

A C-terminal amino acid substitution in the γ -chain caused by a novel heterozygous frameshift mutation (Fibrinogen Matsumoto VII) results in hypofibrinogenemia

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Keywords: hypofibrinogenemia, nucleotide deletion, frameshift mutation, assembly, secretion

Word count (Text): 4999 (including Abstract: 250)

(excluding title page, key word, references, tables, figure legends)

Number of figures: 5

Number of tables: 2

Number of references: 25

This study was supported by a Grant-in-Aid for Science Research from the Ministry of Education, Science, Sports, and Culture of Japan (20930020, 21931032, NF).

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Summary

We found a novel hypofibrinogenemia designated as Matsumoto VII (M-VII), which is caused by a heterozygous nucleotide deletion at position g.7651 in *FGG* and a subsequent frameshift mutation in codon 387 of the γ -chain. This frameshift results in 25 amino acid substitutions, late termination of translation with elongation by 15 amino acids, and the introduction of a canonical glycosylation site. Western blot analysis of the patient's plasma fibrinogen visualized with anti- γ -chain antibody revealed the presence of two extra bands. To identify the extra bands and determine which of the above-mentioned alterations caused the assembly and/or secretion defects in the patient, 11 variant vectors that introduced mutations into the cDNA of the γ -chain or γ' -chain were transfected into CHO cells. *In vitro* expression of transfectants containing $\gamma\Delta 7651A$ and $\gamma\Delta 7651A/399T$ ($\gamma\Delta 7651A$ with an amino acid substitution of 399Asn by Thr and a variant lacking the canonical glycosylation site) demonstrated a reduction in secretion to approximately 20% of the level seen in the transfectants carrying the normal γ -chain. Furthermore, results from other transfectants demonstrated that 8 aberrant residues between 391 and 398 of the M-VII variant, rather than the 15 amino acid extension or the additional glycosylation, are responsible for the reduced levels of assembly and secretion of M-VII variant fibrinogen. Finally, the results of this study and our previous reports demonstrate that the fibrinogen γ -chain C-terminal tail (388-411) is not necessary for protein assembly or secretion, but the aberrant amino acid sequence observed in the M-VII variant (especially 391-398) disturbs these functions.

Keywords: hypofibrinogenemia, nucleotide deletion, frameshift mutation, assembly, secretion

Introduction

Fibrinogen is a 340-kDa plasma glycoprotein, which consists of two copies of three polypeptide chains, A α , B β , and γ , linked by an extensive network of 29 intra- and inter-chain disulfide bonds (1,2). The three chains are synthesized, assembled into a hexameric molecule in hepatocytes, secreted into the blood, and circulated at 1.8-3.5 g/l. Mature polypeptides of the A α -, B β -, and γ -chains are composed of 610, 461, and 411 residues, respectively (1,2), and the genes encoding the individual polypeptides are located on chromosome 4 (4q28.1, 4q28.2, and 4q28.3 for *FGG*, *FGA*, and *FGB*, respectively), as a cluster (3). Furthermore, 10-15% of plasma fibrinogen contains a heterodimeric molecule that carries a variant chain termed the γ' -chain (4), which arises through alternative splicing and polyadenylation of the γ -chain mRNA transcript (5). Hypofibrinogenemia or afibrinogenemia, defined as reduced or negligible levels of fibrinogen in plasma, can be hereditary. In the past decade, genetic abnormalities in patients with these diseases have been found in all three genes and identified as missense, nonsense, or frameshift mutations; splice-site abnormalities; or large deletions (listed at <http://www.geth.org/databaseang/fibrinogen>).

We previously reported on hypofibrinogenemia, which is caused by a missense mutation of γ 153Cys to Arg (6), and found that the assembly of this variant fibrinogen in CHO cells was defective and that the secretion of the variant was impaired. Thereupon, we also synthesized a series of fibrinogen variants with truncated γ -chains terminating between residues γ 379 and the C terminus, γ 411 (7). Only variants with γ -chains longer than 386 residues were secreted into the culture medium, and the synthesis of the variants with 386 residues or less was markedly reduced in CHO cell lysates. Furthermore, to examine the role of fibrinogen γ -chain residue 387Ile in the assembly and secretion of fibrinogen, we synthesized a series of variants where γ 387 was replaced by Arg, Leu, Met, Ala, or Asp (8).

Only variant γ 387Asp showed impaired synthesis in the cells and very low secretion into the medium. These studies demonstrated that the tertiary structure of the γ -chain C-terminal module is important for the assembly of fibrinogen, and more specifically, that the γ 387 residue and/or the conformation of the γ 388-411 residues, but not the length of the γ C-tail, are critical for its assembly and subsequent secretion (7,8).

Recently, we found a novel variant (Matsumoto VII, M-VII) with a frameshift mutation at codon 387 of the γ -chain that results in 25 amino acid substitutions, late termination of translation with elongation by 15 amino acids, and the introduction of a canonical glycosylation site. Furthermore, Western blot analysis of the patient's plasma fibrinogen visualized with anti- γ -chain antibody revealed two extra bands. *In vitro* expression of M-VII and appropriate C-terminal variants associated with M-VII were used to identify the extra bands and to determine which of these alterations causes the assembly and/or secretion defects.

Materials and Methods

Patient

The patient was a 45-year-old woman who suffered from myoma uteri and had no history of bleeding or thrombosis. A routine blood examination showed a low concentration of plasma fibrinogen. After the patient provided informed consent, we collected a blood sample for biochemical and genetic analyses. This study was approved by the ethics committee of Shinshu University, Japan. Although we were not able to analyze other family members, none of her family members had a history of bleeding or thrombotic tendency.

Coagulation screening tests

Nine volumes of blood were collected in plastic tubes containing 1 vol of 3.2% trisodium citrate. The subject's plasma was separated by centrifugation at 1,500g for 10 min at 4°C. The buffy coat was collected and extracted to prepare genomic DNA. Prothrombin time (PT), activated partial thromboplastin time (APTT), and the fibrinogen concentration, which was determined by the thrombin time method, were measured with an MDA II automated analyzer (Biomerieux, Lyon, France). The immunologic fibrinogen concentration was determined by a latex photometric immunoassay using antifibrinogen antibody-coated latex particles (Mitsubishi Chemical Medience Co., Tokyo, Japan).

Polymerase chain reaction (PCR) amplification and DNA sequence analysis

To amplify the 5 exons (exon I through V) of the A α -chain gene, 8 exons (exon I through VIII) of the B β -chain, and 10 exons (exon I through X) of the γ -chain gene, we designed pairs of primers corresponding to the appropriate introns as described previously (6). Genomic DNA was isolated from peripheral blood leukocytes as described previously (9). PCR amplification and direct sequencing of the DNA fragments extracted from the agarose gels were performed as described previously (6,9).

Construction of mutant expression vectors

The fibrinogen γ -chain expression vector, pMLP- γ , and the fibrinogen γ' -chain expression vector, pMLP- γ' , were kindly provided by Lord ST (University of North Carolina) and altered by oligonucleotide-directed mutagenesis using the QuikChange II Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA) (10) and ten pairs of mutagenesis primers (Table 1).

Recombinant protein expression

CHO cell lines that express normal human fibrinogen A α and B β chains, A α B β -CHO cells, were obtained by cotransfecting the plasmids pMLP-A α , pMLP-B β , and pRSVneo into CHO cells. The cells were cultured in Dulbecco modified Eagle's medium Ham nutrient mixture F12 supplemented as described previously (DMEM-F12 medium) (11). Each of the variant pMLP- γ vectors, original pMLP- γ vector, variant pMLP- γ' -vector, and original pMLP- γ' -vector, was cotransfected with the histidinol selection plasmid (pMSVhis) into the A α B β -CHO cell line, using the standard calcium-phosphate coprecipitation method. Colonies were selected using G418 (Gibco BRL Rackville, MD) and histidinol (Aldrich Chemical, Milwaukee, WI). Individual colonies were expanded in DMEM-F12 medium containing G418 and histidinol and examined for fibrinogen synthesis as described previously (12).

Culture medium and cell lysate for immunologic analysis

The fibrinogen concentrations of the cell lysates and culture media were determined by an enzyme-linked immunosorbent assay (ELISA) as described elsewhere (10). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblot analysis were performed using the enhanced chemiluminescence (ECL) detection reagent (Amersham Pharmacia Biotech, Buckinghamshire, United Kingdom) and Hyperfilm-ECL (Amersham Pharmacia Biotech) as described previously (7). The cells were grown to confluence in 60-mm dishes (approximately $1.5-2.0 \times 10^6$ cells), and the conditioned medium was harvested 1 day after confluence (6-8 days after seeding) for immunoblot analysis or ELISA. Cell lysates were prepared from the same cultures in 60-mm dishes. The cells were harvested in trypsin-ethylene diamine tetraacetic acid (EDTA) solution (Sigma, St Louis, MO), washed 3 times with phosphate-buffered saline (PBS), and lysed in either 50 μ L Laemmli sample buffer for immunoblot analysis, or 250 μ L 0.1% IGEPAL CA-630 (nonionic detergent; Sigma) and

10 mM phenylmethylsulfonyl fluoride (PMSF; Sigma) for ELISA. Rabbit anti-human fibrinogen antibody (Ab), anti-human γ -chain monoclonal 2G10 Ab (MAb; specific for γ 15-35) (13), and anti-human γ' -chain MAb (specific for γ' 408-427) (14) were obtained from Dako (Carpinteria, CA), Accurate Chemical and Scientific (Westbury, NY), and Upstate (Lake Placid, NY), respectively. Horseradish peroxidase conjugated-goat anti-rabbit IgG Ab and horseradish peroxidase conjugated-rabbit anti-mouse IgG Ab were purchased from MBL (Nagoya, Japan).

Enzymatic elimination of N-linked oligosaccharides in fibrinogen

Approximately 10-15 μ g of fibrinogen, dissolved in 30 μ l of 1/15M PBS, 12.5 mM EDTA, 1.0% IGEPAL, 0.2% SDS, and 1% 2-mercaptoethanol (pH 7.4), was digested with 0.5-1.0 U of N-Glycosidase F (Roche, Basel, Switzerland) for 48 hrs at 37 °C. We then performed immunoblot analysis using this sample to investigate the carbohydrate modification of various γ - or γ' -chains.

Purification of plasma fibrinogen and factor XIIIa-catalyzed cross-linking of

Matsumoto VII fibrin

The purification of fibrinogen from the plasma was performed by a modified immunoaffinity-chromatography procedure utilizing IF-1 monoclonal antibody, as previously described (9). Eluted fibrinogen was dialyzed against 20 mM N-[2-hydroxyethyl] piperazine-N'-[2-ethanesulfonic acid] (HEPES), pH 7.4, containing 0.12 M NaCl. Factor XIII (FXIII; 50 units/ml) was activated with human α -thrombin (1 unit/ml) for 60 minutes at 37°C in HEPES-buffered saline (HBS) with 5 mM CaCl₂. To examine the cross-linking of fibrin, fibrinogen (final concentration: 4 mg/ml) was incubated at 37°C with FXIIIa (final

concentration: 3.3 units/ml) and human α -thrombin (final concentration: 0.07 units/ml) in HBS and 0.67 mM calcium (8). The reactions were stopped after 1 hour by the addition of equal volumes of SDS-sample buffer and 2-mercaptoethanol and incubation (5 min) at 100°C.

Pulse-chase analysis of protein synthesis using [³⁵S]-methionine

Pulse-chase studies were performed as described previously (6). In brief, for metabolic radiolabeling of methionine (Met) residues, γ N and $\gamma\Delta 7702A$ cell lines were grown to confluence in 60-mm dishes. The medium was replaced with 1 ml of Met-free DMEM supplemented with 1.5 MBq (40 μ Ci) L-[³⁵S]-Met (MP Biomedicals, Inc., Irvine, CA), and the cells were incubated in 5% CO₂ for 60 min at 37°C. After being pulsed, the cells were rinsed twice, before 1 ml of fresh DMEM containing 20 mM unlabeled L- Met (Wako, Osaka, Japan) was added, and the cells were incubated for 0-, 1-, 3-, 6-, and 24-hour chase periods. Then, the culture media were harvested, and cell lysates were prepared in 120 μ l lysis buffer containing 1% IGEPAL CA-630, 150 mM NaCl, 5 mmol/L EDTA, and 10 mM PMSF in 50 mM Tris-HCl buffer at pH 8.0. Each 500 μ l of medium or cell lysate was added to an equivalent amount of 1:1000 diluted rabbit anti-fibrinogen polyclonal Ab (Dako) and incubated overnight at 4°C, before immunocomplexes were precipitated with Protein A-Sepharose (GE Healthcare Ltd., Tokyo, Japan). The precipitates dissolved with Laemmli sample buffer were resolved on SDS-polyacrylamide gradient gels. Radioactive bands were detected with the Bio-Imaging Analyzer BAS1500 System (Fuji Photo Film Co., Tokyo, Japan).

Statistical methods

Data are presented as means \pm SD. Statistical analysis was performed with Welch's *t* test

using StatFlex (Artec, Osaka, Japan). *p* values <0.05 were accepted as statistically significant.

Results

Clinical characterization of the patient

Routine screening assays before the operation showed impaired coagulation, with normal PT and APTT. The subject's PT was 13.2 sec, whereas the normal range is 11.5 to 15.0 sec. Her APTT was 28.7 sec, whereas the normal range is 25.5 to 39.8 sec. Her plasma fibrinogen concentration determined by both the thrombin time method (0.77 g/L) and the immunologic method (0.89 g/L) was markedly lower than the normal range (1.80 to 3.50 g/L).

Nucleotide-sequence analyses of the fibrinogen gene

The sequences of the fibrinogen A α -, B β -, and γ -chain genes from the proposita were determined by direct analysis of PCR-amplified DNA fragments. An aberrant pattern was found in the sequencing fluorogram of the PCR-amplified exon IX of the γ -chain gene from the proposita. We subcloned the DNA fragments and found a heterozygous nucleotide deletion (A) at position 7651 (all nucleotide positions were numbered by taking the starting point of the translation of the γ -chain gene as 1) (GenBank accession number M10014). This deletion caused a frameshift mutation in codon 387 and resulted in 25 amino acid substitutions (γ 387-411), late termination of translation with elongation by 15 amino acids, and induction of a potential glycosylation site at codon 399Asn (Table 2). No other mutation except those at polymorphic sites was found in either the coding region or in any of the exon-intron boundaries.

Immunoblot analysis of fibrinogen in the patient's plasma

To examine how the deletion of $\gamma 7651A$ affects the structure (or construction) of the fibrinogen molecule, we performed immunoblot analysis of the patient's plasma using three kinds of Abs as described in Materials and Methods. No difference in the immunoblot pattern was observed between the healthy volunteer's plasma and the patient's plasma when anti-fibrinogen polyclonal Ab was used as the primary Ab. When immunoblotting was visualized with anti- γ -chain MAb, a small amount of the γ' -chain was present close to the band for the γ -chain in both the healthy volunteer's and patient's plasma. However, two extra bands, with approximately the same molecular size as the $B\beta$ -chain, one of which had a slightly higher molecular weight than the other, were detectable in the patient's plasma using anti- γ -chain MAb. In addition, the latter band was detectable but faint, whereas neither band reacted with anti- γ' -chain MAb, which specifically recognizes the C-terminus of the γ' -chain molecule (14) (**Fig. 1A**). Therefore, we speculate that the former extra band is the elongated γ -chain (γ^*) and the latter is the elongated γ' -chain (γ'^*) (**Fig. 1A**). Densitometric analysis of M-VII plasma fibrinogen visualized by anti- γ -chain monoclonal Ab indicated that the proportions of the γ -, γ^* -, γ' -, and γ'^* -chains were 60 %, 27%, 11%, and 2%, respectively (**Fig. 1A**).

Immunoblot analysis of N-Glycosidase F-treated fibrinogen from the patient's plasma

Since the variant fibrinogen of the M-VII proposita had a new potential N-glycosylation site at codon 399Asn, we next examined whether these extra bands were derived from carbohydrate modification of the fibrinogen molecule on the basis of the deletion of $\gamma 7651A$. For this purpose, we carried out immunoblot analysis using the patient's plasma, treated with N-glycosidase F as described in Materials and Methods. Although the bands of the normal $B\beta$ -chain, γ -chain, and γ' -chain were shifted down to a position ($B\beta^+$, γ^+ , and γ'^+ ,

respectively) corresponding to an approximately 4-kDa lower molecule weight by treatment with N-glycosidase F, no differences in immunoblot patterns were observed between the healthy volunteer's plasma and the patient's plasma, irrespective of the treatment with N-glycosidase F when using anti-fibrinogen polyclonal Ab or anti- γ' -chain monoclonal Ab (**Fig. 1B**). However, two extra bands of higher molecular weight, which were detected in the patient's plasma using anti- γ' -chain monoclonal Ab, disappeared after treatment with N-glycosidase F (**Fig. 1B**). We speculate that the γ^* -chain was shifted down to a similar position (γ^{*+}) to the normal γ' -chain (γ'^+) but that the γ'^* -chain disappeared due to the lower density of its band (**Fig. 1B**).

Factor XIIIa-catalyzed cross-linking of Matsumoto VII fibrin

The variant γ -chain of M-VII was altered at 398Gln and 406Lys residues, both of which are essential for the isopeptide bonding between adjacent fibrin molecules by factor XIIIa. To confirm the cross-linking ability of the proposita's fibrin and determine whether the variant γ -chain has the ability to clarify the relative amount of the three fibrinogen molecules (normal homodimer, variant homodimer, and normal-variant heterodimer) in the proposita's plasma, the factor XIIIa-catalyzed cross-linking of fibrin was performed in the presence of thrombin and Ca^{2+} , and the reaction products were analyzed by SDS-PAGE under reducing conditions. For normal and proposita fibrin (**Fig 1C**), the presence of the γ - γ dimer, the γ' - γ' dimer, and the bands corresponding to a higher molecular weight than that of the γ' - γ' dimer were found not to differ from each other upon Coomassie Brilliant Blue R-250 staining and blotting with anti-human fibrinogen Ab. In contrast, anti-human γ -chain MAb revealed that variant γ -chain, γ^* , did not form isopeptide bonds between neighboring normal and variant fibrin molecules (**Fig 1C**). If variant γ -chain had formed γ - γ^* and/or γ^* - γ^* dimers, the relative

amount of the three fibrinogen molecules would be revealed by immunoblotting using anti-human γ -chain MAb.

Effect of the mutation of the γ -chain on the expression and secretion of fibrinogen

To define the regulatory region of the γ -chain necessary for the synthesis and secretion of fibrinogen, various mutated constructs derived from pMLP- γ (γ N) were transfected into CHO cells followed by the determination of the fibrinogen concentrations in the culture media and cell lysates by ELISA. The mutated constructs prepared in this study are summarized in Table 2. The $\gamma\Delta 7651A/399T$ ($\gamma\Delta 7651A$ with amino acid substitution of 399Asn by Thr) construct was made to lack a potential canonical glycosylation site at codon 399Asn. As shown in **Fig. 2**, both the synthesized fibrinogen in cell lysates (C-Fbg) and the secreted fibrinogen in the culture media (M-Fbg) of the transfectants containing $\gamma\Delta 7651A$ or $\gamma\Delta 7651A/399T$ were significantly reduced compared with those of the transfectants carrying γ N ($p < 0.001$). However, the ratio of the intracellular- to extracellular-fibrinogen content of the transfectants carrying $\gamma\Delta 7651A$ or $\gamma\Delta 7651A/399T$ was similar to that of the transfectants possessing γ N. Similarly, the C-Fbg and M-Fbg of the transfectants carrying $\gamma'\Delta 7651$ were significantly reduced compared with those of the transfectants carrying pMLP- γ' (γ' N) ($p < 0.001$). In contrast, for the other transfectants containing $\gamma\Delta 7689A$, $\gamma\Delta 7713G$, or $\gamma\Delta 7726T$, which contained the same sequence as Matsumoto VII beyond $\gamma 398$, $\gamma 407$, and $\gamma 411$, respectively, the concentrations of intracellular C-Fbgs ($\gamma\Delta 7713G$ and $\gamma\Delta 7726T$) were significantly reduced compared with that of the normal control ($p < 0.001$); however, no statistically significant differences in the concentrations of the extracellular M-Fbgs were observed. In addition, the levels of secreted extracellular M-Fbgs of the transfectants that contained substitutions close to $\gamma 387$ ($\gamma 387S$, $\gamma 389S$, $\gamma 390T$, $\gamma 387S/\gamma 388H$, or $\gamma 389S/\gamma 390T$) were increased rather than

reduced.

Immunoblot analysis of the recombinant fibrinogen derived from transfectants containing $\gamma\Delta 7651A$

Furthermore, we characterized the recombinant fibrinogen derived from the transfectants possessing $\gamma\Delta 7651A$ by immunoblot analysis under non-reducing or reducing conditions. The band of slightly higher molecular weight than plasma fibrinogen and that of recombinant normal fibrinogen reacted with anti-fibrinogen polyclonal Ab and was detected in the lysates of the transfectants carrying $\gamma\Delta 7651A$ under non-reducing conditions. When the immunoblot analysis was performed under reducing conditions, the extra band of variant fibrinogen was hardly detectable using anti-fibrinogen polyclonal Ab. However, as in the immunoblot analysis of the patient's plasma (see Fig. 1A), an extra band of variant fibrinogen with approximately the same molecular weight as the B β -chain was detected when the anti- γ -chain MAb was used. The immunoblot patterns of the culture media were the same as those of the lysates for both Abs (**Fig. 3A**).

To confirm that the extra band of variant fibrinogen was derived from carbohydrate modification of the fibrinogen molecule due to $\gamma\Delta 7651A$, we carried out immunoblot analysis of the synthesized recombinant fibrinogen in these transfectants, after treating them with N-glycosidase F. The bands derived from the recombinant normal B β -chain (56.0 kDa) and γ -chain (47.5 kDa), and the recombinant variant γ -chain containing $\gamma\Delta 7651A/399T$ (50.4 kDa) (γ^{**}), were shifted down to a position (B β^+ , γ^+ , and γ^{**+} , respectively) corresponding to an approximately 4-kDa lower molecule weight; however, the recombinant variant γ -chain carrying $\gamma\Delta 7651A$ (56.0 kDa) (γ^*) was shifted down to a position (γ^{*+}) approximately corresponding to a 10-kDa difference (**Fig. 3B**). The 6-kDa difference in the molecular mass

caused by N-glycosidase F-treatment between the recombinant variant γ -chains containing $\gamma\Delta 7651A/399T$ (possessing one glycosylation site: $\gamma 52Asn$) and $\gamma\Delta 7651A$ (possessing two glycosylation sites: $\gamma 52Asn$ and $\gamma 399Asn$) may have been due to the presence of an additional sugar chain and its tertiary structure or negative charges. The calculated molecular weights of the N-glycosidase F treated-normal γ -chain and -recombinant variant γ -chains carrying $\gamma\Delta 7651A$ or $\gamma\Delta 7651A/399T$ were approximately 43.5 kDa and 46.1 kDa, respectively.

Immunoblot analysis of recombinant fibrinogen derived from transfectants containing $\gamma'\Delta 7651A$

In the experiments using the lysates and culture media from the transfectants containing $\gamma'\Delta 7651A$ under non-reducing conditions, PAGE detected a higher molecular weight band than that of recombinant normal fibrinogen and γ' -chain containing fibrinogen with anti-fibrinogen polyclonal Ab (**Fig. 4A**) (culture medium **data not shown**). Under reducing conditions, the extra band (γ'^*) was detected in a position between the $A\alpha$ -chain and $B\beta$ -chain of normal fibrinogen by immunoblot analysis using anti-fibrinogen polyclonal Ab or anti- γ' -chain MAb (**Fig. 4A**). However, in the transfectants containing $\gamma'\Delta 7651A$, the variant band did not react with anti- γ' -chain MAb (**data not shown**). Similar results were obtained for the culture media (**Fig. 4A**)

Similar to the results for the normal γ -chain, the molecular weight of the normal γ' -chain (53.5 kDa) (γ') was shifted down to a position (γ'^+) corresponding to an approximately 4-kDa lower molecule weight by treatment with N-glycosidase F; however, the variant γ' -chain (γ'^*) (63.2 kDa) derived from the transfectants containing $\gamma'\Delta 7651A$ was shifted down to a position (γ'^{*+}) corresponding to a slightly lower molecular weight than that of the normal $B\beta$ -chain, resulting in an approximately 13-kDa lower molecular weight than the non-treated

γ^* -chain (**Fig. 4B**). The 9-kDa difference in the molecular mass caused by N-glycosidase F-treatment between the recombinant variant γ' -chain containing the $\gamma'\Delta 7651A$ (possessing four glycosylation sites: $\gamma 52Asn$, $\gamma 399Asn$, $\gamma 406Asn$, and $\gamma 417Asn$) and the normal γ' -chain (possessing one glycosylation site: $\gamma 52Asn$) may have been due to the presence of three additional sugar chains and their tertiary structure or negative charges (Table 2).

Pulse-chase analysis of the synthesized fibrinogen

Finally, using the pulse-chase analysis method as described in Materials and Methods, we examined the assembly and secretion of normal and variant fibrinogen containing $\gamma\Delta 7651A$. As shown in **Fig. 5**, the cells were incubated with [^{35}S]-Met for 1 hr, the medium was replaced with unlabeled-Met, and samples were immunoprecipitated at various times up to 24 hr. The specificity and integrity of the immunoprecipitation were verified by the loss of labeled bands when excess purified plasma fibrinogen was added to the samples before immunoprecipitation (**data not shown**). Analysis of the lysates from γN cells showed a band at 340 kDa, as expected for the intact fibrinogen molecule. This band was clearly present prior to the chase, increased in intensity at 1 hour into the chase, and decreased thereafter (**Fig. 5A**). Analysis of the culture media showed that radiolabeled fibrinogen was first detected in the media following the 1-hour chase. Thereafter, the intensity increased at each time point (**Fig 5A**). The increase in radiolabeled fibrinogen in the medium paralleled the decrease in radiolabeled fibrinogen in the cell lysates. Four other immunospecific bands, with relative mobilities corresponding to $A\alpha$ - $B\beta$ - γ complex, and the $A\alpha$, $B\beta$, and γ bands, were detected in cell lysates from 1-3-hour chases (**Fig 5A**). In contrast, analysis of the lysates from $\gamma\Delta 7651A$ cells showed a faint fibrinogen band after 3- and 6-hour chases (**Fig 5B**), and analysis of the culture media showed faint secretion of variant fibrinogen after 24-hour chase (**Fig 5B**),

indicating that the rate of fibrinogen synthesis or assembly was markedly lower in this cell line.

Discussion

We found a novel hypofibrinogenemia caused by a heterozygous nucleotide deletion at *FGG* g.7651 and designated it fibrinogen Matsumoto VII. This nucleotide deletion results in a frameshift mutation in codon 387Ile, 25 amino acid substitutions (γ 387-411), late termination of translation with elongation by 15 amino acids, and the induction of a canonical glycosylation site at codon 399Asn (Table 2). Western blot analysis for plasma fibrinogen revealed that the elongated γ -chain was present in 27% of γ -chains. Moreover, CHO cells expressing *FGG* Δ g.7651 showed reduced secretion of fibrinogen (about 22% of CHO cells expressed the wild type). Pulse-chase analysis indicated that the synthesis, assembly, and secretion of variant fibrinogen by CHO cells that expressed *FGG* Δ g.7651 were markedly impaired. Schmidt *et al.* also found a novel hypofibrinogenemia caused by a heterozygous nucleotide deletion at *FGG*g.7656 and designated it fibrinogen Heidelberg (Schmidt D *et al.*, abstract #59 in XIXth International Fibrinogen Workshop, 2006), in which a frameshift mutation in codon 389Phe was found to cause the same substitution and elongation (γ 389-426) as seen for fibrinogen M-VII (Table 2).

The variant γ -chain of M-VII was altered at 398Gln and 406Lys residues, both of which are essential for γ - γ dimer formation. A factor XIIIa-catalyzed cross-linking experiment demonstrated that variant γ -chain did not form the γ - γ dimer between neighboring normal or variant γ -chains of fibrin molecules. If the mutant genes are transcribed, translated, post-translationally modified, assembled, and secreted at rates similar to normal, we would expect that plasma fibrinogen synthesized from heterozygous genes will contain a 1:2:1

mixture of normal homodimers, heterodimers, and variant homodimers, respectively. One might expect distributions other than 1:2:1 if 1) the variant chain shows impaired assembly (6), 2) the assembled protein with altered polypeptide is not stable in the cell (15,16), or 3) the assembled protein shows reduced secretion into plasma (15,16,17). For the case of M-VII, our results strongly suggest that all three fibrinogen molecules, namely, normal homodimers, heterodimers, and variant homodimers, are present in plasma, but the relative amounts of both heterodimers and variant homodimers might be less than normal homodimers as shown in Fig 1A (ratio of normal γ -chain:variant γ -chain was 60:27). Since ELISA and pulse-chase experiments demonstrated that variant homodimers were assembled in CHO cells and secreted into medium more slowly than normal, we infer that heterodimers are also assembled in hepatocytes and secreted into blood. Furthermore, there is a possibility that normal fibrinogen synthesis and/or assembly are impeded or reduced by those of variant γ -chain or fibrinogen, namely, the so-called dominant-negative phenomenon in the proposita's hepatocytes. It would be interesting to resolve this issue; however, our presented data are inadequate to reach a conclusion.

In order to determine which of these alterations causes the reduced assembly and/or secretion of variant fibrinogen, we expressed 10 C-terminal variants associated with M-VII *in vitro* and compared them with the predicted γ' -chain variant. The *in vitro* expression of these variants demonstrated that the aberrant 8 residues between 391 and 398 of the M-VII variant, DSQLEKDS, rather than the 15 amino acid extension or the new glycosylation site at Asn399, are responsible for the markedly reduced synthesis, assembly, and secretion of M-VII variant fibrinogen. This conclusion was confirmed by the expression of the predicted γ' -chain variant of M-VII (1-435) in comparison to the wild-type γ' -chain (1-427). Furthermore, we have previously reported that the *in vitro* expression of γ Ile387Asp indicated markedly reduced

assembly and secretion of variant fibrinogen. Since the crystal structure of the γ -module of the D region (γ 143-392) shows that γ 387Ieu lies within a β strand composed of residues γ 381-389 and that this strand is inserted in an antiparallel fashion between strands formed by residues γ 189-197 and γ 243-252 (18,19), the loss or alteration of γ 387Ieu results in a change in the γ -module and induces defective fibrinogen assembly and secretion. Therefore, the γ 387Ile residue and/or the conformation of the γ C-tail beyond 388, but not the length of the γ C-tail, are critical for fibrinogen assembly and secretion (8). Since the C-terminal end of the γ -chain is flexible, x-ray diffraction analysis has not provided information about the tertiary structure of γ 391-411 (18,19) except for that of the co-crystal of human γ 398-411 linked to the C-terminal of chicken egg white lysozyme (20). In contrast, an NMR (nuclear magnetic resonance) study of γ 385-411 strongly suggested the presence of multiple-turns or helix-like structures in residues 390 to 402 (21), and Blumenstein *et al.* also indicated the presence of type II β -turn spanning residues 407 to 410 using the γ 392-411 fragment (22). Finally, x-ray diffraction analysis and NMR analysis have not provided enough information about the normal structure of the 8 residues between 391 and 398, which are substituted in M-VII fibrinogen and cause its reduced fibrinogen assembly and secretion.

Although the strong evolutionary and structural association between B β - and γ -chain molecules is well established (3), the functions of the C-terminal regions in fibrinogen assembly and secretion differ. Namely, Vu *et al.* demonstrated that the C-terminal tail of the B β -chain (455-461) is necessary for the secretion of fibrinogen but not for its assembly (23), whereas the C-terminal tail of the γ -chain (387-411) is necessary for the assembly of fibrinogen (7). Two elongation variants in the fibrinogen B β -chain caused by a missense mutation in the termination codon were reported as dysfibrinogenemia and designated as Osaka VI (24) and Magdeburg II (Franke K *et al.*, abstract #T22-48 in XVIIth International

Fibrinogen Workshop, 2002), respectively. Both are elongated by 12 amino acids (B β 462-473), and the first amino acids in these variants are Lys and Trp, respectively, whilst the other 11 amino acids are the same. These B β -chain elongated variants correspond to the recombinant $\gamma\Delta 7726T$ variant, which shows reduced assembly and secretion in comparison to the wild type, and they have a complete C-terminal tail with additional aberrant peptides. Interestingly, studies of these naturally occurring variants indicated that neither their assembly nor secretion processes were impaired, whereas when the extra 472Cys residues form a variant fibrinogen homodimer, the aberrant molecules lead to the formation of fragile clots made of a lacelike structure composed of highly branched fibers that are thinner than those of normal fibrin clots, causing a bleeding tendency in patients (24). On the other hand, naturally occurring truncated variants of the A α -chain indicated that the C-terminal region from residues 328 to 610 of the A α -chain is not necessary for the assembly or secretion of fibrinogen (25).

In conclusion, the results of this study and those of our previous reports demonstrate that the 24 amino acids of the fibrinogen γ -chain C-terminal tail (388-411) are not necessary for protein assembly or secretion, but aberrant amino acids such as those observed in the M-VII variant, especially in the region from 391-398, disturb these functions; however, the aberrant extension and/or additional glycosylation observed in the M-VII variant do not severely disturb these functions.

Acknowledgements

We gratefully acknowledge Professor Susan T Lord (Department of Pathology and Laboratory Medicine, The University of North Carolina at Chapel Hill, NC) for the kind gift of the γ' -chain cDNA containing plasmid, Professor Takayuki Honda (Department of

Laboratory Medicine, Shinshu University School of Medicine, Matsumoto) and Emeritus Professor Tsutomu Katsuyama (Shinshu University School of Medicine, Matsumoto) for their helpful advice, and Shunsuke Kojima (Department of Transfusion, Shinshu University Hospital, Matsumoto) for helping with the recombinant protein production.

References

1. Doolittle RF, Bouma IH, Cottrell BA, *et al.* The covalent structure of human fibrinogen.
In: Bing DH, ed. The chemistry and physiology of the human plasma proteins. New York, NY: Pergamon Press; 1979:77-95.
2. Doolittle RF. Fibrinogen and fibrin. *Sci Amer* 1981;245:92-101.
3. Kant JA, Fornace AJ Jr, Saxe D, *et al.* Evolution and organization of the fibrinogen locus on chromosome 4: gene duplication accompanied by transposition and inversion. *Proc Natl Acad Sci USA* 1985;82:2344-8.
4. Wolfenstein-Todel C, Mosesson MW. Human plasma fibrinogen heterogeneity: evidence for an extended carboxy-terminal sequence in a normal gamma chain variant (γ'). *Proc Natl Acad Sci USA* 1980;77:5069-73.
5. Chung DW, Davie EW. γ and γ' chains of human fibrinogen are produced by alternative mRNA processing. *Biochemistry* 1984;23:4232-36.
6. Terasawa F, Okumura N, Kitano K, *et al.* Hypofibrinogenemia associated with a heterozygous missense mutation γ 153Cys to Arg (Matsumoto IV): In vitro expression demonstrates defective secretion of the variant fibrinogen. *Blood* 1999;94:4122-31.
7. Okumura N, Terasawa F, Tanaka H, *et al.* Analysis of fibrinogen γ -chain truncations shows the C-terminus, particularly γ Ile387, is essential for assembly and secretion of this multichain protein. *Blood* 2002;99:3654-60.
8. Kani S, Terasawa F, Yamauchi K, *et al.* Analysis of fibrinogen variants at γ 387Ile shows that side-chain of γ 387 and the tertiary structure of γ C terminal tail are important for not only assembly and secretion of fibrinogen but also lateral aggregation of protofibrils and XIIIa-catalyzed γ - γ dimer formation. *Blood* 2006;108:1887-94.

9. Okumura N, Terasawa F, Hirota-Kawadobora M, *et al.* A novel variant fibrinogen, deletion of Bbeta111Ser in coiled-coil region, affecting fibrin lateral aggregation. *Clin Chim Acta* 2006;365:160-7.
10. Okumura N, Gorkun OV, Lord ST. Severely impaired polymerization of recombinant fibrinogen γ -364 Asp His, the substitution discovered in a heterozygous individual. *J Biol Chem.* 1997;272:29596-601.
11. Rooney MM, Parise LV, Lord ST. Dissecting clot retraction and platelet aggregation. Clot retraction does not require an intact fibrinogen γ chain C terminus. *J Biol Chem.* 1996;271:8553-5.
12. Binnie CG, Hettasch JM, Strickland E, *et al.* Characterization of purified recombinant fibrinogen: partial phosphorylation of fibrinopeptide A. *Biochemistry.* 1993;32:107-13.
13. Kudryk B, Rohoza A, Ahadi M, *et al.* Antibodies specific for neoantigens expressed on chains or degradation products of fibrin(ogen). In: Mosesson MW, Amrani DL, Siebenlist KR *et al.* eds. *Fibrinogen 3 -Biochemistry, biological functions, gene regulation and expression.* Amsterdam: Elsevier Science Publishers BV; 1988, pp.129-32.
14. Lovely RS, Falls LA, Al-Mondhiry HA, *et al.* Association of γ A/ γ ' fibrinogen levels and coronary artery disease. *Thromb Haemost* 2002;88:26-31.
15. Duga S, Asselta R, Santagostino E, *et al.* Missense mutations in the human β fibrinogen gene cause congenital afibrinogenemia by impairing fibrinogen secretion. *Thromb Haemost* 2002;88:26-31.
16. Brennan SO, Wyatt J, Medicina D, *et al.* Fibrinogen Brescia. Hepatic endoplasmic reticulum storage and hypofibrinogenemia because of a γ 284 Gly \rightarrow Arg mutation. *Am J Pathol* 2000;157:189-96.
17. Kani S, Terasawa F, Lord ST, *et al.* *In vitro* expression demonstrates impaired secretion of

- the γ Asn319,Asp320 deletion variant fibrinogen. *Thromb Haemost* 2005;94:53-9.
18. Yee VC, Pratt KP, Cote HCF, *et al.* Crystal structure of a 30 kDa C-terminal fragment from γ -chain of human fibrinogen. *Structure* 1997;5:125-38.
 19. Spraggon G, Everse SJ, Dollittle RF. Crystal structures of fragment D from human fibrinogen and its crosslinked counterpart from fibrin. *Nature* 1997;389:455-62.
 20. Donahue JP, Patel H, Anderson WF, *et al.* Three-dimensional structure of the platelet integrin recognition segment of the fibrinogen γ chain obtained by carrier protein-driven crystallization. *Proc Natl Acad Sci USA* 1994;91:12178-82.
 - 21 Mayo KH, Burke C, Lindon JN, *et al.* ^1H NMR sequential assignments and secondary structure analysis of human fibrinogen γ -chain C-terminal residues 385-411. *Biochemistry* 1990;29:3277-86.
 22. Blumenstein M, Matsueda GR, Timmons S, *et al.* A β -turn is present in the 392-411 segment of the human fibrinogen γ -chain. Effects of structural changes in this segment on affinity to antibody 4A5. *Biochemistry* 1992;31:10692-8.
 23. Vu D, Di Sanza C, Caille D, *et al.* Quality control of fibrinogen secretion in the molecular pathogenesis of congenital afibrinogenemia. *Hum Mol Genet* 2005;14:3271-80.
 24. Sugo T, Nakamikawa C, Yoshida N, *et al.* End-linked homodimers in fibrinogen Osaka VI with a B β -chain extension lead to fragile clot structure. *Blood* 2000;96:3779-85.
 25. Lefebvre P, Velasco PT, Dear A, *et al.* Severe hypodysfibrinogenemia in compound heterozygotes of the fibrinogen A α IVS4+1G>T mutation and an A α Gln328 truncation (fibrinogen Keokuk). *Blood* 2004;103:2571-6.

Tables

Table 1. Primer pairs used for oligonucleotide-directed mutagenesis

Variant plasmid	Oligonucleotide sequence (5'→3')
$\gamma\Delta 7651A$	CCAATATGAAGATA_TCCCATTC AACAGACTCACAATTGG CCAATTGTGAGTCTGTTGAATGGGA_TATCTTCATAGTGG
$\gamma'\Delta 7651A$	CCAATATGAAGATA_TCCCATTC AACAGACTCACAATTGG CCAATTGTGAGTCTGTTGAATGGGA_TATCTTCATAGTGG
$\gamma\Delta 7651A/399Thr$	GAGAAGGACAGCA <u>C</u> CACCACCTGGGGGG CCCCCAGGTGGT <u>G</u> GTGCTGTCCTTCC
$\gamma\Delta 7689A$	CAATTGGAGAAGGACAGCA_CACCACCTGGGGGG CCCCCAGGTGGT_GGCTGTCCTTCTCCAATTG
$\gamma\Delta 7713G$	CTGGGGGGAGCCAAACA_GCTGGAGACGTTTAAAAG CTTTTAAACGTCTCCAGC_TGTTTGGCTCCCCCAG
$\gamma\Delta 7726T$	CAGGCTGGAGACGTT_AAAAGACCGTTTCAAAG CTTTTGAAACGGTCTTTT_AACGTCTCCAGCCTG
$\gamma 387Ser$	CCACTATGAAGATAA <u>G</u> CCCATTCAACAGACTCACAATTGG CCAATTGTGAGTCTGTTGAATGGG <u>C</u> TTATCTTCATAGTGG
$\gamma 389Ser$	CAAGATAATCCCAT <u>C</u> CAACAGACTCACAATTGGAGAAGGACAGC GCTGTCCTTCTCCAATTGTGAGCTTGT <u>G</u> GATGGGATTATCTTC
$\gamma 390Thr$	CAAGATAATCCCATTCA <u>C</u> CAGACTCACAATTGGAGAAGGACAGC GCTGTCCTTCTCCAATTGTGAGTCTG <u>G</u> TGAATGGGATTATCTTC
$\gamma 387Ser/\gamma 388His$	CCACTATGAAGATAA <u>G</u> CC <u>A</u> CTTCAACAGACTCACAATTGGAG CTCCAATTGTGAGTCTGTTGAAG <u>T</u> GG <u>C</u> TTATCTTCATAGTGG
$\gamma 389Ser/\gamma 390Thr$	GAAGATAATCCCAT <u>C</u> CA <u>C</u> CAGACTCACAATTGGAGAAGGACAGC GCTGTCCTTCTCCAATTGTGAGTCTG <u>G</u> T <u>G</u> GATGGGATTATCTTC

The 5'-primer is presented above and the 3'-primer below for each mutagenesis. The gaps and underlined positions indicate deleted nucleotides, and the bold and underlined letters indicate substituted nucleotides. Mutagenesis was performed using 9 pairs of primers and the pMLP- γ vector, whereas mutagenesis for $\gamma'\Delta 7651A$ was performed using the primer pairs and the pMLP- γ' vector, and that for $\gamma\Delta 7651A/399T$ was performed using the primer pairs and the pMLP- $\gamma\Delta 7651A$ vector. In addition, the same primer pairs were used for mutation of $\gamma\Delta 7651A$ and $\gamma'\Delta 7651A$

Table2. Amino acid sequences of patient and recombinant Fbg γ -chain

fibrinogen	amino acid sequences of Fbg γ -chain C-terminal tail					
number of residues	381	391	399	401	411	421
γ N(=wild type)	↓	↓	↓	↓	↓	↓
Heidelberg	KTTMKIIPFN RL TIGEGQQH HLGGAKQAGD V (411)					
$\gamma\Delta 7651A(=M-VII)$	KTTMKIIPST DSQLEKDS NT TWGEPNRLET FKRPFQKRFT FLKDFI (426)					
$\gamma\Delta 7651A/399Thr$	KTTMKI SHST DSQLEKDS NT TWGEPNRLET FKRPFQKRFT FLKDFI (426)					
$\gamma\Delta 7689A$	KTTMKIIPFN RL TIGEGQ HT TWGEPNRLET FKRPFQKRFT FLKDFI (426)					
$\gamma\Delta 7713G$	KTTMKIIPFN RL TIGEGQQH HLGGAK QLET FKRPFQKRFT FLKDFI (426)					
$\gamma\Delta 7726T$	KTTMKIIPFN RL TIGEGQQH HLGGAKQAGD V FKRPFQKRFT FLKDFI (426)					
$\gamma 387Ser$	KTTMKI S PFN RL TIGEGQQH HLGGAKQAGD V (411)					
$\gamma 389Ser$	KTTMKIIP S N RL TIGEGQQH HLGGAKQAGD V (411)					
$\gamma 390Thr$	KTTMKIIPF T RL TIGEGQQH HLGGAKQAGD V (411)					
$\gamma 387Ser / \gamma 388His$	KTTMKI SH FN RL TIGEGQQH HLGGAKQAGD V (411)					
$\gamma 389Ser / \gamma 390Thr$	KTTMKIIP ST RL TIGEGQQH HLGGAKQAGD V (411)					
$\gamma'N(=wild type)$	KTTMKIIPFN RL TIGEGQQH HLGGAKQ VRP EHPAET EYDS LYPEDDL (427)					
$\gamma'\Delta 7651A$	KTTMKI SHST DSQLEKDS NT TWGEP NRS DQ STLRKQ NMT H FTLRMICRKL TANFY (435)					

The underlined, framed rectangle, and letters with a gray background indicate substituted or additional amino acids, a canonical glycosylation site, and the alternative splicing amino acids of the γ' -chain. Bold letters indicate amino acids that are the same as those found in M-VII fibrinogen. Heidelberg is a fibrinogen with a similar frameshift mutation. The numbers in parentheses indicate the numbers of amino acids in the whole chain. The $\gamma\Delta 7651A/399T$ ($\gamma\Delta 7651A$ with amino acid substitution of 399Asn by Thr) construct was made to lack a potential canonical glycosylation site at codon 399Asn. Top line of fibrinogen indicates the number of γ -chain residues.

Figures

A

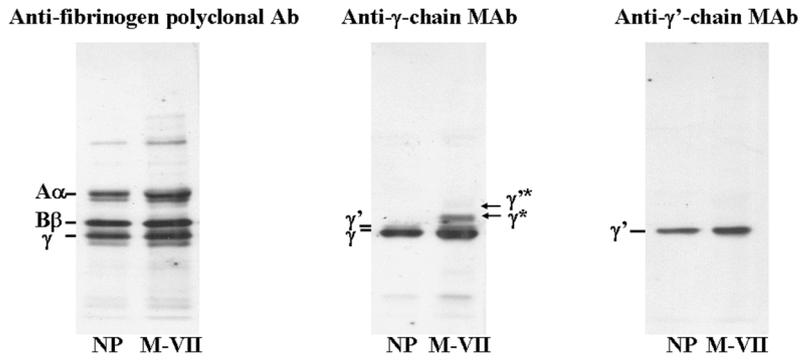


Figure 1A

B

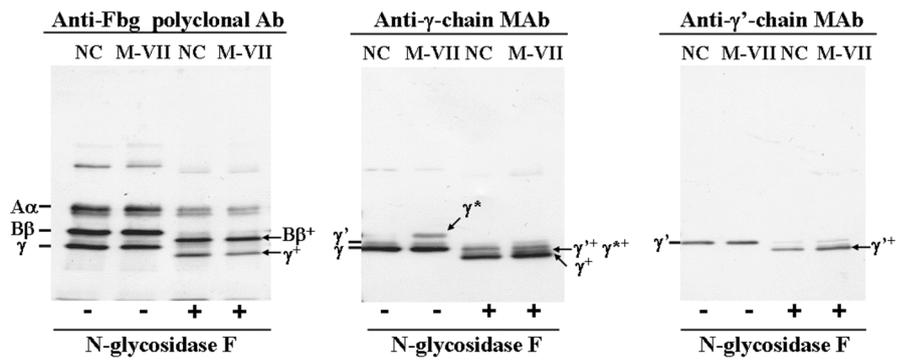


Figure 1B

C

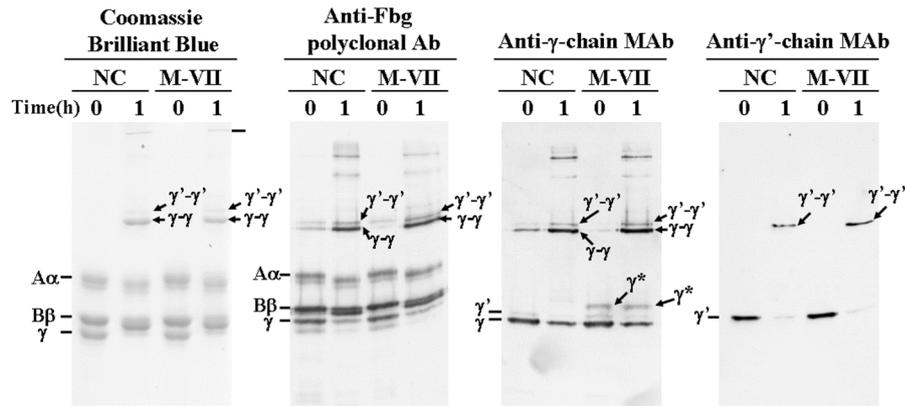


Figure 1C

Figure 1. Immunoblot analysis of plasma fibrinogen. Healthy volunteer's plasma (NP) and the patient's plasma (M-VII) treated without (-) or with N-glycosidase F (+) were subjected to 8% SDS-PAGE under reducing conditions (B). Blots were developed with an anti-fibrinogen polyclonal antibody, an anti- γ -chain monoclonal antibody (2G10), or an anti- γ' -chain monoclonal antibody as described in Materials and Methods. The bands derived from normal fibrinogen are indicated as $A\alpha$, $B\beta$, γ , and γ' , and the extra bands derived from the patient's fibrinogen are indicated as γ^* and γ'^* . In addition, the N-glycosidase F-treated bands are indicated as $B\beta^+$, γ^+ , γ'^+ , and γ'^* . Factor XIIIa-catalyzed cross-linking was performed using purified fibrinogen, Factor XIIIa, and α -thrombin. Samples equivalent to 4 μg or 40 ng fibrinogen were separated on 8% SDS-PAGE under reducing conditions and the former were stained with Coomassie Brilliant Blue R-250 and the latter were developed by immunoblotting using rabbit anti-human fibrinogen Ab, anti-human γ -chain MAb, and anti-human γ' -chain MAb (C). The bands derived from normal fibrin are indicated as α , β , γ , γ' , γ - γ dimer, and γ' - γ' dimer and the extra bands derived from the patient's fibrinogen are indicated as γ^* .

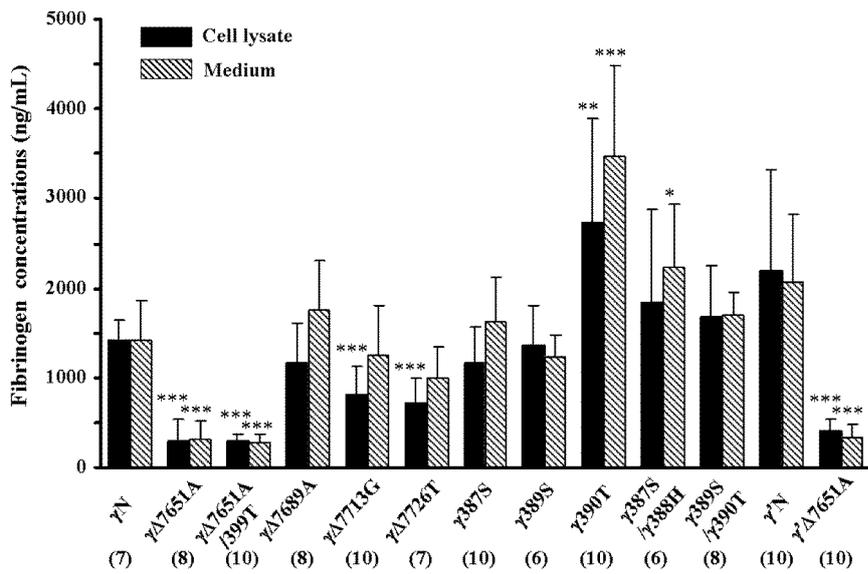
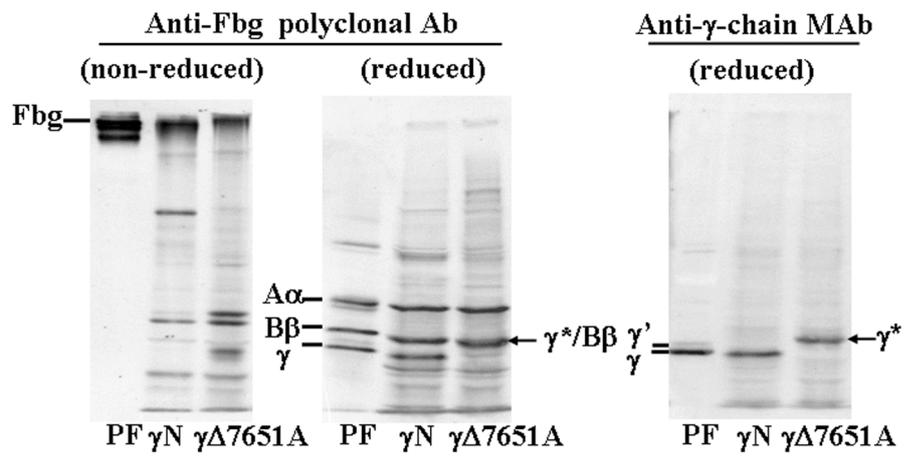


Figure 2

Figure 2. The concentrations of fibrinogen derived from various transflectants. The concentrations of fibrinogen in the cell lysates and the culture media from various transflectants were determined by ELISA as described in Materials and Methods. At least 7 independent clones were tested, and each experiment was performed in triplicate. The numbers in parentheses indicate the clone numbers used. The $\gamma\Delta7651A/399T$ ($\gamma\Delta7651A$ with amino acid substitution of 399Asn by Thr) construct was made to lack a potential canonical glycosylation site at codon 399Asn. Data are expressed as mean \pm SD. *: $p < 0.05$, **: $p < 0.01$, and ***: $p < 0.001$ compared with the normal γ transflectant (γN) or the γ' transflectant ($\gamma'N$) according to Welch's t test.

A

Cell lysates



Media

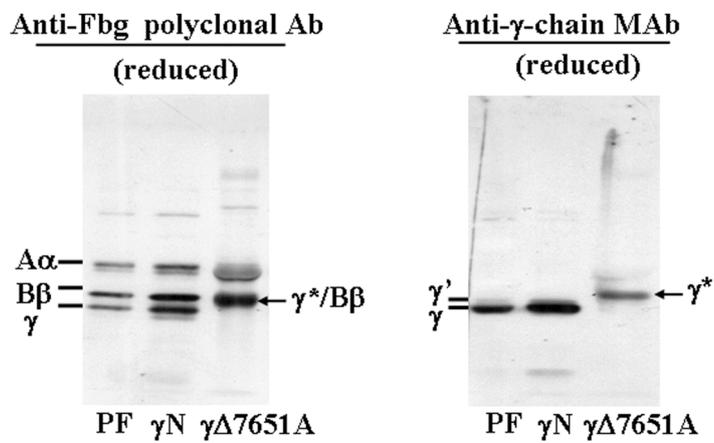


Figure 3A

B

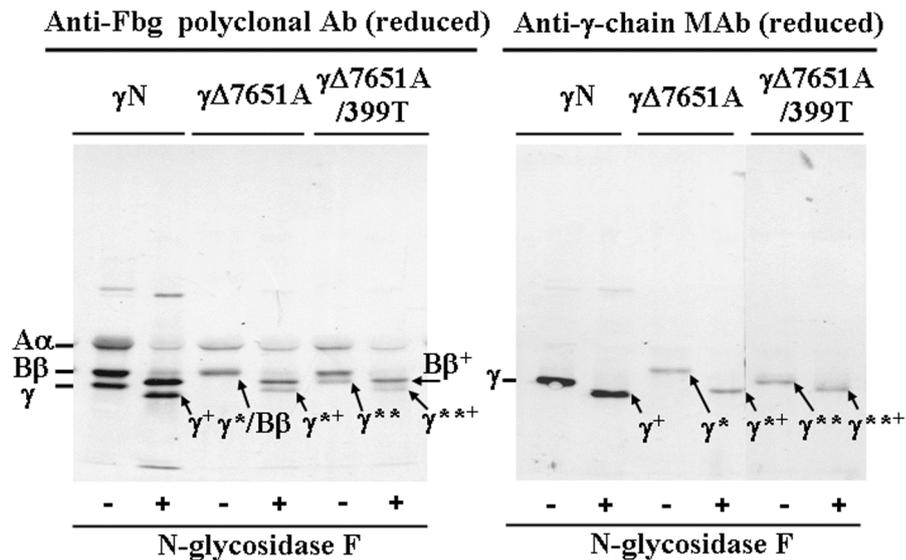


Figure 3B

Figure 3. Immunoblot analysis of the recombinant fibrinogen derived from the transfectants containing $\gamma\Delta 7651A$ and $\gamma\Delta 7651A/399T$. Immunoblot analysis of the recombinant fibrinogen in CHO cell lysates or in culture media transfected with the normal γ -chain (γN), $\gamma\Delta 7651A$, or $\gamma\Delta 7651A/399T$ plasmid. (A) The prepared samples were subjected to 8% SDS-PAGE under non-reducing or reducing conditions. Blots were developed with an anti-Fbg polyclonal Ab or an anti- γ -chain MAb (2G10). (B) The samples were treated with N-glycosidase F as described in Materials and Methods and then analyzed by immunoblot analysis as described above. Lane PF indicates purified plasma fibrinogen. The bands derived

from normal fibrinogen are indicated as $A\alpha$, $B\beta$, and γ ; the variant γ -chains derived from the transfectants are indicated as γ^* ($\gamma\Delta 7651A$) and γ^{**} ($\gamma\Delta 7651A/399T$), and the N-glycosidase F-treated bands are indicated as $B\beta^+$, γ^{*+} , and γ^{**+} .

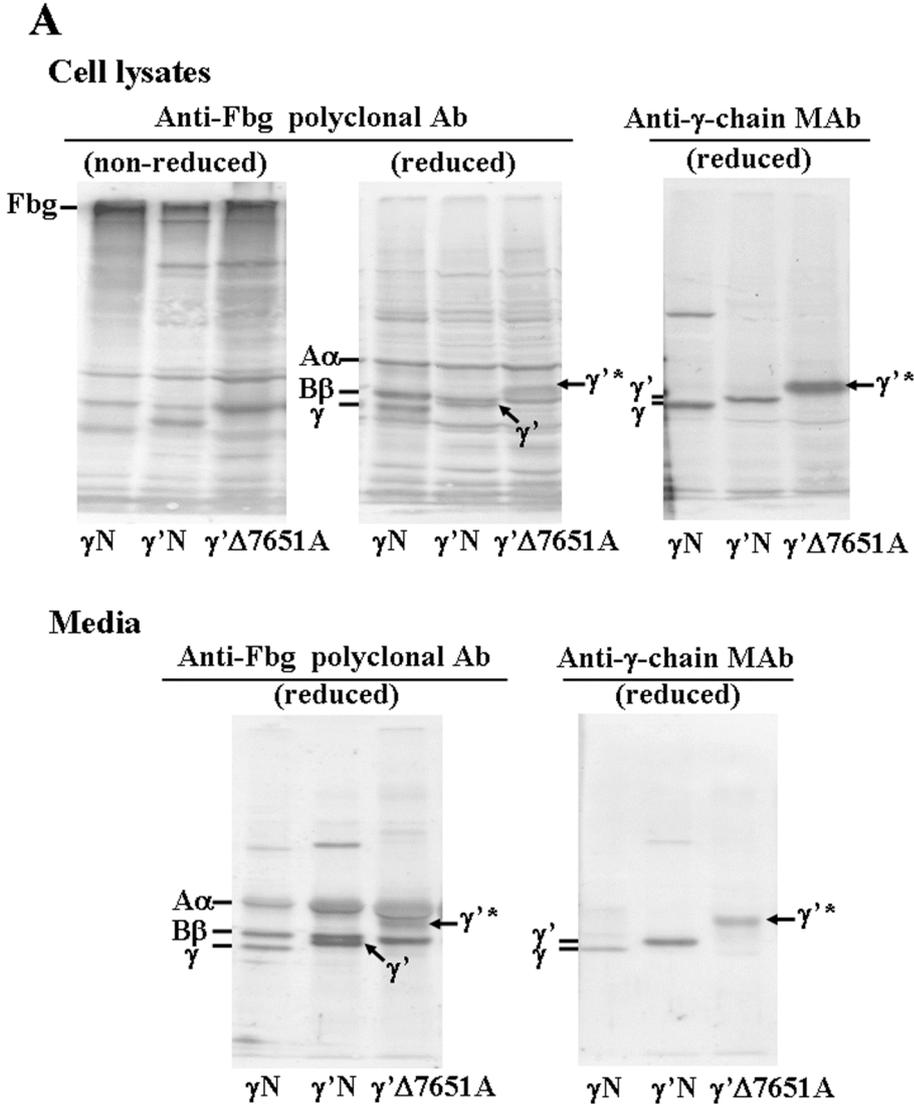


Figure 4A

B

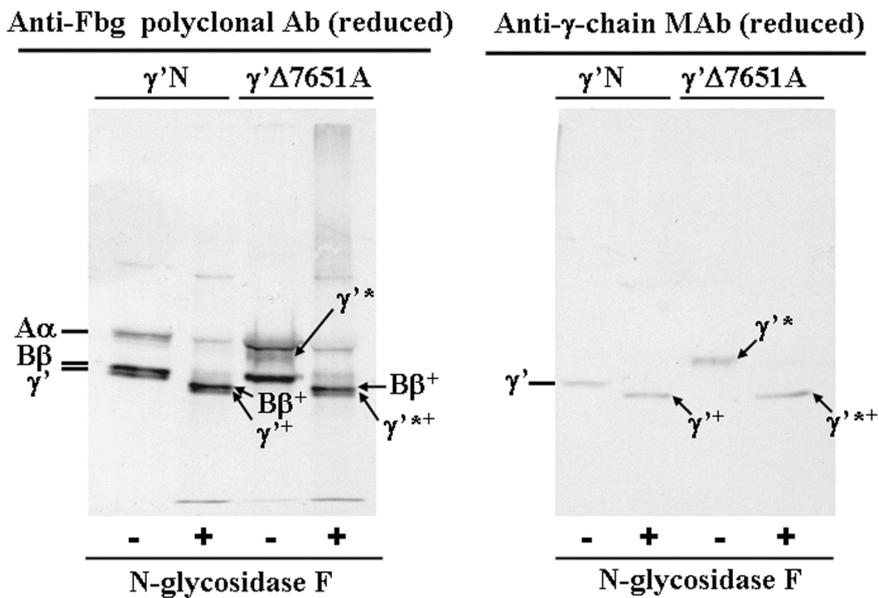
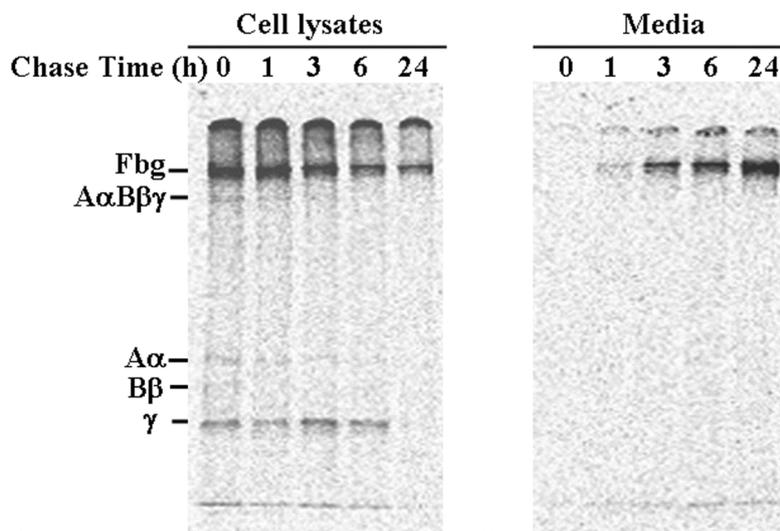


Figure 4B

Figure 4. Immunoblot analysis of the recombinant fibrinogen derived from the transfectants containing γ' Δ 7651A. Immunoblot analysis of the recombinant fibrinogen in CHO cell lysates or in culture media transfected with the normal γ -chain (γ N), normal γ' -chain (γ' N), or γ' Δ 7651A plasmid. (A) The prepared samples were subjected to 8% SDS-PAGE under non-reducing or reducing conditions. Blots were developed with an anti-Fbg polyclonal Ab or an anti- γ -chain MAb (2G10). (B) The samples were treated with N-glycosidase F as described in Materials and Methods and then analyzed by immunoblot analysis as described above. The bands derived from normal fibrinogen are indicated as A α ,

B β , and γ' ; the variant γ -chain derived from the transfectants is indicated as γ'^* , and N-glycosidase F-treated bands are indicated as B β^+ , γ'^+ , and γ'^{**} .

A: γ N-CHO cells



B: $\gamma\Delta 7651A$ -CHO cells

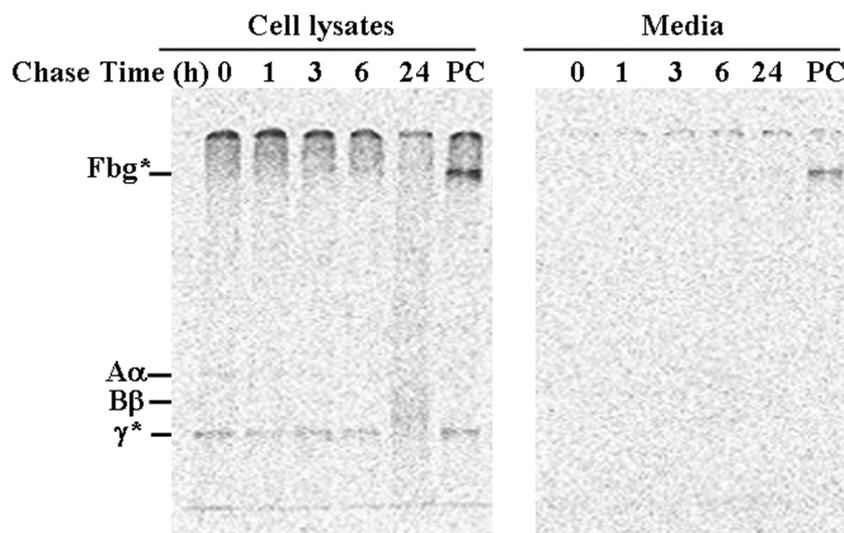


Figure 5. Pulse-chase analysis of the synthesized fibrinogen. Pulse-chase analysis was performed as described in Materials and Methods. The cells were labeled for 1 hour with [35 S]-methionine and chased for the indicated periods with an excess of unlabeled methionine. Immunoprecipitates from the cell lysates or conditioned media were analyzed on 4% to 12% gradient SDS-PAGE under nonreducing conditions, and labeled bands were detected by

autoradiography. Lane PC in variant was conditioned medium at the 24-hour chase of γ N-CHO cells as a positive control. Immunoprecipitate bands derived from γ N-CHO cells are indicated as Fbg, $A\alpha B\beta\gamma$ (intermediate complex), $A\alpha$, $B\beta$, and γ , and those from $\gamma\Delta 7651A$ -CHO cells are indicated as Fbg* (variant fibrinogen) and γ^* (variant γ -chain).