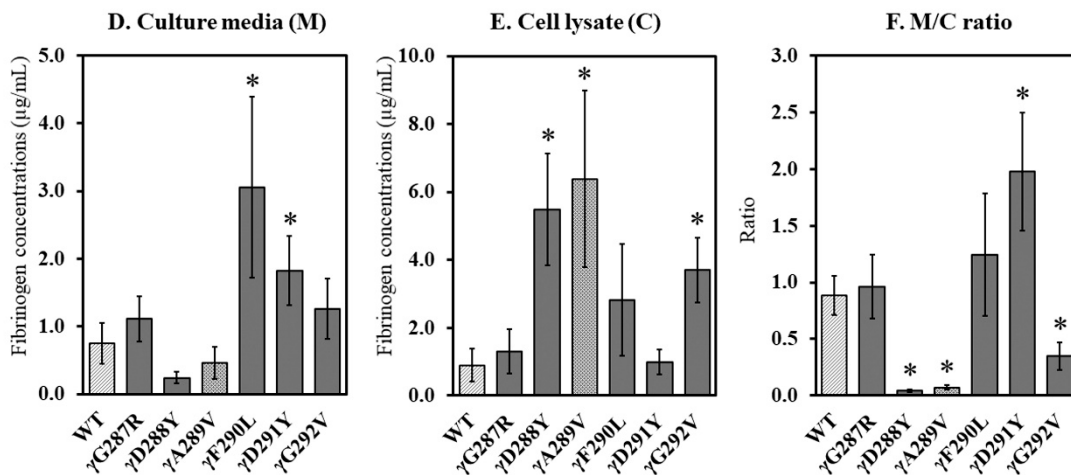Fig. 1

Immunoblotting analysis
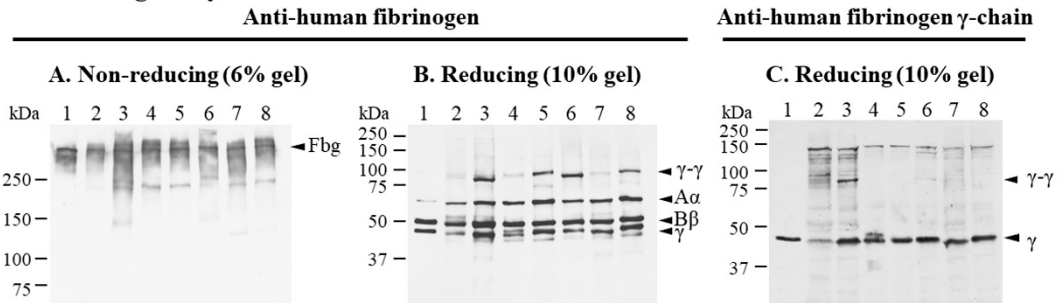
Figure 1. Immunoblotting was performed using anti-human fibrinogen antibodies in non-reducing (Panel A) or reducing conditions (Panel B) and anti-human fibrinogen γ-chain antibodies in reducing conditions (Panel C). 1: purified wild-type fibrinogen, 2: wild-type-, 3: γG287R-, 4: γD288Y-, 5: γA289V-, 6: γF290L-, 7: γD291Y-, 8: γG292V-fibrinogen-producing CHO cells.

Fibrinogen concentrations in culture media (Panel D) and cell lysates (Panel E) were measured by ELISA. Panel F shows the ratio in the medium to the cell lysate. Mean values

are presented with standard deviations. Concentrations were assessed for clones expressing wild-type (WT, n=11 clones), γG287R (n=12), γD288Y (n=10), γA289V (n=12), γF290L (n=5), γD291Y (n=12), and γG292V (n=11). The significance of differences between WT and variant fibrinogen-producing cells was shown. *p <0.001.
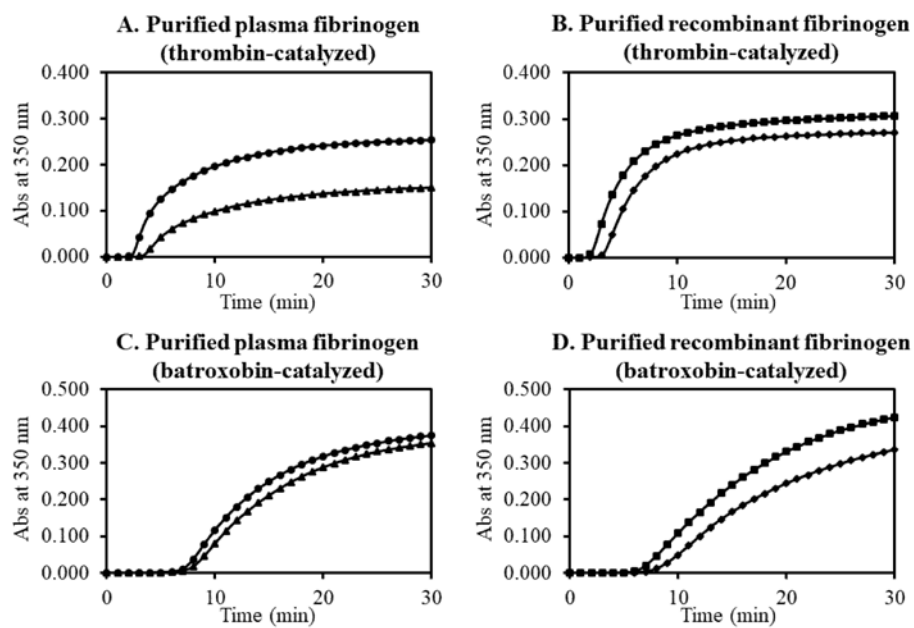
Figure 2. The polymerization of plasma fibrinogens (0.18 mg/mL, Panel A, C) or recombinant fibrinogens (0.18 mg/mL, Panel B, D) was initiated with thrombin (0.05 U/mL, Panel A, B) or batroxobin (0.05 U/mL, Panel C, D). ●: normal plasma fibrinogen, ▲: Kanazawa III (γA289V) plasma fibrinogen, ■: recombinant wild-type fibrinogen, ◆: recombinant γF290L fibrinogen.
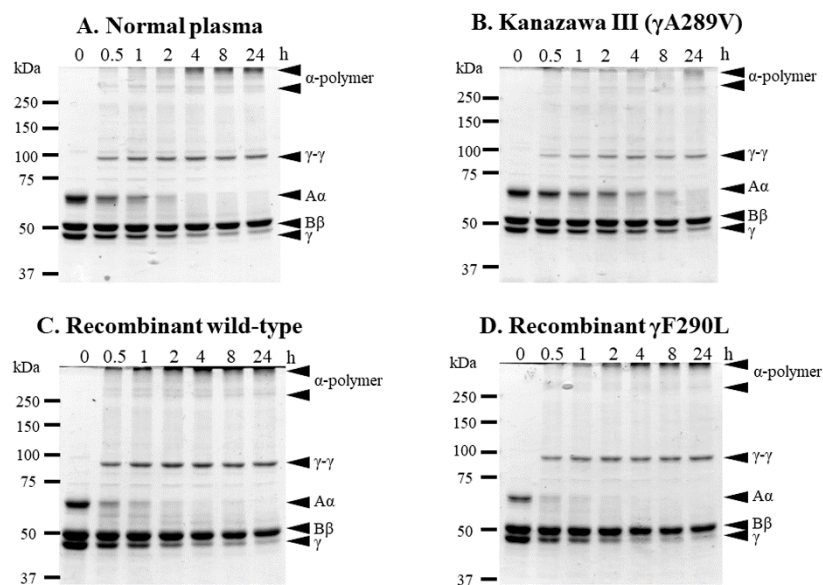
Figure 3. Fibrinogens (Panel A: normal plasma fibrinogen, Panel B: Kanazawa III plasma

fibrinogen, Panel C: recombinant wild-type fibrinogen, Panel D: recombinant γF290L

fibrinogen) were cross-linked by FXIIIa in the presence of the thrombin inhibitor, hirudin,

and analyzed on an 8% SDS-PAGE gel in reducing conditions and stained with CBB.
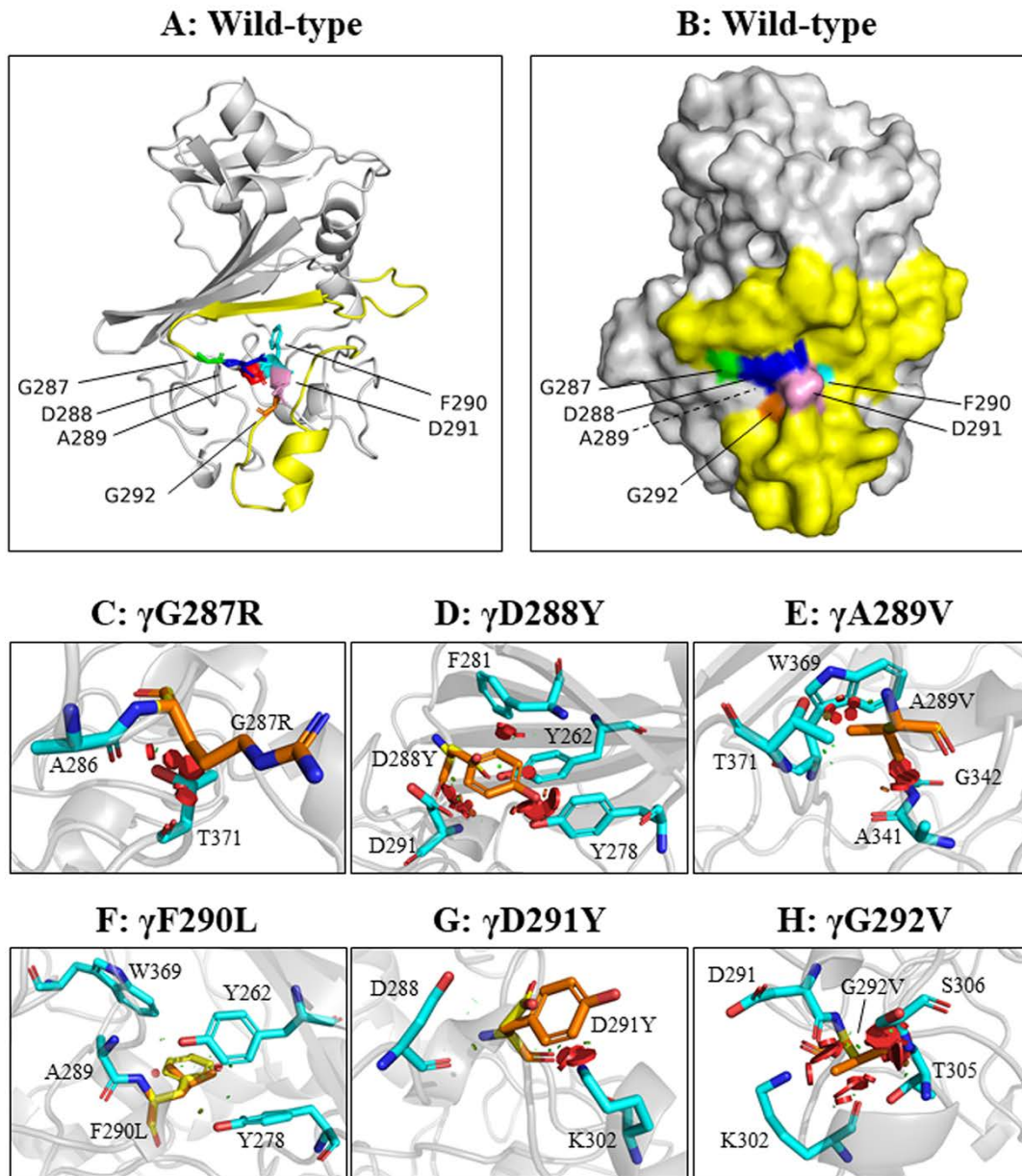
Fig. 4

A: Wild-type

B: Wild-type

C: γG287R

D: γD288Y

E: γA289V

F: γF290L

G: γD291Y

H: γG292V

Figure 4. Panels A and B show the tertiary structure and surface structure of the wild-type γ-module, respectively. Yellow indicates the "D:D" interface. Each mutation site is shown in different colors (γG287: green, γD288: blue, γA289: red, γF290: cyan, γD291: pink, and γG292: orange). In the surface structure, the γA289 residue is invisible because

it is located on the inside of the molecule. Panel C-H: Residues of the wild-type and mutant are shown as yellow and orange sticks, respectively. The residues of the cyan stick show contact or bumps with the substituted residue. The disks represent pairwise overlaps of atomic van der Waals radii. Green lines and disks indicate close contact or slight overlaps, whereas red disks show significant overlaps.

Table and figure caption list

Table 1. Three parameters of thrombin- or batroxobin-catalyzed fibrin polymerization.

Figure 1. Synthesis and secretion of fibrinogen in CHO cells.

Figure 2. Thrombin- or batroxobin-catalyzed fibrin polymerization.

Figure 3. FXIIIa-catalyzed cross-linking for fibrinogen.

Figure 4. Predicted steric clashes by each substitution in the γ-module.