Published in final edited form as:
Thromb Res. 2009 July ; 124(3): 356–363. doi:10.1016/j.thromres.2008.11.016.

The presence of γ' chain impairs fibrin polymerization

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Abstract

Introduction—A fraction of fibrinogen molecules contain an alternatively spliced variant chain called γ'. Plasma levels of this variant have been associated with both myocardial infarction and venous thrombosis. Because clot structure has been associated with cardiovascular risk, we examined the effect of γ' chain on clot structure.

Materials and Methods—We expressed three fibrinogen variants in Chinese hamster ovary (CHO) cells: γ/γ homodimer, γ/γ' heterodimer, and γ'/γ' homodimer. We observed thrombin-catalyzed fibrinopeptide release by HPLC, fibrin polymerization by turbidity, and clot structure by scanning electron microscopy. We characterized post-translational modifications by mass spectrometry.

Results—Fibrinopeptide A was released at the same rate for all three fibrinogens, while fibrinopeptide B was released faster from the γ'/γ' homodimer. The rise in turbidity was slower and final absorbance was lower during polymerization of γ'-containing fibrinogens than for γ/γ fibrinogen. Micrographs showed that γ'/γ' fibrin clots are composed of very thin fibers, while the diameter of γ/γ' fibers is similar to γ/γ fibers. Further, the fiber networks formed from γ'-containing samples were non-uniform. Mass spectrometry showed heterogeneous addition of N-glycans and tyrosine sulfation in the γ' chain.

Conclusions—The presence of γ' chains slows lateral aggregation and alters fibrin structure. We suggest these changes are likely due to charge-charge repulsion, such that polymerization of the γ'/γ' homodimer is more impaired than the heterodimer since these repulsions are partially offset by incorporation of γ chains in the γ/γ' heterodimer.

Keywords

Blood Coagulation; Fibrinogen; γ' Chain; Polymerization

Fibrinogen is a 340,000 Da dimeric glycoprotein with two copies each of three polypeptide chains, Aα, Bβ, and γ. A splice variant of the γ chain mRNA that retains the last intron is

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translated into a polypeptide chain called γ' [1,2]. This alternate chain ends with the 20 residues VRPEHPAETEYDSLYPEDDL, in place of the last four γ chain amino acids, AGDV [3]. About 10% of fibrinogen molecules incorporate the negatively-charged γ' chains, forming γ/γ' heterodimers [4,5]. Recent epidemiological studies have shown that elevated levels of γ' are associated with an increased risk for coronary artery disease, myocardial infarction, and stroke [6-9], while reduced levels have been correlated with deep vein thrombosis and thrombotic microangiopathy [10,11]. Biochemical studies have shown the γ' chain is a high affinity binding site for thrombin [12,13], accounting for the antithrombin activity of a fibrin clot [14] as well as the reported role of the γ' chain in protection from thrombosis [10,11]. The γ' chain may also serve as a binding site for plasma factor XIII, such that FXIII circulates in a complex with fibrinogen [15,16].

The conversion of fibrinogen to a fibrin clot is initiated by thrombin-catalyzed cleavage of two fibrinopeptides, FpA and FpB, to generate fibrin monomers. Fibrin monomers polymerize spontaneously, forming double-stranded protofibrils that assemble into branched fibrin fibers. Three groups have examined fibrinopeptide release and fibrin formation in the presence of the γ' chain, with little consensus in their findings [17-19]. For example, when fibrin clots were examined by scanning electron microscopy, Collet *et al.* showed no difference in morphology between γ'-containing fibrin clots and those with only γ chains [18]. In contrast, both Cooper *et al*. and Siebenlist *et al*. showed clots of γ'-containing fibrin had thinner fibers and more branch points than clots with only γ chains [17,19]. While these discrepancies may be due to differing experimental conditions, it is likely that the source of γ'-containing fibrinogen contributed to the disparate results. Two groups fractionated plasma fibrinogen into a γ chain only sample and a sample with both γ and γ' chains. Because plasma-derived fibrinogen is a complex mixture of molecules due to post-translational modifications and/or genetic differences among individuals, it is reasonable to anticipate that the basis for separating these samples also partitions other fibrinogen characteristics. In addition, plasma proteins, particularly FXIII, may co-purify selectively with the γ or γ' fractions [15,16]. The third group, Collet *et al*., performed experiments using recombinant human fibrinogens that contained only γ or only γ' chains. Thus, studies with the recombinant proteins compared homodimeric molecules, while those with plasma fibrinogen compared γ chain homodimers to γ/γ' heterodimers.

To better determine the role of the γ' chain in polymerization, we synthesized three recombinant fibrinogens: γ/γ homodimer, γ/γ' heterodimer, and γ'/γ' homodimer. Using these proteins, we were able to compare molecules that differed only by the presence or absence of the γ' chain in one or both half-molecules. This is the first study that includes recombinant γ/γ' heterodimer, the main species found *in vivo*. Herein we characterize the thrombin-catalyzed polymerization of these fibrinogens.

Materials and methods

Materials

Chemicals were obtained from Fisher (Hampton, NH) or Sigma-Aldrich (St. Louis, MO) unless otherwise described. Restriction enzymes were purchased from New England Biolabs (Ipswich, MA), and Taq polymerase, T4 DNA ligase, and penicillin/streptomycin from Invitrogen (Carlsbad, CA). Expression vectors, DMEM-F12, Nu serum, calf serum, and aprotinin were previously described [20]. Insulin-transferrin-sodium selenite was from Roche (Indianapolis, IN) or Sigma-Aldrich. Antibodies were: rabbit anti-human fibrinogen and HRP-conjugated goat anti-rabbit IgG from DAKO (Carpinteria, CA); goat anti-human fibrinogen and HRP-conjugated goat anti-human fibrinogen from Cappel (Cochraville, PA); IF-1 calcium-dependent monoclonal anti-fibrinogen from Kamiya Biomedical (Seattle, WA); 4A5 monoclonal anti-γ chain a kind gift from Dr. Gary Matsueda (Bristol Myers Squibb, Princeton,

NJ); 2G2H9 monoclonal anti-γ' chain a kind gift from Dr. David Farrell (Oregon Health and Sciences University) and purchased from Upstate (Charlottesville, VA). Sepharose Q Fast Flow Columns and CNBr-activated Sepharose were purchased from Amersham Biosciences (Piscataway, NJ).

Vector construction

We constructed an expression vector for the γ' chain by replacing a fragment in the γ cDNA expression plasmid [20] with the analogous γ' chain fragment. The plasmid was digested with BstXI and NotI, the products separated on an agarose gel, and the larger fragment purified using a QIAquick Gel Extraction Kit (Qiagen, Valencia, CA). The γ' codons were PCR amplified from human liver cDNA (Stratagene, La Jolla, CA) using the forward primer CAC GCT GGC CAT CTC AAT GGA GTT TA, including a BstXI site (underlined), and reverse primer, AGC ATT TGC GGC CGC CTA CAA ATC ATC CTC A, including a NotI site (underlined). Oligonucleotides were prepared by the UNC-Chapel Hill Pathology Department Oligonucleotide Synthesis Facility. The PCR product (0.05 μg) was digested using BstXI and NotI, purified as described above, and ligated to the expression plasmid fragment (0.05 μg) with T4 DNA ligase. The ligated DNA was transformed into competent DH5α cells. The entire cDNA in candidate clones was sequenced by Taq Dye Terminator Sequencing at the UNC Genome Analysis Facility.

Expression of γ'-containing fibrinogens

CHO cell lines expressing γ'-containing fibrinogens were prepared essentially as described [20]. For the γ'/γ' clone 18 μg of plasmid pMLP-γ' was transfected into CHO cells that expressed the Aα and Bβ chains of fibrinogen; for the γ/γ' clone transfections contained 18 μg of pMLP-γ' and 36 μg of pMLP-γ. Clones were screened by ELISA, measuring the fibrinogen secreted into serum-free medium, and those expressing the highest levels of fibrinogen were subcloned by infinite dilution. In addition to fibrinogen concentration, clones expressing γ/γ' fibrinogen were also screened for the ratio of γ to γ' chains by Western blot of media concentrated with 100 kDa MWCO Centricon Eppendorf concentrators to ≤ 0.01 μg/μL fibrinogen. SDS-PAGE was run under reducing conditions at 60-80V on an 11% gel with a tall stacking gel. Blots were developed using a polyclonal anti-fibrinogen antibody, and the ratio of γ: γ' chains quantified by densitometry using Scion Image (SCIONCorp, Frederick, MD). Clones expressing the highest levels of fibrinogen with near equal ratios of γ and γ' chains were subcloned; this subclone had 63% γ' chains. Thus, the anticipated percentage of heterodimers (2*[γ chains]*[γ' chains]) was 47%.

Fibrinogens were synthesized as described [20], with the following modifications. Microcarrier beads were omitted, cells were grown for six days in serum-free media, and 50 mL of fibrinogen-containing media were collected and replaced each day until cell death about 3 weeks later. Before freezing at -20°C, 0.01% NaN_3 and protease inhibitors (30 μM benzamidine, 6μM EDTA, 0.15mM PMSF) were added. Regular γ/γ homodimer fibrinogen was synthesized from the described cell line [20].

Fibrinogen purification

All fibrinogens, γ/γ homodimers, γ/γ' heterodimers, and γ'/γ' homodimers, were purified from media using an IF-1 calcium-dependent monoclonal anti-fibrinogen immunoaffinity column as described [21]. Eluted fibrinogen was dialyzed into HBS (20 mM HEPES pH 7.4, 150 mM NaCl).

Fibrinogen from the γ/γ' heterodimer clone medium was further purified, to separate the desired heterodimer from the two contaminating homodimers, as all three variants (γ/γ and γ'/γ' homodimers, as well as γ/γ' heterodimers) were produced by the γ/γ' cell line. We prepared

an immunoaffinity column (4 mL) by coupling γ chain-specific monoclonal antibody 4A5 to CNBr-activated Sepharose according to the manufacturer's protocol. The column was equilibrated in 50 mM Tris-HCl pH 8.0, 150 mM NaCl, 1mM $CaCl_2$ (LB), and 2 mg fibrinogen (a mixture of γ/γ, γ/γ', and γ'/γ') diluted to 1 mg/mL in LB was applied to the column at 10 mL/hour. Homodimer γ'/γ' fibrinogen flowed through during loading. The column was washed at 50 mL/hour for 10 minutes with LB. Bound fibrinogens (γ/γ and γ/γ') were eluted with 1M guanidinium-HCl in 50 mM Tris-HCl pH 7.4, 150 mM NaCl (TBS) at 20 mL/hour, and the fractions containing fibrinogen were immediately dialyzed against 1L LB and subsequently 3X 1L of TBS. Dialyzed samples were concentrated to 1-4 mg/mL (Amicon Ultra 100,000 MWCO Centrifugal Filter Devices) and further separated by loading 0.2 - 0.5 mgs onto a 1 mL Sepharose Q Fast Flow ion-exchange column equilibrated in buffer A (39 mM Tris pH 8.5 with H_3PO_4) at 1 mL/minute. Proteins were eluted with a linear gradient of 0 - 100% buffer B (190 mM Tris pH 2.4 with H_3PO_4) over 40 minutes, a procedure analogous to that used to separate γ/γ from γ/γ' fibrinogen in plasma [15]. The fractions with γ/γ' heterodimers, which eluted from our column at 55% buffer B, were collected, extensively dialyzed into HBS, and concentrated.

Fibrinogen concentration was measured spectrophotometrically (A_{280} = 1.51 for 1 mg/mL). The purity and chain composition were analyzed by SDS-PAGE.

Mass spectrometry

Peak 2 fibrinogen from human plasma (Enzyme Research Laboratories, South Bend, IN) was reduced and the γ and γ' chains isolated as a single fraction by HPLC (Shimazdu) essentially as described [13], but using a Supelco Discovery C-18 column and eluting the chains with a linear gradient from 40-60% buffer B, where buffer A was 0.1% TFA and B was 0.1% TFA in acetonitrile. Recombinant fibrinogens, 300 μL of 1 mg/ml in 8 M urea, 0.1 M Tris-HCl, pH 8.0, were reduced with 20 mM dithiothreitol for 4 hrs at 37° C. Acetic acid was added to 10% and 100 μL of sample was fractioned by HPLC as above. In contrast to plasma fibrinogen, only two peaks were resolved. These were collected, analyzed by SDS-PAGE, and found to contain α chains and a mixture of β and γ chains or a mixture of β and γ' chains.

Mass spectrometry was performed by the UNC-Duke Michael Hooker Proteomics Center. Samples containing the mixed chains were lyophilized and dissolved in 10 μL DI water. A one-μL aliquot was diluted with 5 μL of methanol:water:acetic acid, 75:23:2, and 5 μL placed into an ES381 Au/Pd-coated borosilicate nanospray needle (Proxeon Biosystems, Odense, Denmark). Samples were analyzed by nano-electrospray on an AB Q-Star Pulsar QqTOF (Applied Biosystems, Foster City, CA) mass spectrometer operating in the positive ion mode. Spectra were acquired over the mass range 400-2000 Da, scanned every 2 s, with an ionspray voltage of 1000-1200 V. The resulting ion signals were averaged over a maximum of 5 minutes. Calibration was done using the renin substrate MS/MS spectrum, acquired over this same mass range. Data were acquired and processed using AB's BioAnalyst software and deconvoluted to singly-charged species using the maximum entropy (MaxEnt) software provided by the manufacturer.

Fibrinopeptide release

Fibrinopeptide release was performed as described [20] with 0.1 mg/ml fibrinogen in HBS, pH 7.4 with 1.0 mM $CaCl_2$ catalyzed by 0.10 nM (0.01 U/mL) thrombin. Fibrinopeptides were analyzed by HPLC with peak areas compared to synthetic FpA and FpB standards. Data were plotted as % fibrinopeptide released and first order rate constants were determined as described [20].

Fibrin polymerization

Polymerization was monitored by turbidity at 350 nm as described [22], with the following modifications. Reactions, 100 μL, were performed in Corning half-area untreated polystyrene microtiter plates (sample pathlength of 0.56 cm) with 0.15 mg/mL fibrinogen in HBS at three concentrations of calcium, 10 μM, 2.0 mM, or 5.0 mM. The NaCl concentration was adjusted to maintain ionic strength of 0.15. Polymerization was initiated by the addition of 1 nM (0.1 U/mL) thrombin.

Scanning electron microscopy

Clots were prepared in HBS + 2.0 mM $CaCl_2$, by incubating 1.0 mg/mL fibrinogen and 0.10 U/mL thrombin at room temperature for 1 hour. Samples were prepared for microscopy as described, including fixing, dehydration, critical point drying, and sputter coating in gold-palladium [23]. Images were acquired digitally on a Phillips XL20 scanning electron microscope (FEI Company, Hillsboro, OR). Fiber diameters were measured in Image J v.1.37 (Wayne Rasaband, NIH) by using a line to bisect foreground fibers in 10,000X magnification images.

Results

Expression, purification, and characterization of recombinant fibrinogens

To our knowledge, this is the first time in which γ/γ' fibrinogen heterodimers have been expressed by CHO cells and isolated from the contaminating homodimers found in the media. To determine whether fibrinogen function was impaired by 4A5 chromatography, we did control experiments with recombinant γ/γ fibrinogen. We found that polymerization of γ/γ fibrinogen was not altered by this step (data not shown). Purified fibrinogens were characterized by SDS-PAGE under reducing conditions (Figure 1). Comparing the band patterns for the γ'/γ' homodimer to the γ/γ homodimer confirmed that migration of the γ' chain was intermediate between the Bβ and γ chains, as previously shown in plasma fibrinogen [24]. Densitometry showed approximately equal amounts of γ and γ' chains in the purified γ/γ' heterodimer. The SDS-PAGE also showed all three fibrinogens were pure. Each fibrinogen had some Aα chain degradation, with more for the heterodimer relative to either of the homodimers. This level of Aα degradation is common, especially with plasma fibrinogen [21].

Because tyrosine sulfation of the γ' chain is important for thrombin binding [25,26], we examined modification of the recombinant γ' chain by mass spectrometry. As a control, we analyzed plasma fibrinogen peak 2 by electrospray mass spectrometry. We separated chains by HPLC and analyzed a mixture of γ and γ' chains. The mass of the mono-sialylated form of the γ chain was 48,367 Da, which is close to the calculated value of 48,383 Da. The mass of the monosialylated form of the γ' chain was 50,562 Da, which is 164 Da greater than the value calculated for the non-sulfated chain (50,398), indicating both tyrosines are sulfated (80 Da per sulfate group). This finding is consistent with previous reports [13]. We then analyzed both γ/γ and γ'/γ' fibrinogens, separating the reduced chains using the same HPLC procedure as for plasma fibrinogen. With both proteins, the Bβ and γ chains eluted together, as shown by SDS-PAGE (data not shown). The mixtures were analyzed by electrospray mass spectrometry as described in methods. The spectra contained multiple peaks for each chain (Figure 2), consistent with heterogeneous addition of N-glycans as previously reported for human proteins synthesized in CHO cells [27]. For γ fibrinogen the two most abundant peaks were 48,079 Da and 47,916 Da, or 1611 and 1448 Da more than the mass predicted from the amino acid sequence of the γ chain (46,468 Da). These masses are consistent with the biantennary glycans $Gal_1GlaNAc_4Man_3Fuc_1$ and $GlaNAc_4Man_3Fuc_1$, respectively. All the Bβ chain peaks were less abundant than those for γ; the two most abundant were 53,915 Da and 54,079 Da, 1600

Da and 1764 Da higher than the mass calculated from the sequence of the encoded Bβ isoform (52,315 Da). These masses are consistent with the addition of $Gal_2GlaNAc_4Man_3Fuc_1$ and $Gal_1GlaNAc_4Man_3Fuc_1$. For γ' fibrinogen the most abundant peak was that from the Bβ chain with a mass of 54,091 Da, 1776 Da higher than the Bβ chain indicating addition of $Gal_2GlaNAc_4Man_3Fuc_1$. The next peaks of approximately equal abundance were 50,422 Da and 50,340 Da, 1939 Da and 1857 Da higher than the calculated mass of the γ' chain (48,483 Da), indicating addition of $Gal_2GlaNAc_4Man_3Fuc_1$ and 2 or 1 sulfates, respectively. Thus the γ' chains are sulfated, with about half having sulfate on both tyrosine residues.

Fibrinopeptide release

We measured the kinetics of fibrinopeptide release using HPLC to quantify changes in peptide concentration with time. As shown in Figure 3, the rate of thrombin-catalyzed FpA release was similar for all three variants, while FpB release from γ'/γ' fibrinogen was faster than from either γ/γ or γ/γ' fibrinogen. We fit the data to two sequential first-order rate equations to determine the rate constants [20]. FpA release constants were the same for all three variants (γ/γ = 12 +/- 1 × 10^{-3} s^{-1}; γ/γ' = 12 +/- 1 × 10^{-3} s^{-1}; γ'/γ' = 13 +/- 1 × 10^{-3} s^{-1}). FpB release from γ'/γ' fibrinogen was 1.5-fold faster than FpB released from γ/γ or γ/γ' fibrinogen (γ/γ = 7.8 +/- 0.6 × 10^{-3} s^{-1}; γ/γ' = 8.6 +/- 0.5 × 10^{-3} s^{-1}; γ'/γ' = 12 +/- 1 × 10^{-3} s^{-1}; $p < 0.002$).

Fibrin polymerization

We monitored thrombin-catalyzed polymerization by turbidity. From the curves shown in the upper row of Figure 4, we determined the lag time, maximal slope and final absorbance as described [22]; the data are shown in Table 1. Polymerization of both γ' variants clearly differed from γ/γ fibrinogen. The maximal slope and final absorbance were lower for the heterodimer with one copy of the γ' chain than for γ/γ fibrinogen, and in turn, these were lower for γ'/γ' fibrinogen with two copies of the γ' chain than for the heterodimer. These findings suggest the γ' chain slowed lateral aggregation and reduced fiber diameter. In contrast, with one exception, the γ' chain did not influence the lag times (lower row of Figure 4). This exception was γ'/γ' fibrinogen at low calcium concentration, where the lag time was significantly longer. This finding suggests that at the low Ca^{2+} concentration without cations to offset their electrostatic repulsion, the additional negative charges of two γ' chains hindered monomer assembly into protofibrils.

Because calcium affects fibrinogen polymerization, we monitored the variants at three Ca^{2+} concentrations: 10 μM, where calcium is bound only in the high affinity sites, 2.0 mM, which is near physiological, and 5.0 mM, which is similar to that used in previous γ' studies [17, 18]. An increase in calcium from 10 μM to 2 mM increased both the rate and the final absorbance of γ/γ fibrinogen polymerization, but further increasing calcium to 5 mM did not enhance these values. The same trend was found for both γ/γ' and γ'/γ' fibrinogens. With the exception noted above, the changes in calcium did not impact lag times.

Scanning electron microscopy

We prepared clots for microscopy according to the method of Weisel and Nagaswami [23]. Representative low magnification images from each sample are shown in Figure 5A while high magnification images are shown in Figure 5B. We measured fiber diameters from ten 10,000X magnification images. As expected from the turbidity results, γ'/γ' fibrin clots were composed of significantly thinner fibers (average fiber diameter = 0.17 +/- 0.04 μm from 500 fibers measured) than γ/γ fibrin clots (average fiber diameter = 0.25 +/- 0.06 μm from 447 fibers measured; $p < 0.01$). The γ'/γ' clots were also more tightly packed with smaller pores and more branching. In contrast to the uniform structure of the γ/γ clots, the thin γ'/γ' fibers appeared to loosely form into bundles that produced large pores in addition to the smaller pores of the clots (see arrows in Figure 5A). Contrary to our expectations from the turbidity data, the γ/γ' fibrin

clots (average fiber diameter = 0.27 +/- 0.05 μm from 291 fibers measured) did not have thinner fibers than γ/γ fibrin clots; in fact they were slightly thicker ($p < 0.01$). However, turbidity is related to fiber diameter only if the fibers are uniformly distributed in the clot. In this case, the fiber arrangement was decidedly non-uniform. The γ/γ' clots had bundles of fibers and pores like the super-structure of the γ'/γ' clots. The large open spaces in these clots could account for the decrease in turbidity for γ/γ' fibrin compared to γ/γ fibrin. In addition, the γ/γ' clots contained a large number of free fiber ends (see arrows in Figure 5B). Fiber ends are normally found at low levels in scanning electron micrographs of fibrin clots, and have been suggested to arise from early truncation of protofibril formation or lateral aggregation [28,29].

Discussion

Our investigation is the first to study all three possible combinations of γ and γ' chains: γ/γ homodimer, γ/γ' heterodimer, and γ'/γ' homodimer. By using these recombinant fibrinogens rather than the comparable species from plasma, we avoided inter-individual variability, as well as contamination by co-purifying plasma proteins. Importantly, our common method of synthesis allowed comparison of fibrinogens that differed only in the region of interest: the alternative forms of the C-terminus of the γ chain. Although the γ'/γ' homodimer is only found in < 1% of plasma fibrinogen molecules [24], studying it here provided us with a way to isolate the role of the γ' chain in fibrin clot structure.

We have shown by electrospray mass spectrometry that the γ' chain is expressed by CHO cells with the biantennary N-glycan $Gal_2GlaNAc_4Man_3Fuc_1$ and one or two sulfates. Our data show that the γ' chains are sulfated, with about half doubly sulfated. Because tyrosine sulfation is important for thrombin binding and yet binding can still take place even when only one tyrosine is sulfated [25,26], we conclude that our recombinant γ' fibrinogen is a good model for plasma γ' fibrinogen.

Although binding of thrombin to the γ' chain has been described as inhibitory to its activity [14], we found the γ' chain had no effect on fibrinopeptide A release since all three variants displayed identical kinetics for release of FpA. Fibrinopeptide B was released more quickly from γ'/γ' than from either γ/γ or γ/γ' fibrinogens. Previous studies suggest FpB is likely released from E nodule when it is bound to the DD interface in protofibrils [30,31]. As the γ' chain contains a high-affinity thrombin binding site [12], our data suggest that the juxtaposition of two γ' chains at the DD interface in γ'/γ' protofibrils brings a high concentration of thrombin to this locale enhancing FpB release. The binding of thrombin to the γ' chain has previously been suggested as a way to localize thrombin to the fibrin clot as blood continues to flow [32].

Measuring polymerization by turbidity, we found the lag time was not influenced by the γ' chain, except at the lowest concentration of calcium (10 μM). We conclude that the γ' chain does not alter the rate of protofibril formation at physiological calcium concentration. In contrast, we found the maximal slope and final absorbance were reduced in the presence of the γ' chain. When compared to the kinetic model of polymerization developed by Weisel and Nagaswami [23], these differences suggest the γ' chain leads to an increase in the rate of fiber initiation and thus a decrease in lateral aggregation and formation of thinner fibers. This interpretation is consistent with the SEM images of clots prepared from the γ'/γ' homodimer that do, indeed, exhibit thinner fibers compared to the γ/γ homodimer. While the γ/γ' heterodimer turbidity profile suggests a rate of lateral aggregation and final turbidity intermediate between the two homodimers, the micrographs showed heterodimer fiber diameters are of similar width as the γ/γ homodimer. Fibers in the γ/γ' clots were much more loosely and non-uniformly packed, which would account for the decreased turbidity. We propose that the γ' chain impairs lateral aggregation of the γ'/γ' clot by charge-charge repulsion,

but in the heterodimer clot, the interspersing of γ and γ' chains allows fibers of normal width to form. However, periodically, the alignment gets disturbed when multiple γ' ends come together. The proximity of these negatively charged regions caps the fiber and terminates polymerization. A similar model was proposed by Woodhead and colleagues to explain why fibrinogen Caracas II (a mutation that affects the αC domain of 50% of fibrinogen molecules in affected individuals) forms fibrin networks with large and small pores and many free fiber ends [28].

Although our studies contribute novel data, they do not, unfortunately, resolve the inconsistencies reported in previous work. Previous studies of γ'-containing fibrinogens were performed under a variety of conditions and with different sources of fibrinogen (Table 2). Internal inconsistencies make it impossible to reconcile all of the results, even if we were to repeat each of the previous experiments with our recombinant fibrinogen. Our fibrinopeptide release results are not consistent with either of the other two fibrinopeptide release studies, which were also inconsistent with one another. These previous studies examined fibrinogens fractionated from plasma so cleavage of γ'/γ' homodimers was not measured. Thus our finding, the increased rate of FpB release from the γ'/γ' homodimer, is novel. In contrast, our finding of normal rates for both FpA and FpB release from γ/γ' heterodimer conflicts with the published data. Comparing the γ/γ' heterodimer to γ/γ homodimer, Cooper *et al*. found normal FpA and delayed FpB release while Siebenlist *et al*. found FpA and FpB release were both delayed. The differences among the three reports likely reflect experimental conditions as our experiments were completed in the absence of FXIII and with 1 mM $CaCl_2$, Siebenlist's in the presence of FXIII but without added calcium, and Cooper's in the absence of added FXIII or calcium. One can speculate that FXIII, which is also a thrombin substrate, might inhibit release of both fibrinopeptides as was observed by Siebenlist *et al*.; and that the lack of calcium might slow FpB release, as release of this fibrinopeptide depends on the rate of polymerization which is slower at μM concentrations of calcium.

Our clot morphology data also differ from the published studies. We found the heterodimer forms clots with the same fiber diameter as the γ/γ homodimer, while Cooper *et al*. and Siebenlist *et al.* both found these fibers were thinner. Heterogeneities unrelated to the γ' chain that partitioned during purification of fractions from plasma could explain these differences. Another possibility is that the γ' containing fibrinogens prepared from plasma may be contaminated with bound Factor XIII that affects fibrin polymerization. We also found clots of the γ'/γ' homodimer had thinner fibers than those formed from γ/γ homodimer, while Collet *et al*. found fibers in these two clots prepared from recombinant fibrinogens were not different. Although Collet's experiments included FXIII, used a slightly higher calcium concentration than we did (5 mM vs. 2 mM), and a substantially higher thrombin concentration (0.9 U/mL vs 0.1 U/mL), it is unclear how these different conditions account for the discrepancies between these two studies.

In conclusion, these studies with recombinant fibrinogens allowed direct comparison of all three γ' chain variants, the two homodimers and the heterodimer. Our data have shown that the γ' chain *per se* modulates polymerization, in particular it slows lateral aggregation of protofibrils. Moreover, our microscopy data showed the presence of the γ' chain altered clot structure, introducing a non-uniform structure. Because clot structure has been correlated with thrombotic risk, our findings can be related to the potential role of γ' in disease. Individuals with increased γ' chain levels will have increased proportions of both γ/γ' heterodimers and γ'/γ' homodimers. Our findings predict a clot with more γ' chains would be more heterogeneous than one composed of fewer γ'-containing molecules. One could expect not only more areas with thinner fibers and tighter pores, structures associated with increased thrombotic risk, but also more areas with bundled fibers and larger pores, structures associated with reduced risk [33]. Our results are therefore consistent with the apparently conflicting epidemiologic studies

that suggest elevated γ'/γ ratios may be either a risk factor for or protection from thrombotic disease. The heterogeneous structures we have observed may lead to a heterogeneous phenotype. Further *in vivo* studies are necessary to fully understand the contribution of γ' chains to the etiology of thrombotic disease.

Acknowledgements

We thank Lifang Ping and Riley Carter for technical assistance, Mihaela Mocanu and Dr. Carol Parker of the UNC-Duke Michael Hooker Proteomics Center for assistance with mass spec experiments, and Drs. Rustem Litvinov and Irina Chernysh for critically reading the manuscript. This work was supported by NIH grants HL31048 (STL) and HL30954 (JWW).

Abbreviations

CHO, Chinese hamster ovary; FpA, fibrinopeptide A; FpB, fibrinopeptide B; FXIII, factor XIII.

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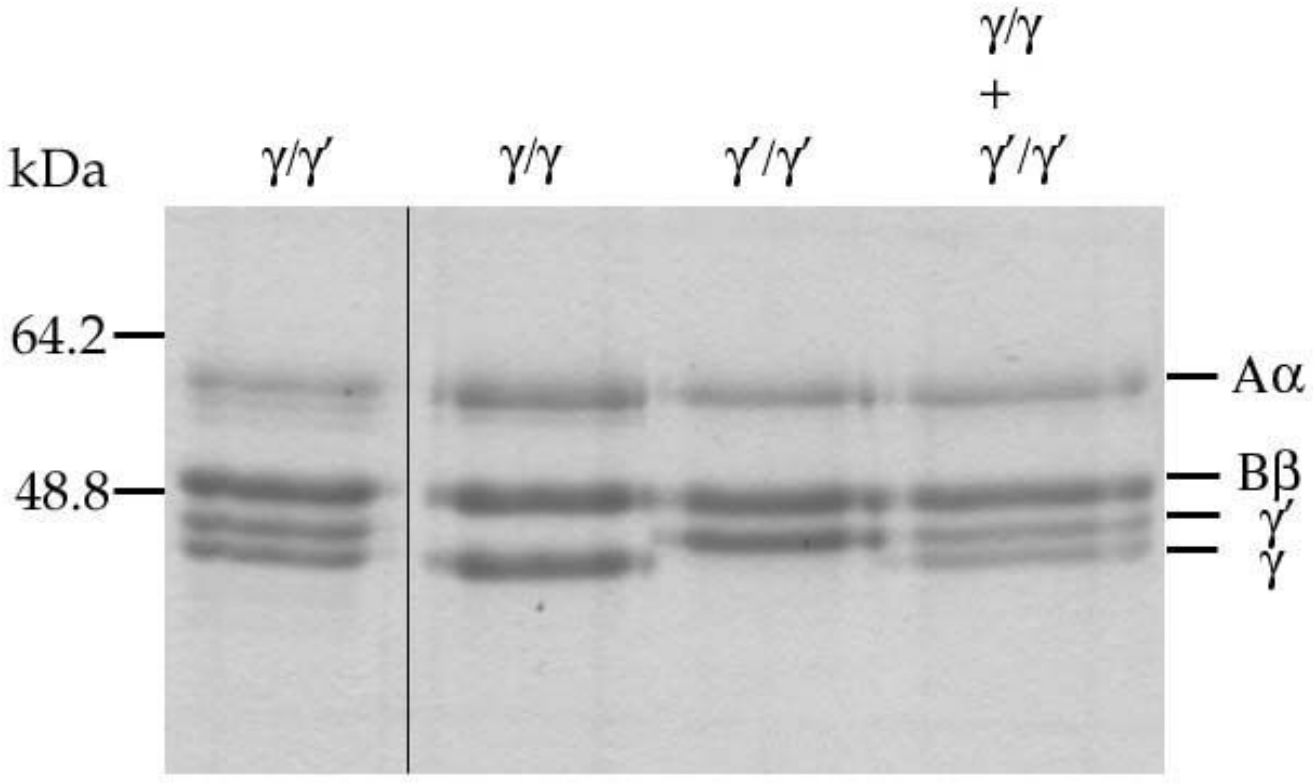

Figure 1. SDS-PAGE of Purified Fibrinogens

Samples (3 μg) of γ/γ', γ/γ, and γ'/γ' fibrinogen and an equimolar (1.5 μg of each) mixture of γ/γ and γ'/γ' fibrinogen were electrophoresed on an 11% SDS-PAGE gel under reducing conditions. Bar shows the excision of empty lanes between samples.

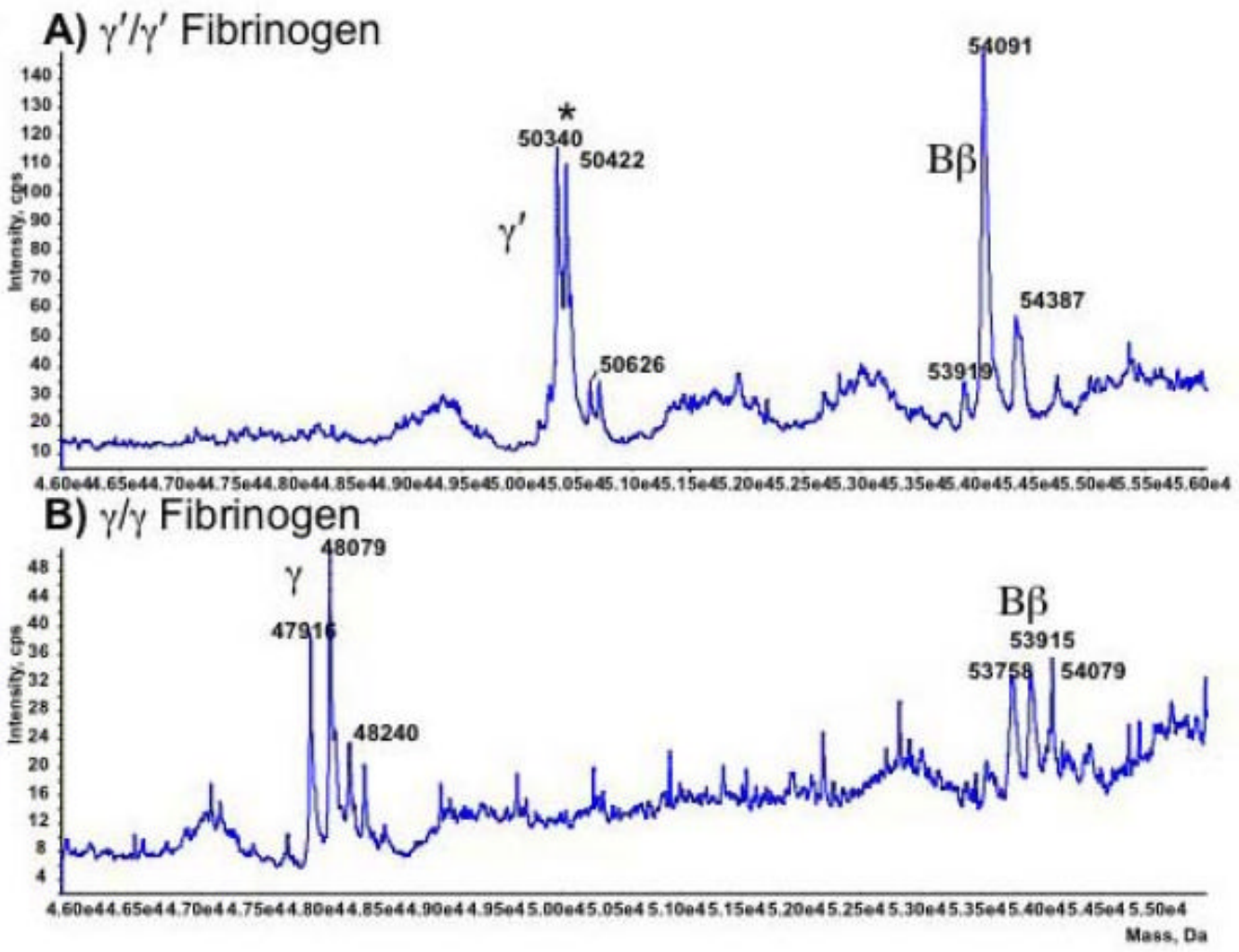

Figure 2. Mass spectra of recombinant fibrinogens

Samples containing a mixture of Bβ and γ chains isolated from γ′/γ′-(panel A) and γ/γ-(panel B) fibrinogens were analyzed by electrospray mass spectrometry. In panel A, the pair of peaks marked by the * are consistent with tyrosine sulfation of one (50,340 Da) or both (50,422 Da) tyrosines in the γ′ chains. The mixture of γ chain peaks in panel B (48,079 Da and 47,916 Da) arises from heterogeneous addition of N-glycans to the chain.

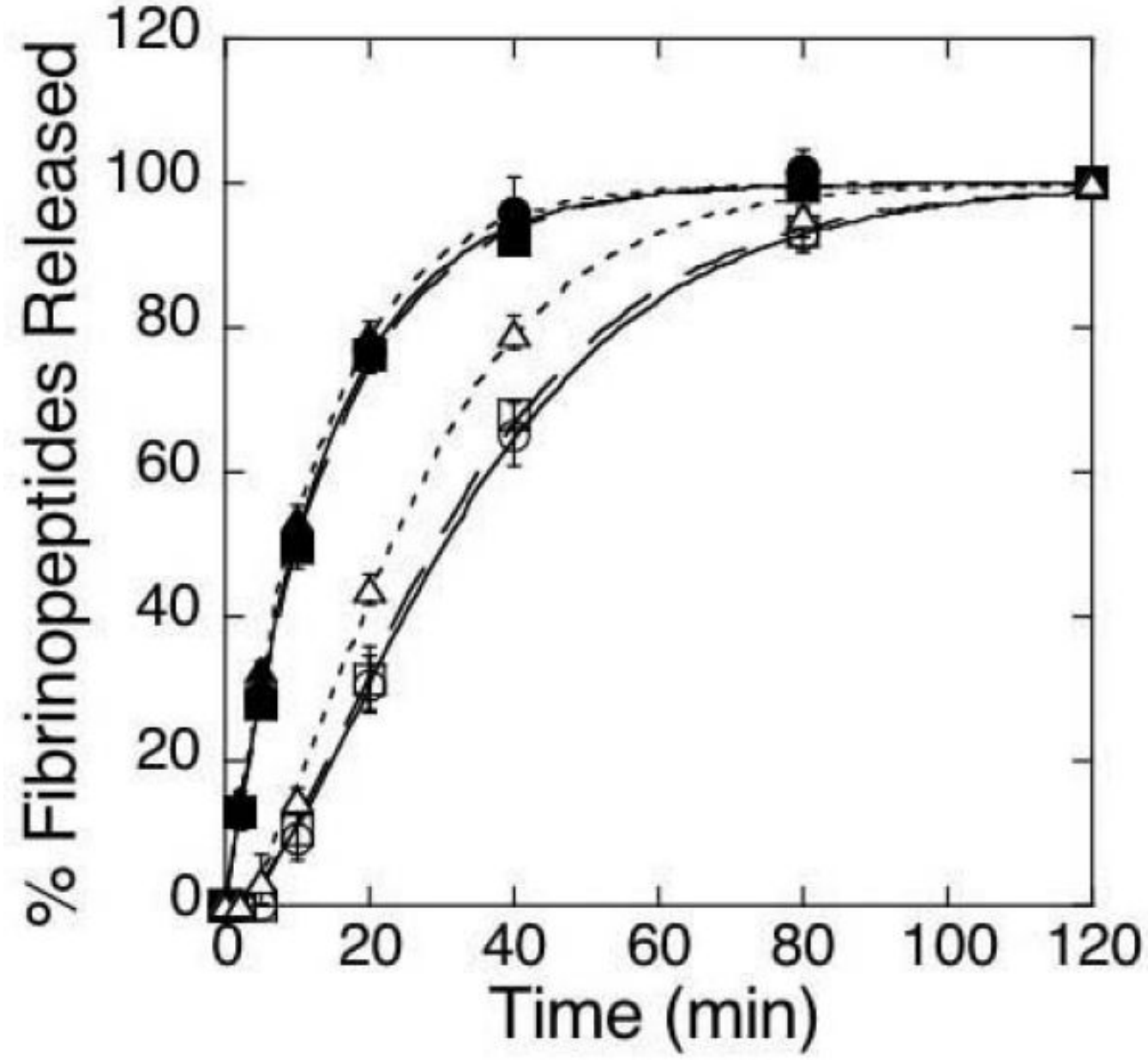

Figure 3. Kinetics of Fibrinopeptide Release

FpA (closed symbols) and FpB (open symbols) release from fibrinogen γ/γ (circles), γ/γ' (squares), and γ'/γ' (triangles). Reactions contained 0.1 nM (0.01 U/mL) thrombin and 0.1 mg/ml fibrinogen in HBS with 1 mM $CaCl_2$, as described in Materials and methods.

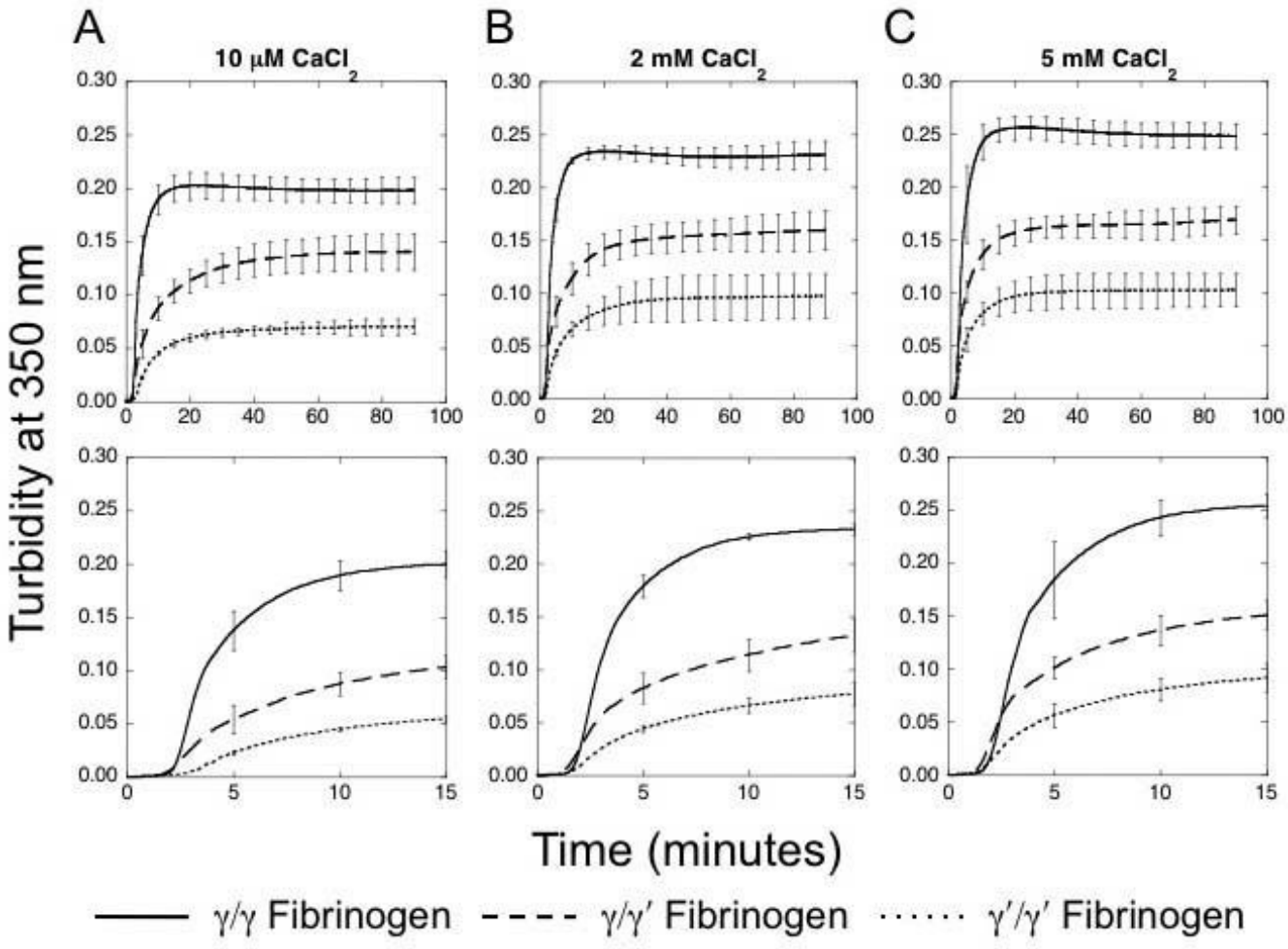

Figure 4. Averaged Polymerization Curves

Polymerization was initiated by addition of 1.0 nM (0.10 U/mL) thrombin to 0.15 mg/mL fibrinogen at three concentrations $CaCl_2$, 10 μM (A), 2.0 mM (B), and 5.0 mM (C). Turbidity was monitored at 350 nm for 90 minutes (upper panels); the first 13 min are shown in greater detail in the lower panels. Curves are the mean of 3 experiments. Error bars are ± 1 standard deviation.

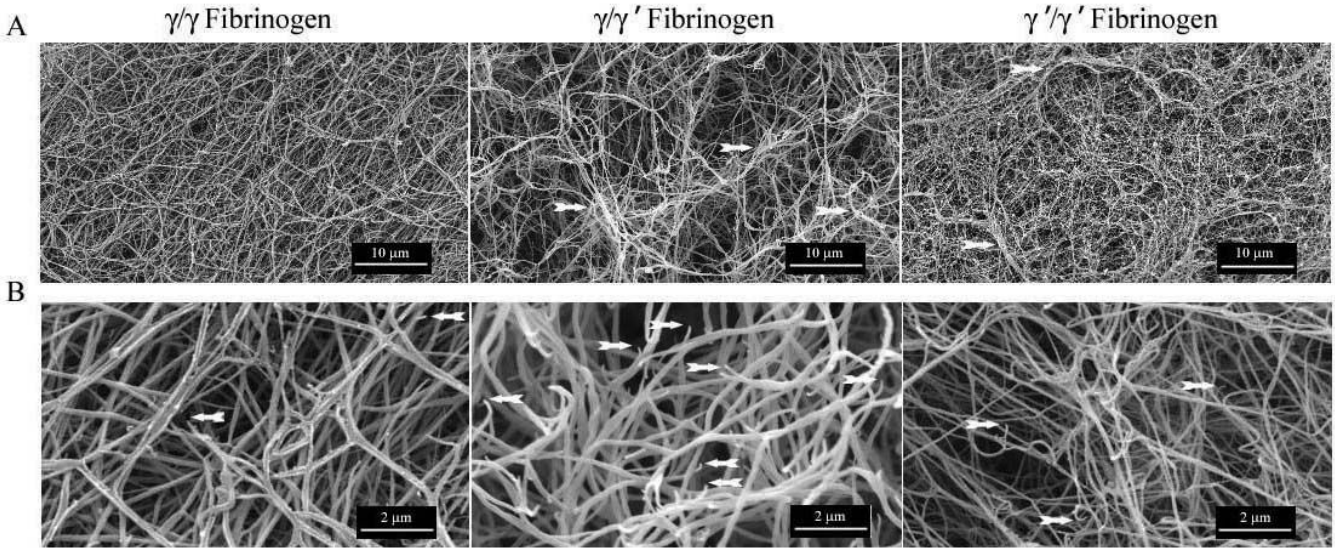

Figure 5. Scanning Electron Micrographs

Clots were prepared from γ/γ homodimer, γ/γ' heterodimer, and γ'/γ' homodimer by addition of 0.10 U/mL thrombin to 1.0 mg/mL fibrinogen in HBS + 2.0 mM $CaCl_2$ (final concentration). Samples were prepared for microscopy as described in Materials and methods. Images were acquired digitally on a Phillips XL20 scanning electron microscope (FEI Company, Hillsboro, OR). In low magnification images (A) the scale bar indicates 10 μm and arrows point to fiber bundles. In high magnification images (B) the scale bar indicates 2 μm and arrows point to free fiber ends.

Table 1

Polymerization of γ/γ, γ/γ ', and γ'/γ' Fibrin

Polymerization was monitored by turbidity at 350 nm for each variant as described in Materials and methods.

	γ/γ	γ/γ'	γ'/γ'
Lag Time, sec			
10 μM $CaCl_2$	117 +/- 12	120 +/- 30	175 +/- 7 *†
2.0 mM $CaCl_2$	100 +/- 20	83 +/- 25	97 +/- 12
5.0 mM $CaCl_2$	103 +/- 25	75 +/- 7	90 +/- 17
Maximal Rate, milliunits/min			
10 μM $CaCl_2$	83 +/- 8 †	26 +/- 6 *†	11 +/- 0.2 *†
2.0 mM $CaCl_2$	108 +/- 7	40 +/- 8 *	19 +/- 4 *
5.0 mM $CaCl_2$	108 +/- 17	50 +/- 0.2 *	25 +/- 9 *
Final OD, 350 nm			
10 μM $CaCl_2$	0.198 +/- 0.012 †	0.140 +/- 0.017 *	0.070 +/- 0.007 *
2.0 mM $CaCl_2$	0.231 +/- 0.014	0.160 +/- 0.019 *	0.097 +/- 0.021 *
5.0 mM $CaCl_2$	0.248 +/- 0.011	0.169 +/- 0.013 *	0.103 +/- 0.015 *

* p values < 0.05 compared to γ/γ fibrinogen.

† p values < 0.05 for 10 μM or 5.0 mM compared to 2.0 mM $CaCl_2$.

Table 2

Influence of γ' chain: data from this and previous studies

The conditions used in and results of this and previous studies are as shown.

Study	*Protein Source*	*FpA Release*	*FpB Release*	*Turbidity*	*Morphology*[*]
Current *Results*	**Recombinant homo- and heterodimers**	**Normal**	**Normal (γ/γ˚F) or enhanced (γ˚F/γ˚F)**	**Slower/ lower**	**Free fiber ends Bundles and pores Thinner fibers (homodimer)**
Conditions		[Fgn] = 0.1 mg/mL [IIa] = 0.01 U/mL [$CaCl_2$] = 1 mM [FXIII] = 0	[Fgn] = 0.1 mg/mL [IIa] = 0.01 U/mL [$CaCl_2$] = 1 mM [FXIII] = 0	[Fgn] = 0.15 mg/mL [IIa] = 0.1 U/mL [$CaCl_2$] = 10 μM, 2 mM, 5 mM [FXIII] = 0	[Fgn] = 1.0 mg/mL [IIa] = 0.1 U/mL [$CaCl_2$] = 2 mM [FXIII] = 0
Cooper 2003[17] *Results*	**Plasma fractionated**	**Normal**	**Slower**	**Slower/lower**	**Thinner fibers More branch points**
Conditions		[Fgn] = 0.77 mg/mL [IIa] = 1 IU/mL [$CaCl_2$] = 0 [FXIII] = 0	[Fgn] = 0.77 mg/mL [IIa] = 1 IU/mL [$CaCl_2$] = 0 [FXIII] = 0	[Fgn] = 1 mg/mL [IIa] = 0.5 IU/mL [$CaCl_2$] = 10 mM [FXIII] = 0	[Fgn] = 0.7 mg/mL [IIa] = 1 IU/mL [$CaCl_2$] = 10 mM [FXIII] = 0
Collet 2004[18] *Results*	**Recombinant homodimers**	**ND**	**ND**	**ND**	**No significant differences**
Conditions					[Fgn] = 1.5 mg/mL [IIa] = 0.9 IU/mL [$CaCl_2$] = 5 mM [FXIII] = 1 IU/mL
Siebenlist 2005[19] *Results*	**Plasma fractionated**	**Slower**	**Slower**	**ND**	**Thinner fibers More branch points**
Conditions		[Fgn] = 3 mg/mL [IIa] = 0.5 U/mL [$CaCl_2$] = 0 [FXIII] = 1 mg/mL[o]	[Fgn] = 3 mg/mL [IIa] = 0.5 U/mL [$CaCl_2$] = 0 [FXIII] = 1 mg/mL[*]		[Fgn] = 0.18 mg/mL [IIa] = 0.1 U/mL [$CaCl_2$] = 0 [FXIII] = 0

ND: Not determined

[*] Measured by SEM

[o] According to methods of this paper [19], FXIII preparations are between 2100 - 2300 Loewy units/mg, so Fp release experiments use 2100 - 2300 Loewy units/mL = 19 - 21 Units/mL.