Recombinant γ′ Fibrinogen and its Interaction with Factor XIII

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ABSTRACT

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(Under the direction of Susan T. Lord)

Because the γ′ chain of fibrinogen has been hypothesized as a binding site for factor XIII zymogen, I prepared two recombinant fibrinogen variants, a γ′/γ′ fibrinogen homodimer and a γ/γ′ fibrinogen heterodimer. Characterization of their expression in CHO cells revealed the following: 1) γ′ chain protein is produced in about the same proportion as γ′ mRNA in any given cell line. 2) γ and γ′ chains assemble into homodimeric fibrinogen molecules as readily as they do heterodimers. 3) Mass spectrometry studies indicate that one of two tyrosine residues in the recombinant γ′ chain is sulfated.

I next characterized the polymerization of purified recombinant γ′/γ′ and γ/γ′ fibrinogen. As measured by turbidity, the presence of the γ′ chain leads to decreased lateral aggregation at all calcium concentrations studied. I monitored fibrinopeptide release by HPLC and found that release of fibrinopeptide A occurs at the same rate regardless of the presence of a γ′ chain, but fibrinopeptide B release is faster from γ′/γ′ than γ/γ or γ/γ′ fibrinogen. Factor XIIIa-catalyzed cross-linking of the variants starts at a slower rate for fibrinogens with a γ′ chain than it does for those without.

I then used the γ′/γ′ and γ/γ′ fibrinogen variants in an ELISA assay to measure factor XIII binding to fibrinogen and fibrin. I studied binding of factor XIII zymogen, recombinant factor XIII composed of just A subunits, and active-site-inhibited, activated recombinant factor XIIIa. No matter what form the factor XIII is in, or whether fibrinogen or fibrin is used, these experiments clearly showed that the γ′ chain has no role in factor XIII binding to fibrinogen.

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CHAPTER ONE

INTRODUCTION

Upon blood vessel injury, endothelium ruptures and exposes the membrane protein tissue factor to blood from within the vessel. Tissue factor forms a complex with factor VIIa that catalyzes the activation of factor IX into IXa and factor X into Xa [1]. On the surface of the tissue-factor bearing cell, Xa participates with Va in formation of a prothrombinase complex to convert prothrombin to thrombin on a small scale. This thrombin then converts XI to XIa, dissociates the VIII/von Willebrand factor complex to activate VIIIa, converts V to Va, and activates platelets [2].

IXa that has been activated by the TF:VIIa complex dissociates from the surface of the tissue-factor bearing cell, travels through the blood, and binds to negatively-charged phospholipids on the surface of the activated platelet. It then forms the tenase complex with VIIIa to convert larger quantities of X to Xa on the platelet surface. In turn, Xa complexes with Va to form the prothrombinase complex that generates large quantities of active thrombin on the platelet surface [2]. This thrombin then converts fibrinogen to fibrin to form a clot; it also converts factor XIII to active factor XIIIa. Factor XIIIa covalently cross-links fibrin fibers to mechanically stabilize the clot. It is these last two steps of the coagulation pathway, fibrin formation and cross-linking by factor XIIIa that are the subject of my studies.

Fibrinogen

The fibrin clot's precursor is the protein fibrinogen, a 340 kDa glycoprotein. Human plasma fibrinogen is synthesized in the liver at a rate of 1.7 – 5.0 g per day and circulates at a concentration of approximately 2.5 mg/mL [3]. Fibrinogen is composed of two copies of each of three polypeptide chains, termed the Aα chain (66.5 kDa), the Bβ chain (52 kDa), and the γ chain (46.5 kDa). Alternative splicing of the γ chain mRNA leads to an extended γ

chain termed the γ′ chain, present in about 10% of plasma fibrinogen molecules. This chain has a molecular weight of 51.5 kDa due to the replacement of the last four amino acids of the γ chain (Ala – Gly – Asp – Val) with a negatively charged 20 amino acid residue sequence [4]. This replacement results from translation of the last intron up to a stop codon, eliminating translation of the final exon [5]. Due to the high density of negatively charged residues on the γ′ chain, this polypeptide chain has significantly different biochemical characteristics than does the normally spliced γ chain.

An early observation of fibrinogen by electron microscopy suggested a tridodular model [6]. A more complete picture of the molecule's three-dimensional structure was determined by a combination of electron microscopy and low resolution X-ray crystallography [7]. Fibrinogen has a rod-like structure with the N-termini of all six chains held together by disulfide bonds in the center of the molecule in a region known as the E region. The chains extend out from this region as half-molecules, first forming two coiled-coils. At the end of each coiled-coil the C-termini of the more mobile Aα chains double back onto the coiled-coil. The C-termini of the Bβ and γ chains, however, each form individual globular domains that compose the D regions at each end of the molecule [7].

Later, low resolution structures were determined by X-ray crystallography for bovine and chicken fibrinogen [8], [9]. Crystallization of the entire human fibrinogen molecule is difficult due to the mobility of the C-termini of the Aα chains (termed the αC regions). However, crystal structures of D fragments isolated from both plasma and recombinant fibrinogen have recently been published [10], [11].

Conversion of fibrinogen into fibrin occurs when thrombin cleaves fibrinopeptides from the N-termini of the Aα and Bβ chains of fibrinogen to expose polymerization sites A

and B, respectively. Once exposed, these polymerization sites are capable of interaction with the constitutively available a and b polymerization sites in the D region of another fibrin(ogen) molecule. These results were determined by affinity chromatography of fibrin(ogen) fragments over a column composed of fibrinogen and fibrin monomers coupled to sepharose [12]. The a site is a pocket located at the C-terminus of the γ chain while the b site pocket is located at the C-terminus of the (B)β chain [13],[14]. A-a interactions between fibrin monomers lead to formation of a double-stranded, half-staggered protofibril, as deomonstrated by electron micrographs of fibrin trimers [15]. Further studies with high resolution electron micrographs demonstrate that protofibrils exist as twisted structures [16]. Light scattering experiments performed to study the kinetics of clot formation suggest that once protofibrils have grown to an appropriate length they laterally aggregate. Lateral aggregation requires preformed protofibrils because the individual interactions involved are weak on their own, but become significant as protofibrils become longer. During this process, longer protofibrils branch out as well as laterally aggregate with two protofibrils, and the network of fibers that composes a clot will form [17]. The results of the light scattering experiments from which this model was proposed were confirmed by electron microscopy [18]. Finally, the noncovalent clot is stabilized by factor XIIIa-catalyzed covalent cross-linking [19].

The process of clot formation follows strict kinetics. HPLC was used to measure the release of fibrinopeptides from bovine fibrinogen. This study showed that fibrinopeptide A (FpA) is released first while fibrinopeptide B (FpB) release is very slow at the start of the reaction, but speeds up after FpA release is complete [20]. Experiments with mutant fibrinogen Detroit, from which FpA is not cleaved, confirm that cleavage of FpA is required

for efficient FpB cleavage [21]. Fibrinopeptides were released at an equal rate from a recombinant fibrinogen in which the FpB sequence was replaced by sequence for FpA, showing that the difference in kinetics between FpA and FpB release is due to thrombin's specificity for the sequence of the fibrinopeptide [22]. Conversion of fibrinogen to fibrin at neutral pH leads to dissociation of the αC domains from the center part of the molecule, as demonstrated by electron microscopy comparisons of fibrinogen and fibrin monomer. Other experiments in this same study showed the interaction of αC fragments with one another, suggesting the possibility that they may be involved with intermolecular interactions *in vivo* [23]. These domains are hypothesized to be involved with lateral aggregation, according to one model of fibrin polymerization. Electron microscopy and turbidity studies of fibrinogen fragment X, from which the αC domains are removed, form clots with altered ability to laterally aggregate [24].

Factor XIII

Factor XIIIa catalyzes the formation of N^{ε}(γ-glutamyl)lysine cross-links between two γ chains, multiple α chains, or a γ and an α chain of neighboring fibrin molecules within the clot. The plasma-derived form of this enzyme is composed of two A and two B subunits [25]. Plasma factor XIII has a molecular weight of 325.8 kDa; the A subunit weighs 83.2 kDa while the B subunit weighs 79.7 kDa. These subunits noncovalently associate into a tetramer [26]. A combination of ELISA assays showed that the plasma concentration of B subunits was 0.26 - 0.28 μmol/L while the concentration of A subunits was 0.13 – 0.16 μmol/L. These ELISA assays also indicated that all of the A subunits in plasma were complexed with B subunits while half of the B subunits existed free in solution [27]. In

addition to the plasma form of the enzyme, platelet factor XIII exists as an A_2 dimer with no B subunits. These two forms of the enzyme have an equal share of total factor XIII activity in blood [28].

Factor XIII's active site is located within the A subunit. The crystal structure of this subunit has been solved and shows the presence of a beta sandwich domain, the catalytic core, and two beta barrel domains [29]. The active site holds a catalytic triad of Cys314-His373-Asp396. Substrate access to this catalytic core is blocked by a 37-residue activation peptide located at the N-terminus of the A-chain. The two A subunits of the molecule associate as a dimer with the activation peptide from one subunit blocking access to the other subunit's active site [29].

Unlike the A subunits of factor XIII, factor XIII B subunits have not been crystallized so much less is known regarding their structure. While the hydrophobic A subunit is carbohydrate-free, the B subunit is highly glycosylated [25]. B subunits act as regulatory and carrier subunits for the A subunits as they travel through the plasma [30].

Activation of plasma factor XIII is a multistep process. This process is initiated by thrombin-catalyzed cleavage of the activation peptide [31]. Even though there are two active sites present per factor XIII molecule, only one activation peptide must be cleaved for full factor XIIIa activity, as demonstrated by the incorporation of only one equivalent of iodoacetamide into the active site of each molecule [32]. Crystallographic evidence suggests that after cleavage this peptide may not immediately dissociate from its native position and conformation because the activation peptide is still in place in the crystal structure of thrombin-cleaved factor XIII A_2 dimer [33]. The next step in activation involves dissociation of the carrier B subunits from the A_2 dimer, and the process of factor XIIIa generation is

completed when the A_2 dimer presents the active site cysteine residue, presumably through a conformational change [34]. Because the thrombin-cleaved form of the molecule did not crystallize in the active conformation, the exact structural changes necessary to produce this state are still a matter of speculation [33]. However, the crystal structure of the inactive molecule suggests that in the presence of calcium, one or both of the beta barrel domains would move to provide access to the active site [29].

This activation process requires two cofactors. The first of these cofactors is calcium. Calcium is required to be present for conversion of proteolytically cleaved factor XIII (FXIII′) to active factor XIII (FXIIIa). The concentration of calcium required for 50% conversion at physiological pH and ionic strength was determined to be 25 m*M* by [^{14}C]-iodoacetamide incorporation [34]. It has been suggested that *in vivo*, calcium acts as an allosteric effector to cause dissociation of the B subunits and/or the conformational change in the A_2 dimer that exposes the active site [35].

The other important cofactor in thrombin-catalyzed factor XIII activation is fibrin(ogen). Fibrinogen has been shown to lower to physiological levels the concentration of calcium required for factor XIIIa generation, as determined by incorporation of iodoacetamide into the active site of the molecule [36]. The formation of a complex between thrombin, factor XIII A_2B_2, and des A,B-fibrin led to a 100-fold increase in the factor XIIIa activity in a [^{3}H]-putrescine incorporation assay compared to thrombin and factor XIII alone. Factor XIIIa generation from platelet factor XIII that lacked B subunits required 50% less thrombin than did factor XIII zymogen, but this thrombin requirement moved to the level of plasma factor XIII when platelet factor XIII was incubated with pure B subunits. Addition of fibrin to the mixture of platelet A_2B_2 factor XIII and thrombin produced factor XIIIa activity

equal to A_2 platelet factor XIII and thrombin alone. These results suggest that fibrin can remove the inhibitory effect of B subunits from factor XIIIa generation [37]. Kinetic HPLC studies of fibrinopeptide and activation peptide release showed that inclusion of fibrinogen with thrombin and factor XIII provided an approximately 35-fold increase in the rate of activation peptide release [38]. Fibrin-catalyzed promotion of activity keeps factor XIII from becoming active before substrate is available. The cofactor role of fibrin serves to regulate factor XIIIa activity to just those times when it can be useful [39]. Figure 1.1 shows the steps of factor XIII activation and necessary cofactors.

Once activated, factor XIII is ready to catalyze a transglutaminase reaction. The active site cysteine of factor XIII forms a thioester bond with a glutamine side chain located on the protein substrate (generally fibrin) [30]. Next the enzyme-substrate complex binds to the side chain of a lysine residue on another protein molecule, as shown by the inhibition of cross-linking by a synthetic lysine methyl ester [40]. A rearrangement quickly takes place liberating the enzyme and creating an isopeptide bond between the two proteins [41]. See Figure 1.2.

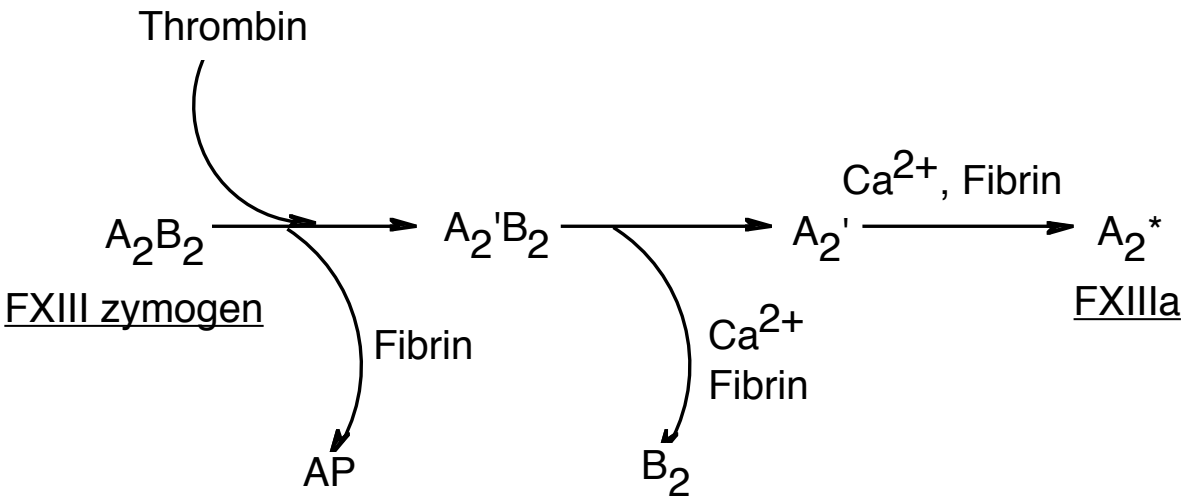

Figure 1.1. Activation of factor XIII is a three-step process that requires thrombin for initiation as well as fibrin(ogen) and calcium as cofactors.

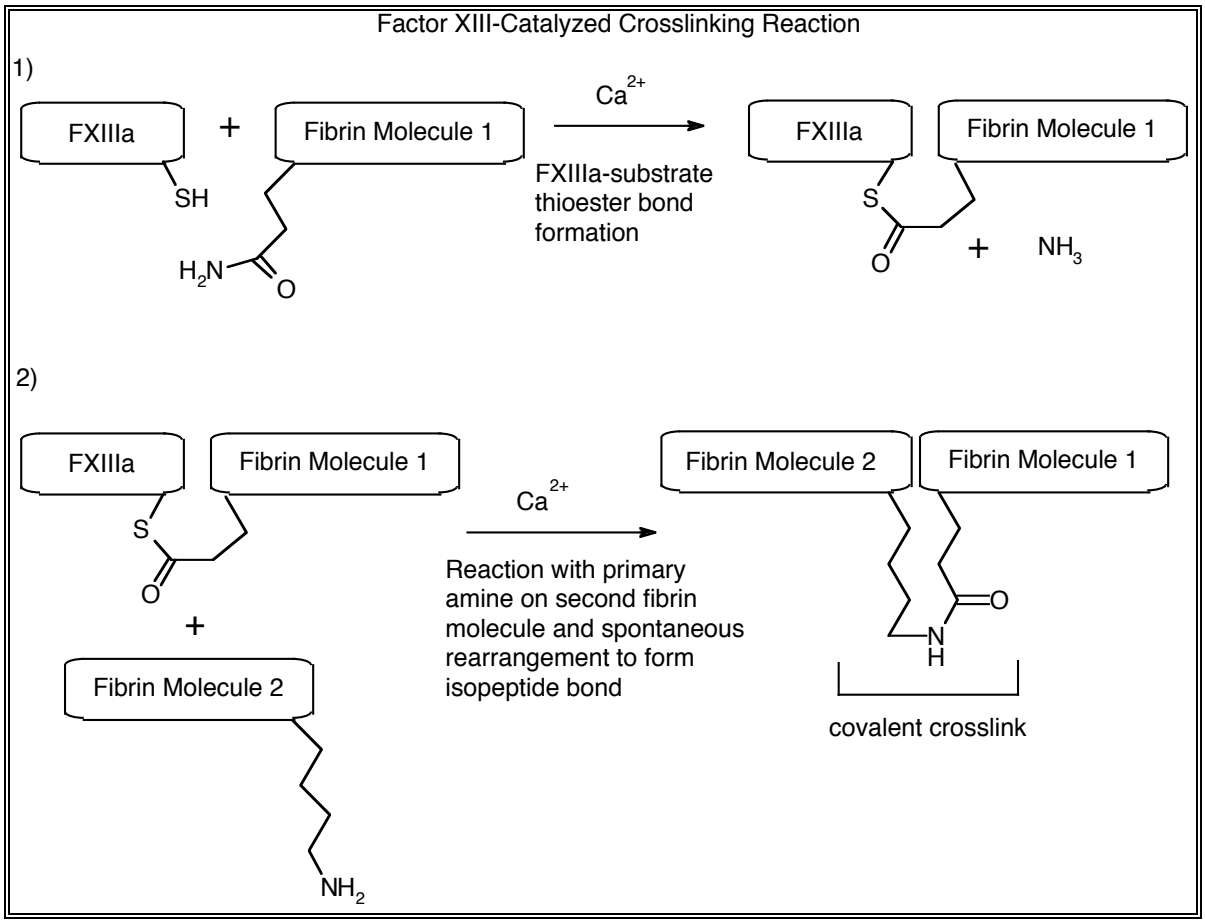

Figure 1.2. Transglutaminase reaction catalyzed by factor XIIIa. In this case, two molecules of fibrin are being cross-linked.

During fibrin clot formation, the transglutaminase reaction first takes place between γ chains of fibrin. Between each set of two aligned fibrin molecules, two cross-links can form. The glutamine acceptors are Gln 398 or Gln 399; the lysine donor on each molecule is Lys 406. Two cross-links can form between each pair of D regions; from the lysine on each molecule to either of the glutamines on the other [42], [43]. The orientation of these cross-links within the protofibril has been a subject of recent debate. Some put forth an argument that cross-links are situated transversely between fibrin molecules on two adjacent fibrin molecules [44]. A more widely accepted model based on structural evidence obtained from crystallography and electron microscopy suggests that these cross-links are formed longitudinally between fibrin molecules aligned end-to-end [45].

After γ-γ dimer formation has begun to occur, cross-links between α chains of fibrin start to form. Four glutamine acceptors are available on the α chain for cross-link formation (Gln 221, Gln 237, Gln 328, and Gln 366) along with a host of lysine donors. Unlike with γ chains, this availability leads to the formation of cross-linked α polymers, rather than just dimers [46], [47], [48], [49].

In addition to the γ and α chains of fibrin, factor XIII is responsible for cross-linking many other proteins available in the blood. It cross-links α2-antiplasmin to the α chain of fibrin to regulate plasmin-catalyzed fibrinolysis [50]. Another substrate for factor XIII is fibronectin which is cross-linked to fibrin during the process of wound healing. Once attached to fibrin, fibronectin is further cross-linked to collagen to assist in clot attachment to the blood vessel wall [51].

Factor XIII has also been shown through both *in vitro* and *in vivo* studies to play a role in up-regulating angiogenesis [52]. Factor XIII binds to the surface of an endothelial

cell and cross-links the integrin $\alpha_V\beta_3$ to vascular endothelial growth factor A (VEGF-A). This interaction then leads to a signaling cascade which eventually triggers cell proliferation and migration resulting in angiogenesis [53].

Fibrinogen and Factor XIII

Early efforts to purify factor XIII from plasma were complicated by the fact that factor XIII coprecipitates with fibrinogen and can only be separated by heat denaturation of the fibrinogen [54]. Greenberg and Shuman mixed radiolabeled factor XIII with fibrinogen immobilized on beads and discovered that plasma factor XIII does, indeed, bind to fibrinogen with a binding constant of 1.4 +/- 0.06 x $10^{-8}M$. Addition of unlabeled platelet factor XIII to the ^{125}I-plasma factor XIII / fibrinogen bead mixture could completely inhibit ^{125}I-plasma factor XIII binding to fibrinogen, just as could unlabeled plasma factor XIII. From this result, Greenberg and Shuman concluded that the fibrinogen binding site on factor XIII is located on the A subunits [55]. In contrast, Hornyak and Shafer showed that radiolabeled factor XIII zymogen did not disrupt binding of radiolabeled, active-site blocked factor XIIIa to fibrin. In their system, fibrinogen and blocked factor XIIIa were added to acid-solubilized fibrin that was then allowed to clot and spun down. From these experiments, they concluded that the factor XIII zymogen / fibrinogen interaction is mediated through the B subunits [56].

In 1996, Siebenlist and coworkers suspected that the γ′ chain of fibrinogen may play a role in binding to factor XIII zymogen. They separated human plasma fibrinogen into a γ/γ fraction and a γ/γ′ fraction by ion-exchange chromatography. Because they had previously observed factor XIII activity in the γ/γ′ fraction, they performed an ammonium sulfate precipitation on this fraction only. γ/γ′ Fibrinogen containing factor XIII was precipitated,

while γ/γ′ fibrinogen without factor XIII remained soluble. To perform their experiments, Siebenlist *et al*. mixed either γ/γ or γ/γ′ fibrinogen with factor XIII and analyzed binding by ion-exchange and size-exclusion chromatography. When mixed with γ/γ′ fibrinogen and subjected to ion-exchange chromatography, factor XIII had a different elution position; this effect was not observed with γ/γ fibrinogen. Size-exclusion chromatography also showed that factor XIII co-eluted with γ/γ′ fibrinogen, but not with γ/γ fibrinogen. From these experiments, they concluded that the γ′ chain provides a binding site for factor XIII zymogen [57].

Localization of factor XIII on the γ′ chain of fibrinogen is supported by the observed resistance to lysis of fibrin clots composed of γ/γ′ fibrinogen compared with γ/γ clots. Falls and Farrell purified γ/γ and γ/γ′ fibrinogen from plasma fibrinogen using much the same method as described above for Siebenlist, though notably, they omitted the ammonium sulfate fractionation to remove bound factor XIII. They measured clot lysis by combining γ/γ or γ/γ′ fibrinogen with thrombin, tPA, and plasminogen in a microtiter plate and monitoring the formation and dissolution of the clot. They discovered that γ/γ and γ/γ′ fibrin lyse at the same rate, but when factor XIII is added to the reaction, γ/γ′ fibrin lyses more slowly than γ/γ and results in more extensively cross-linked D-dimer lysis products. They hypothesized that this difference in lysis is due to γ/γ′ fibrinogen binding factor XIII and localizing it to the clot [58].

Moaddel and coworkers extended the studies described above by measuring the strength of the factor XIII zymogen / fibrinogen interaction by sedimentation equilibrium analytical ultracentrifugation. The affinity of factor XIII for γ/γ′ fibrinogen was 20 times stronger than factor XIII's affinity for γ/γ fibrinogen. The stoichiometry of the complexes

formed in the analytical ultracentrifuge was determined to be two fibrinogen molecules and one molecule of factor XIII. One attractive possibility for how this complex forms may be envisioned as shown in Figure 1.3 [59].

Activation of factor XIII leads to dissociation of the B subunits and therefore the loss of this proposed B:γ′ interaction. Thus, active A subunits must bind elsewhere. Procyk *et al*. examined the binding of active factor XIII to fibrinogen using recombinant factor XIII A subunits [60]. The recombinant A_2 was radiolabeled with ^{125}I, activated, and added to small columns containing fibrin clots. The column was washed with buffer, and the total amount of radioactivity released from the clot was measured to determine the amount of factor XIII A_2 bound to the clot. Binding could be blocked by an antibody to the C-terminus of the Aα chain, suggesting that the binding site for factor XIIIa is located somewhere between residues 389-402. Unactivated recombinant factor XIII (A_2) did not bind significantly to fibrin [60].

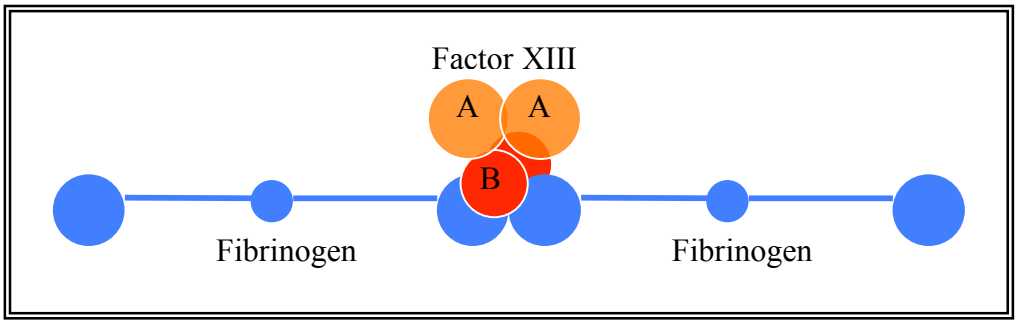

Figure 1.3. Schematic diagram of how factor XIII may interact with fibrinogen. Fibrinogen is shown in blue, while factor XIII is shown in red (B subunits) and orange (A subunits). This arrangement is one way in which the B subunits of one factor XIII molecule may simultaneously interact with two fibrinogen molecules. Based on a figure by Moaddel *et al*. [59].

Summary of Dissertation Research

While these experiments have provided initial information regarding the binding sites on fibrinogen for factor XIII, each has some drawbacks. In the initial experiments suggesting the γ′ chain as a binding site for factor XIII [57], plasma fibrinogen was separated by ion-exchange chromatography to yield the two populations γ/γ and γ/γ′. While this method separates γ/γ′ from γ/γ based on their charge difference, another modification may be present elsewhere in the molecule that contributes to their separation. Other changes to a fraction of the fibrinogen molecules such as glycosylation or post-translational modifications would have no relation to the γ′ chain, but could still affect charge and chromatographic separation. To confirm that the γ′ chain is indeed involved in binding to factor XIII, I have generated recombinant fibrinogen with a γ′ chain for use in binding studies. I prepared two recombinant fibrinogen variants: one with γ′ chains on both ends of the molecule (γ′/γ′), and the other with one γ′ chain and one normally spliced γ chain (γ/γ′). The γ/γ′ variant resembles the form in which the γ′ chain is found physiologically.

While synthesizing the recombinant fibrinogen variants, I studied the expression of the γ and γ′ chains in CHO cells to determine if they are expressed with equal efficiency and whether γ/γ′ heterodimers form as easily as do homodimers. I also compared the polymerization of γ/γ′ and γ′/γ′ fibrinogen compared to normal recombinant fibrinogen.

Regarding the binding of factor XIII to fibrinogen, the Moaddel model derived from ultracentrifugation experiments is a useful starting point, but care must be taken in interpretation of these data. This investigation has received some criticism regarding the possibility of factor XIII zymogen cross-linking fibrinogen during the extended

ultracentrifugation [61]. To account for these considerations, I performed binding studies using alternative methods such as ELISA.

The binding studies included in this work are designed to determine whether or not the γ′ chain has a role in binding to factor XIII. If the Moaddel model of two fibrinogen molecules interacting end to end with one factor XIII molecule holds true, then this variant provides a range of interaction sites for factor XIII: two γ chains, two γ′ chains, or one γ and one γ′ chain. The γ′/γ′ variant does not exist at significant concentrations in human plasma, but provides an excellent positive control with only γ′ chains available for binding. Factor XIII binding to these two molecules was compared with binding to normal recombinant fibrinogen that contains only γ chains. My experiments also address the question of how this proposed binding site functions when factor XIII is in its zymogen form compared to its active form and were performed with factor XIII zymogen, recombinant factor XIII A_2, and active-site inhibited recombinant factor XIIIai.

Fibrinogen's role as cofactor for factor XIII activation was also explored using my γ′/γ′ and γ/γ′ variants. After exposure to variant cofactors, factor XIII activity was monitored by the extent of cross-link formation within each variant catalyzed by factor XIII activated *in situ* compared to pre-activated factor XIIIa. In addition, I compared how the presence of factor XIII affects polymerization of the γ′/γ′ and γ/γ′ variants.

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CHAPTER TWO

EXPRESSION OF γ′/γ′ AND γ/γ′ FIBRINOGEN IN CHO CELLS

Summary

Circulating fibrinogen is highly heterogeneous, with several sites of variability in each chain [1]. The γ/γ′ variant with one normally spliced γ chain and one γ′ chain is present in around 10% of fibrinogen molecules in a normal individual's plasma [2]. The γ′/γ′ homodimer is much more rare physiologically. We prepared each of these recombinant fibrinogen variants and expressed them in CHO cells to provide material for studying the putative factor XIII binding site on the γ′ chain.

In the course of generating the γ/γ′ heterodimer variant, we produced a set of clones that expressed differing ratios of γ′ to γ chains. Using these clones, we determined the correlation between the ratio of γ/γ′ mRNA produced and the ratio of γ/γ′ protein secreted by the cells. Using immunoprecipitation, we also compared the ratio of γ/γ′ heterodimers formed compared to γ/γ and γ′/γ′ homodimers.

In vivo, the γ′ chain contains two sulfated tyrosine residues, Tyr 418 and 422 [3]. We used mass spectrometry to demonstrate that the γ′ chains expressed in CHO cells have this posttranslational modification.

Introduction

γ/γ′ Fibrinogen in Plasma

In a study of normal human blood samples, the concentration of γ/γ′ fibrinogen in plasma was found to be independent of age, gender, or total fibrinogen level [4]. The authors extended their study to individuals undergoing cardiac catheterizations, and found higher levels of γ/γ′ fibrinogen among patients diagnosed with coronary artery disease compared to their healthy cohorts. Little is known, however, about the factors that influence an

individual's level of γ/γ′ fibrinogen compared to γ/γ fibrinogen and whether elevated γ/γ′ fibrinogen is a cause, result, or epiphenomenon of coronary artery disease [4]. It is also unknown whether γ/γ′ heterodimers are assembled with much greater frequency than the rare γ′/γ′ homodimer simply due to probability or whether there is some mechanism that favors γ/γ′ production.

Expression of Recombinant Fibrinogen

The process of recombinant fibrinogen production in CHO cells was developed in the Lord lab in 1993, and is shown in Figure 2.1. The first step in this procedure is to transfect plasmids for two normal chains of fibrinogen (Aα and Bβ in this case) into Chinese hamster ovary cells, along with pRSVneo, a selection plasmid for neomycin resistance. In the second step, we transfect cDNA encoding the mutated third chain (γ′ in this case) and a second resistance plasmid, pMSVhis, for histidinol resistance. Neomycin and histidinol resistance are then used to select for CHO cell lines that carry the three chains of fibrinogen. Assembled fibrinogen is secreted into the culture medium, and collected once that medium has been changed to serum-free [5], [6]. Normal recombinant fibrinogen appears nearly identical to plasma fibrinogen by SDS-PAGE and electron microscopy and exhibits similar behavior during polymerization and FXIIIa-catalyzed cross-linking [5], [7].

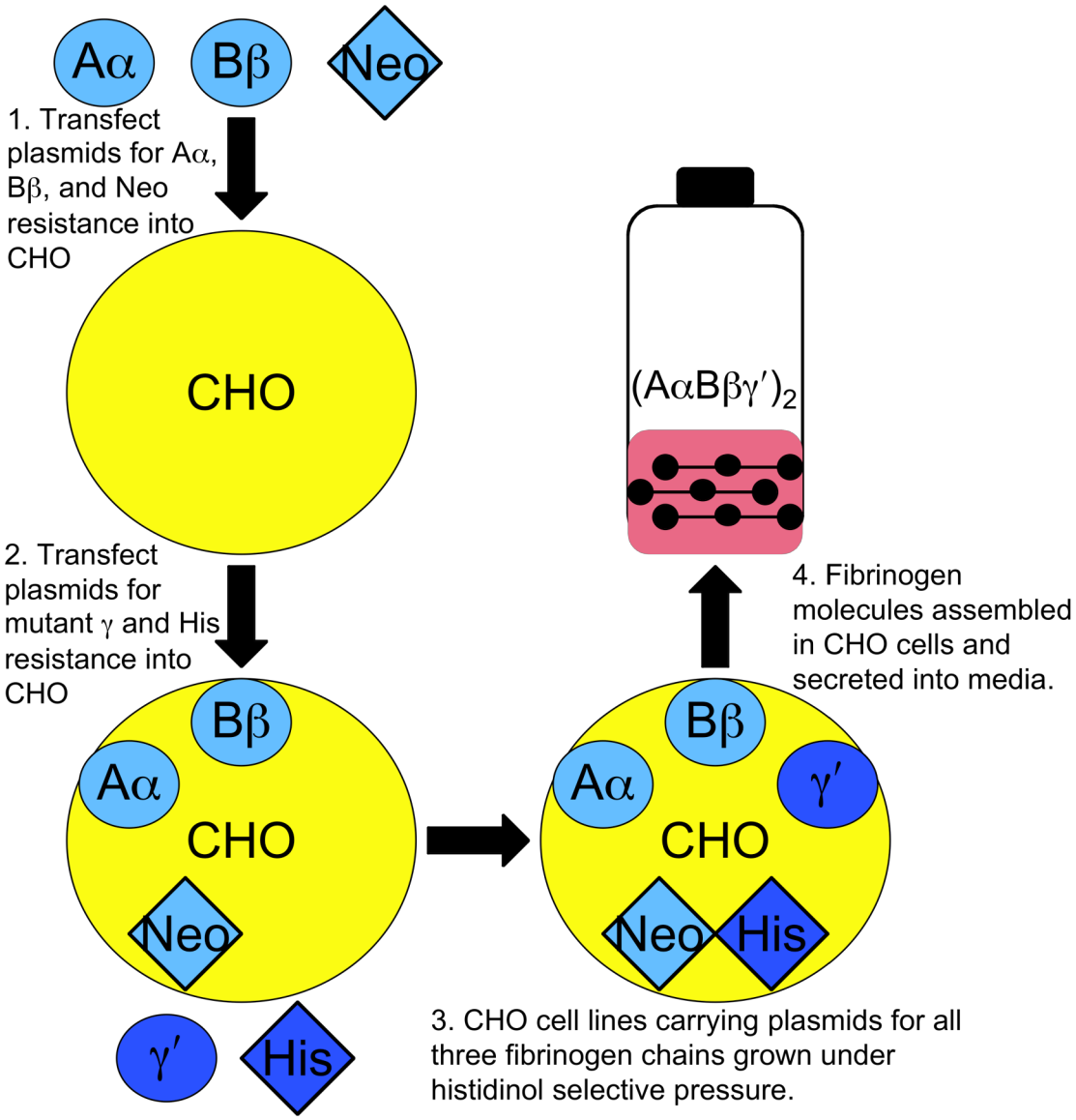


Figure 2.1: Recombinant fibrinogen is expressed in CHO cells by a two-step transfection procedure. This protocol was developed by Binne *et al*. [5].

Tyrosine Sulfation of Fibrinogen γ′ Chains

Unlike the human Aα and Bβ chains, the human fibrinogen γ′ chain contains sulfated tyrosines. This sulfation was demonstrated by $^{35}SO_4$ incorporation into fibrinogen expressed from primary cultures of rat and human hepatocytes. Radioactivity corresponded to the γ′ chain only, as shown by electrophoresis, and was released as tyrosine sulfate by carboxypeptidase Y digestion or base hydrolysis [8], [9]. Sulfation of the γ′ chain was also demonstrated in recombinant fibrinogen expressed in BHK cells by $^{35}SO_4$ incorporation [10]. The sulfation sites on human fibrinogen were localized to tyrosines 418 and 422 by $^{35}SO_4$ labeling of recombinant fibrinogen mutants [3].

Sulfation of tyrosine occurs in the Golgi apparatus [8]. Transfer of a sulfate from the donor PAPS (3′-phospho-adenosine 5′-phosphosulfate) to tyrosine is catalyzed by the enzyme TPST (tyrosylprotein sulfotransferase). There are three characteristics that are considered consensus features for tyrosine sulfation sites:

1) Acidic amino acids are prevalent; typically one is found at position -1 and several are found between -5 and +5 while few basic amino acids are located in this region.
2) A turn-inducing amino acid must be present at the site, typically a proline or glycine.
3) Steric hindrance is minimized due to a lack of disulfide bonds and glycosylations in the region [11].

Until now, the γ′ chain has never been expressed in CHO cells, although other tyrosine-sulfated coagulation proteins have been. Recombinant factor VIII expressed in CHO cells was found with two of three putative tyrosine-sulfation sites completely sulfated; the third was partially sulfated. This diminished sulfation was attributed to limited accessibility of the sulfation site [12]. Recombinant factor V expressed in CHO cells displayed complete

sulfation of the two predicted sites [13]. These results suggest that CHO cells are capable of tyrosine sulfation in general; in particular, sulfation of the γ′ chain of fibrinogen has been confirmed by our mass spectrometry studies.

Materials and Methods

Materials

All chemicals and supplies were obtained from Fisher (Hampton, NH) or Sigma-Aldrich (St. Louis, MO) unless otherwise described. Human adult female liver first strand cDNA was purchased from Stratagene (La Jolla, CA). Restriction enzymes and buffers were purchased from New England Biolabs (Ipswich, MA). Taq polymerase, T4 DNA ligase, Superscript II RNaseH - reverse transcriptase, and penicillin/streptomycin were purchased from Invitrogen (Carlsbad, CA). Expression vectors, DMEM-F12, Nu serum, bovine calf serum, and aprotinin have been previously described [5]. Antibodies were obtained from the following sources: rabbit polyclonal anti-human fibrinogen and HRP-conjugated goat anti-rabbit IgG from DAKO (Carpinteria, CA); rabbit polyclonal anti-fibrinogen γ chain (NC-15) custom antisera from Hazleton Research Products (Princeton, NJ); monoclonal anti-human fibrinogen Aα-chain N-terminus (Y-18) from Dr. W. Nieuwenhiuzen (Foundation of Liver Cell and Endothelial Cell Technology, Netherlands); polyclonal goat anti-human fibrinogen and HRP-conjugated goat anti-human fibrinogen from Cappel (Cochraville, PA); IF-1 monoclonal calcium-dependent anti-fibrinogen antibody from Kamiya Biomedical (Seattle, WA); 4A5 monoclonal mouse anti-human fibrinogen γ chain a kind gift from from Dr. Gary Matsueda at Bristol Myers Squibb (Princeton, NJ); and 2G2H9 mouse monoclonal anti-human fibrinogen γ′ chain a kind gift from Dr. David Farrell (Oregon Health and Sciences

University) and also purchased from Upstate (Charlottesville, VA). Powdered insulin-transferrin-sodium selenite was obtained from Roche (Indianapolis, IN) or Sigma-Aldrich. Sepharose Q Fast Flow Columns and CNBr-activated sepharose were purchased from Amersham Biosciences (Piscataway, NJ). Absolute QPCR Reaction Mix, RT-PCR Tube Strips and UltraClear Cap Strips were purchased from ABGene (Rochester, NY). RNase-free water was obtained from Promega (Fitchburg, WI).

Cloning the γ′ Chain from Human Liver cDNA

Unmodified pMLP-γ expression vector carrying normal γ chain (p674) was transformed into competent DH5α cells and the plasmid DNA isolated by mini-prep. We confirmed the identity of the plasmid by digestion with AflIII. The plasmid was then digested with BstXI and NotI, run on an agarose gel to separate the digested product, and purified using a QIAquick Gel Extraction Kit (Qiagen, Valencia, CA) according to manufacturer's protocol.

We used PCR to amplify sequence corresponding to the γ′ chain from human liver first strand cDNA using the following primers, prepared by the UNC Pathology Department Oligonucleotide Synthesis Facility:

Forward: CAC GCT GGC CAT CTC AAT GGA GTT TA
underlined bases are BstXI recognition site

Reverse: AGC ATT TGC GGC CGC CTA CAA ATC ATC CTC A
underlined bases are NotI recognition site

This PCR product was digested using BstXI and NotI and purified as described above.

Digested, purified PCR product (0.05 μg) and digested, purified vector (0.05 μg) were incubated with 1 unit of T4 DNA ligase for 1 hour at room temperature, and the ligated DNA was transformed into competent DH5α cells. We identified the presence of the γ′

insert by digestion with AflIII alone and AflIII + Bsu36I, and confirmed the resulting γ′ plasmid sequence by Taq Dye Terminator Sequencing at the UNC Genome Analysis Facility. No inadvertent mutations arose during DNA manipulation. Both the γ′ plasmid DNA and the p674 γ chain vector DNA were prepared in large quantities for transfection using the Qiagen Plasmid Maxi-prep kit (Qiagen, Valencia, CA) according to manufacturer's protocol.

Expression of γ′/γ′ and γ/γ′ Fibrinogen in CHO Cells

Transfection of γ′ plasmid into CHO cells carrying plasmids for the Aα and Bβ chains of fibrinogen was performed essentially as described [5]. Briefly, γ′ plasmid and 2 μg of histidinol resistance plasmid were incubated in the presence of calcium and then added to CHO cells that contained the Aα and Bβ chains of fibrinogen as well as a *neo* resistance gene for the antibiotic G418. After four hours, we shocked the cells with 10% glycerol in DMEM-F12 media for three minutes and then washed with G418 media (0.25 mM L-histidinol dihydrochloride, 40 mg/mL Geneticin (G418), in DMEM/F12 media with 5% Nu serum, 5% Calf Serum, and 0.01% Pen/Strep) and allowed them to recover. For the γ′/γ′ clone, we transfected 18 μg of γ′ plasmid; for the γ/γ′ clone 18 μg of γ′ plasmid and 36 μg of p674 γ chain plasmid were mixed before transfection. Appropriate clones for both γ′/γ′ and γ/γ′ were subcloned by infinite dilution.

The best clones for expression in roller bottles were selected based on fibrinogen production. ELISA was used to measure the amount of fibrinogen secreted into serum-free media (0.01% Pen/Strep, 10 μg/mL insulin-transferrin-sodium selenite, 10 U/mL aprotinin in DMEM/F12) by each new γ′ clone. In addition to the amount of fibrinogen expressed, selecting the best clone for γ/γ′ heterodimer roller bottles was done by measuring of the ratio of γ to γ′ chains produced. This ratio was determined by Western blot. Media samples for

Western blot were concentrated with a 100 kDa MWCO Centricon Eppendorf concentrator so that 0.25 μg of fibrinogen could be loaded onto the gel in 20 μL or less. Samples were reduced by the method of Laemmli [14], and run at 60-80V on an 11% gel with a tall stacking gel. This method of running the gel provided optimal separation between the γ and γ′ chains. Western blots were developed using a polyclonal anti-fibrinogen antibody. I used Scion Image (SCIONCorp, Frederick, MD) to quantify the ratio of γ : γ′ chains by densitometry.

Recombinant fibrinogen was expressed by growing cells to confluence in roller bottles, essentially as described [6], with the following modifications: Microcarrier beads were omitted in later expressions once it was determined that they did not significantly improve yield. After cells grew for six days in serum-free media, I collected media every day until the cells died, about 3 weeks later. 50 mL of fibrinogen-containing media was removed from the total 200 mL each day and proteases were inhibited by addition of 30 μ*M* benzamidine, 6μ*M* EDTA, 0.15% PMSF, and 0.01% NaN_3 to collected media before freezing.

Purification of Recombinant γ′/γ′ and γ/γ′ Fibrinogen

Recombinant fibrinogen was purified as described by Gorkun *et al* [7]. Briefly, an inhibitor cocktail was added to thawed media containing ~10 mg of fibrinogen. Ammonium sulfate (either in saturated solution or granular form) was added gradually to precipitate the protein, the sample centrifuged, and precipitate was redissolved in pellet buffer. The fibrinogen sample was purified on an IF-1 immunoaffinity column, a calcium-dependent monoclonal anti-fibrinogen antibody. The resulting fibrinogen was dialyzed against one change of calcium-containing loading buffer, followed by extensive changes of HBS (20 m*M*

HEPES pH 7.4, 150 m*M* NaCl). Concentration was measured spectrophotometrically using an ε_{280} of 1.51 for a 1 mg/mL solution, and the sample was analyzed by SDS-PAGE.

IF-1 purification was sufficient for γ′/γ′ fibrinogen, but γ/γ′ fibrinogen required further purification to isolate heterodimers. I prepared a 4A5 anti-γ chain immunoaffinity column from CNBr-activated sepharose according to the manufacturer's coupling protocol. The column was equilibrated in 4A5 loading buffer (50 m*M* Tris pH 8.0, 150 m*M* NaCl, 1m*M* $CaCl_2$). A sample of approximately 2.5 mg of IF-1 purified γ/γ′ fibrinogen was diluted to around 1 mg/mL in 4A5 loading buffer and applied to the column at 10 mL/hour. During loading, absorbance increased as γ′/γ′ fibrinogen flowed through the column. After loading, I continued the flow of 4A5 loading buffer until column effluent absorbance returned to zero. I increased flow rate to 50 mL/hour for 10 minutes to wash the column. Fibrinogen containing γ chains was eluted with 1*M* guanidinium-HCl in TBS at 20 mL/hour, and fractions were immediately dialyzed against one change of 4A5 loading buffer to remove guanidinium and three changes of TBS (50 m*M* Tris pH 7.4, 150 m*M* NaCl), the last overnight.

Samples were removed from dialysis and concentrated to between 1 and 4 mg/mL in Amicon Ultra 100,000 MWCO Centrifugal Filter Devices. Aliquots of 0.2 – 0.5 mg were loaded onto a Sepharose Q Fast Flow ion-exchange column equilibrated in IEX buffer A (39 m*M* Tris pH 8.5 with H_3PO_4). Proteins were eluted in increasing concentrations of IEX buffer B (190 m*M* Tris pH 2.43 with H_3PO_4) using the following program:

0-15 minutes: 0% B (equilibrate)

15-55 minutes: 0 – 100% B (gradient)

55-65 minutes: 100% B (wash)

65-66 minutes: 100 – 0% B (regenerate)

66-76 minutes: 0% B

γ/γ′ Heterodimers eluted at 55% buffer B; fractions were collected, dialyzed into HBS, concentrated as described above, and analyzed by SDS-PAGE.

Mass Spectrometry

Pure recombinant γ′/γ′ fibrinogen was dialyzed into 50 m*M* ammonium bicarbonate, pH 7.4 at a concentration of 0.68 mg/mL. Protein digestion and mass spectrometry were performed by the UNC Proteomics Core Facility, essentially as described [15]. In brief, each sample was digested with trypsin in solution and analyzed by QTOF, followed by MS/MS fragmentation analysis. A UNC Proteomics in-house version of the MASCOT search engine was used to search for matching fragments, with tyrosine sulfation selected as a possible post-translational modification.

Quantitation of γ and γ′ mRNA

Quantitative RT-PCR was used to determine the relative levels of γ and γ′ message in each of seven CHO cell lines. Cell line A is the normal recombinant fibrinogen cell line that expresses only γ chains, while cell line B is the γ′/γ′ clone that expresses only γ′ chains. The remaining cell lines all express both γ and γ′ chains in different ratios. Cells from each line expressed fibrinogen into serum-free media in a 10 cm cell culture dish for one week. Media was collected and saved for analysis while RNA was harvested from the cells by the UNC Gene Expression facility according to the protocol of Kim *et al*. [16].

Primers and probes are described in the text and were prepared by the UNC Pathology Department Oligonucleotide Sequencing Facility. RT-PCR reactions were set up in 30 μL in 1 m*M* EDTA prepared in RNase-free water as follows:

10 μL of RNA diluted between 1:5 and 1:25

15 μL QPCR mix containing 5 U reverse transcriptase

0.5 μL of 0.1 μg/μL forward primer

0.5 μL of 0.1 μg/μL reverse primer

1 μL of 20 μ*M* probe

The ABI Prism 7700 program ran as follows:

1) 48°C for 30 minutes – reverse transcription
2) 95°C for 10 minutes – polymerase activation
3) 95°C for 15 seconds – double-stranded DNA denaturation
4) 60°C for 1 minute – annealing and extension

Steps 3 and 4 are repeated 40 times. Fluorescence of the reporter FAM is monitored.

A threshold above which the fluorescence exceeds background noise was determined for each experiment, and the number of cycles required for each sample's fluorescence to reach this threshold (C_t) was recorded. C_t is inversely proportional to the amount of message originally present in the sample. C_t values for γ and γ′ message within each cell line were compared using the equation: $2^{(\gamma C_t - \gamma' C_t)}$.

Although it is common to run a control RT-PCR reaction with GAPDH, β-actin, or another housekeeping gene, that was deemed unnecessary in these experiments since the ratio of γ′ to γ chains for each clone is being compared, not their actual amounts. Several preliminary experiments were performed to determine the optimal amount of mRNA template to use for each individual set of primers.

Quantitation of γ and γ′ Protein

ELISA assays were used to determine the ratio of γ′ to γ chains expressed in each clone essentially as described above, but using chain-specific capture antibodies. Microtiter plate wells were coated with either 120 μL of 1:1000 4A5 to determine γ chain concentration or 120 μL of 1:333 2G2H9 to determine γ′ chain concentration. Standard curves were prepared with serial dilutions of either γ/γ clone A media (4A5 plates) or γ′/γ′ clone B media (2G2H9 plates). Samples were prepared from the serum-free media collected from the cells whose RNA was harvested for RT-PCR; media was diluted between 1:4 and 1:20 to yield optimal signal.

Immunoprecipitation

4A5-sepharose beads were prepared in Eppendorf tubes from 0.03 g CNBr-activated sepharose. Beads were incubated for 10 minutes in 1 mL washing solution (1 m*M* HCl) to swell. They were washed with 6 mL of coupling buffer (0.1 *M* $NaHCO_3/Na_2CO_3$ pH 8.3, 0.5 *M* NaCl) and allowed to rotate for 1 hour at room temperature with 4A5 ascites fluid diluted 1:500 in 0.5 mL coupling buffer. The beads were again washed with coupling buffer (7.5 mL) and then allowed to sit for 2 hours in 1 mL blocking buffer (1 *M* ethanolamine in H_2O, pH 8.0) to quench reactive sites. Finally, the beads were washed alternatively three times each with 1.5 mL washing buffer (0.1 *M* $NaCH_3CO_2$ pH 4.0, 0.5 *M* NaCl) and Tris buffer (0.1 *M* Tris-HCl pH 8.0, 0.5 *M* NaCl).

For immunoprecipitation, each batch of beads was split into five tubes. Each tube was blocked with 100 μL of 10% BSA for 30 minutes, and then 1 mL of 1 μg/mL protein was added. The beads were incubated at 4°C for 1 – 2 hours while rotating, the supernatant was removed, and beads were washed four times with 1 mL ice-cold wash buffer (50 m*M*

Tris pH 7.4, 300 m*M* NaCl, 5 m*M* EDTA, 0.1% Triton-X) and once with HBS. Supernatant was removed and replaced with 3 μL HBS, 8 μL 6X Laemmli buffer, and 2 μL BME. Samples were loaded onto an 11% gel and run as described above for optimal γ/γ′ chain separation. Bands were transferred to nitrocellulose, the Western blot developed with polyclonal anti-fibrinogen antibody, and the intensity of bands was quantified by densitometry using SCION Image.

Results

Construction of the γ′ Chain Plasmid

I constructed an expression vector for the γ′ chain of fibrinogen from the pMLP-γ expression vector, p674. This vector was digested with BstXI and NotI. The γ′ insert was amplified from human liver first strand cDNA using primers that incorporated the BstXI and NotI restriction sites. The insert was digested with these enzymes and ligated into the γ chain vector. Initially, I identified samples with the correct construction of the γ′ chain plasmid by restriction digestion with the enzymes AflIII and Bsu36I. As shown in Figure 2.2, digestion of a correctly constructed p674 vector + γ′ insert plasmid will yield a characteristic banding pattern absent if the γ′ insert is not incorporated. These bands will be 3004 bp, 1279 bp, 721 bp, and 658 bp. An incorrectly ligated vector will lack the Bsu36I site and thus only be digested into fragments of 4283 bp, 721 bp, and 658 bp. Figure 2.3 shows the difference between positive and negative clones in the initial ligation transformation. Colony 1 did not contain the γ′ insert because its digestion pattern did not change when Bsu36I was added to AflIII. Colony 2 likely did contain this sequence because two smaller bands were produced upon addition of Bsu36I.

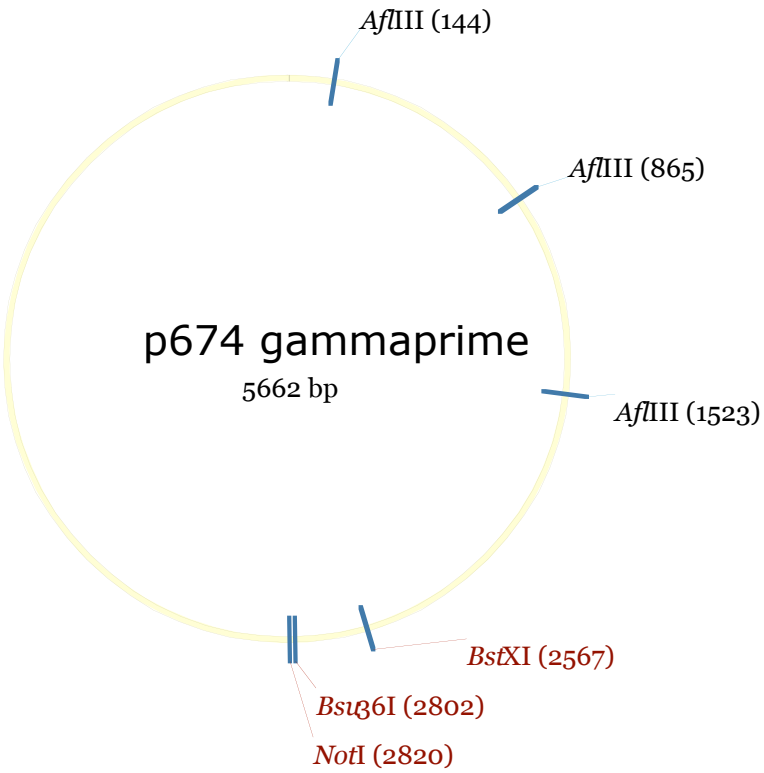


Figure 2.2. Sequence coding for the γ′ extension to the γ chain was inserted into vector p674 using BstXI and NotI. Addition of the γ′ sequence introduces a unique Bsu36I site that allows for identification of positive clones when the plasmid is digested with a combination of Bsu36I and AflIII.

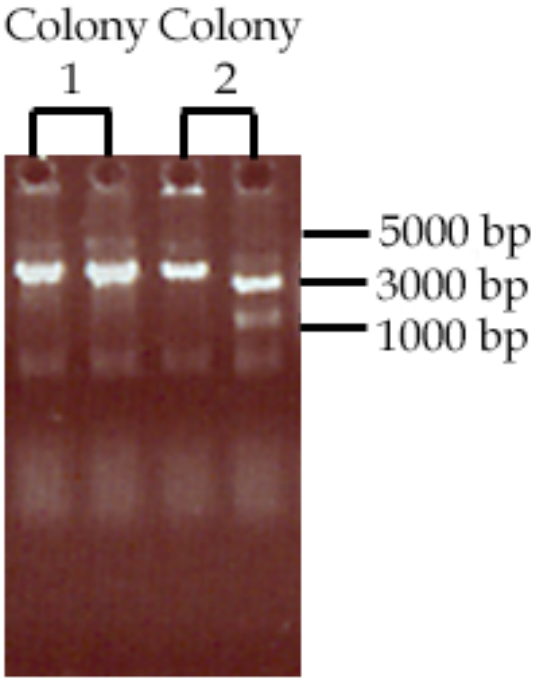


Figure 2.3. 1% agarose gel showing different digestion patterns for positive and negative clones. The band on the left for each clone is digestion with AflIII only; the band on the right is digestion by AflIII + Bsu36I. Colony 1 shows no difference between AflIII and AflIII + Bsu36I digestion patterns. Colony 2 shows that the largest band (~4000 bp) is split in two with the addition of Bsu36I.

A positive clone was selected and sequenced in both the forward and reverse directions through the length of the coding region. The results confirmed insertion of the γ′ sequence with no change in the remaining cDNA.

Expression of γ′ Variants by CHO Cells

After sequencing, I transfected the γ′ plasmid into CHO cells that already carried plasmids for the Aα and Bβ chains of fibrinogen. This transfection produces γ′/γ′ homodimer protein. I grew 24 clones of this transfected cell line and selected the clone with the highest level of fibrinogen secretion to be grown in roller bottles. This clone is also clone B, used for the γ/γ′ ratio quantitation experiments. To prepare my heterodimer protein, I transfected both γ and γ′ plasmids into the CHO cell line with Aα and Bβ chains. Five of the clones from this transfection became cell lines C-G, used in the γ/γ′ ratio quantitation experiments (cell line A comes from normal, recombinant γ/γ fibrinogen). I chose the clone with the highest level of fibrinogen production that also expressed a relatively equal ratio of γ and γ′ chains to be used in roller bottle expression.

Because fibrinogen expression levels were low, I subcloned the two CHO cell lines used for γ′ variant expression. Subcloning led to a distinct improvement in the expression of the γ′/γ′ fibrinogen clone. Prior to subcloning, media was expressed with fibrinogen at a level of around 1.4 μg/mL and after subcloning it was expressed at 2.6 μg/mL. Subcloning did not change the expression level of the γ/γ′ clone as drastically (2.3 μg/mL to 2.8 μg/mL); however, it did allow for selection of a clone with a γ/γ′ ratio closer to 50%. As determined by Western blot, in the new γ/γ′ clone, 63% of the total γ and γ′ chains were γ′, while 85% were γ′ in the old clone. This change translates to a significant difference in the possible

percentage of heterodimers; 47% of molecules could form heterodimers with the new clone versus only 26% for the old.

In early attempts at expressing the γ′ variants, a significant percentage of the Aα chain was proteolytically degraded. To minimize this degradation, I made several adjustments to the usual expression protocol [6]. Instead of collecting 100 mL of media every 2-3 days, I collected 50 mL every day to keep fibrinogen levels low. A cocktail of protease inhibitors (30 μ*M* benzamidine, 6μ*M* EDTA, 0.15% PMSF, and 0.01% NaN_3) was also added to the media right after collection, rather than 0.15% PMSF alone. Figure 2.4 shows a Western blot performed with an antibody specific to the Aα chain (Y-18) that demonstrates an early collection of fibrinogen with significant Aα chain degradation, while fractions collected with the modifications described above have barely detectable degradation bands.

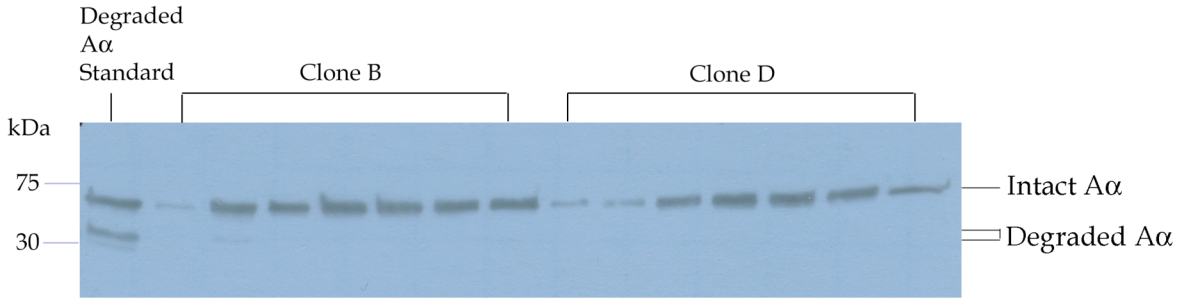


Figure 2.4: Western blot using anti-Aα chain N-terminus antibody (Y-18). The degraded Aα sample (from a degraded preparation of γ/γ fibrinogen) shows the full-length Aα band as well as two degradation products below it. The bands to the right of this standard are every third collection from two clones (B and D) expressed with adjustments to decrease proteolytic degradation. Media samples were concentrated in 100kDa MWCO Eppendorf concentrators and concentrated samples were prepared to load 0.25 μg of fibrinogen.

Purification of Recombinant γ′/γ′ and γ/γ′ Fibrinogen

Recombinant fibrinogens were purified from culture media by immunoaffinity chromatography as described [7]. While this method was sufficient for γ′/γ′ fibrinogen, it did not separate γ/γ′ heterodimers from the γ/γ and γ′/γ′ homodimers that were all produced by the heterodimer cell line. The IF-1 antibody bound to all three of these fibrinogens. Others [17] have developed a DE-52 ion-exchange column to separate γ/γ′ heterodimers from γ/γ homodimers in plasma fibrinogen based on their charge difference. However, this method did not work for our mixture of heterodimers and homodimers because it was unable to separate γ/γ′ heterodimers from γ′/γ′ homodimers. Therefore, I developed a purification protocol that pairs ion-exchange chromatography with immunoaffinity chromatography (Figure 2.5). Unfractionated IF-1 purified γ/γ′ fibrinogen (with γ/γ homodimers, γ′/γ′ homodimers, and γ/γ′ heterodimers) was subjected to a 4A5 immunoaffinity column. As shown in Figure 2.6, γ′/γ′ homodimer fibrinogen did not bind to this column. It eluted in the flow-through (2.6B), while a mixture of γ/γ and γ/γ′ fibrinogen specifically eluted in 1 *M* guanidine-HCl in TBS.

The product of the 4A5 column was applied to an ion-exchange column to separate the γ/γ homodimers from the γ/γ′ heterodimers. The heterodimers were collected from the column, dialyzed, and concentrated. Figure 2.7 shows a characteristic chromatogram from the ion-exchange column and pure γ/γ′ heterodimers as demonstrated on SDS-PAGE. Densitometry showed the presence of approximately 50% each of γ and γ′ chains in the purified γ/γ′ heterodimer sample.

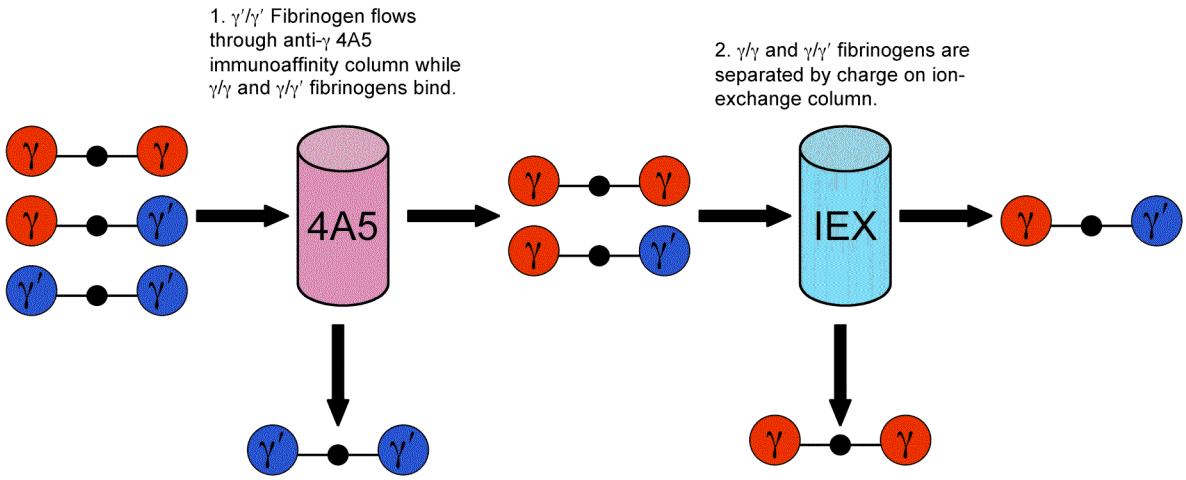


Figure 2.5: Purification of γ/γ′ heterodimers is a two-step process.

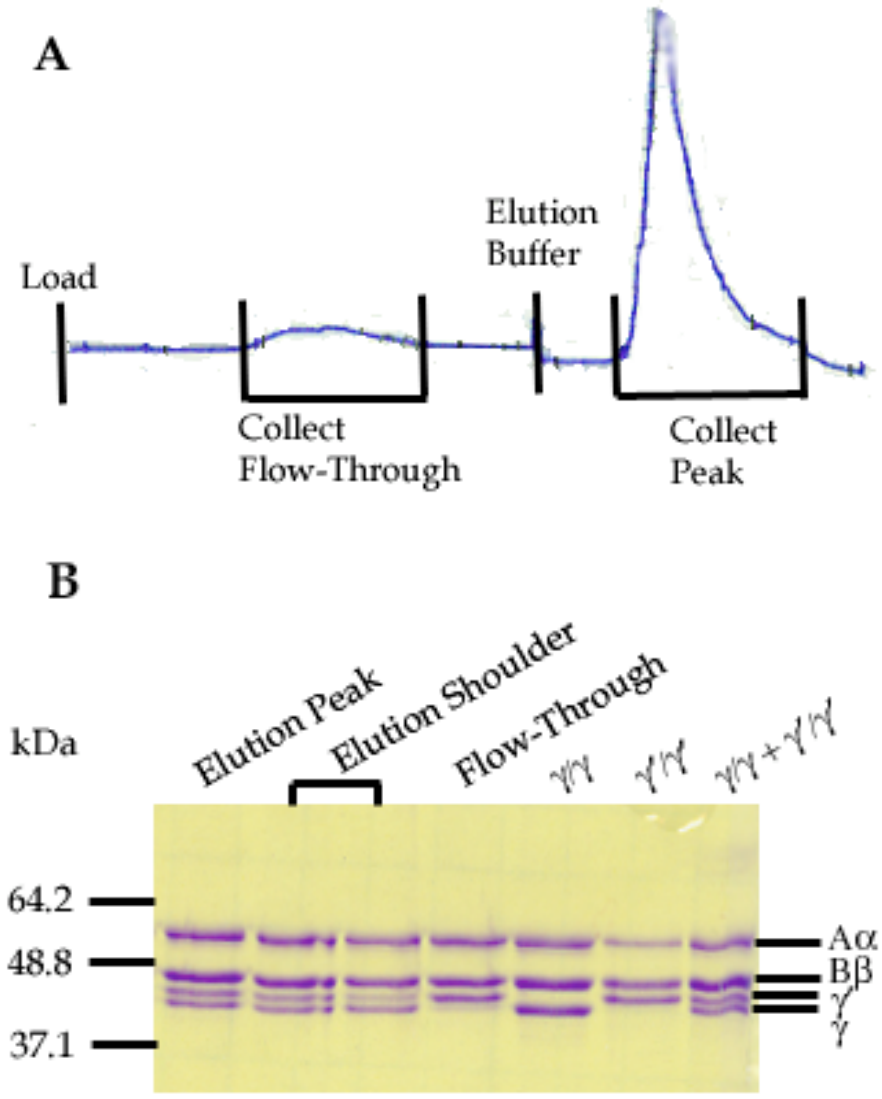


Figure 2.6: A) Elution profile for 4A5 immunoaffinity column. IF-1 purified γ/γ′ fibrinogen is loaded in 50 m*M* Tris pH 8.0, 150 m*M* NaCl, 1m*M* $CaCl_2$. Bound fibrinogen is eluted with 1 *M* guanidine-HCl in TBS and immediately dialyzed. B) 11% reducing gel of 4A5 column fractions. Molecular weight markers are noted on the left of the gel and band identifications are on the right. Elution peak and shoulder are in the first three wells, followed by flow-through from column loading. The last three wells are standards: pure γ/γ fibrinogen, pure γ′/γ′ fibrinogen, and an equimolar mix of γ/γ and γ′/γ′ fibrinogen.

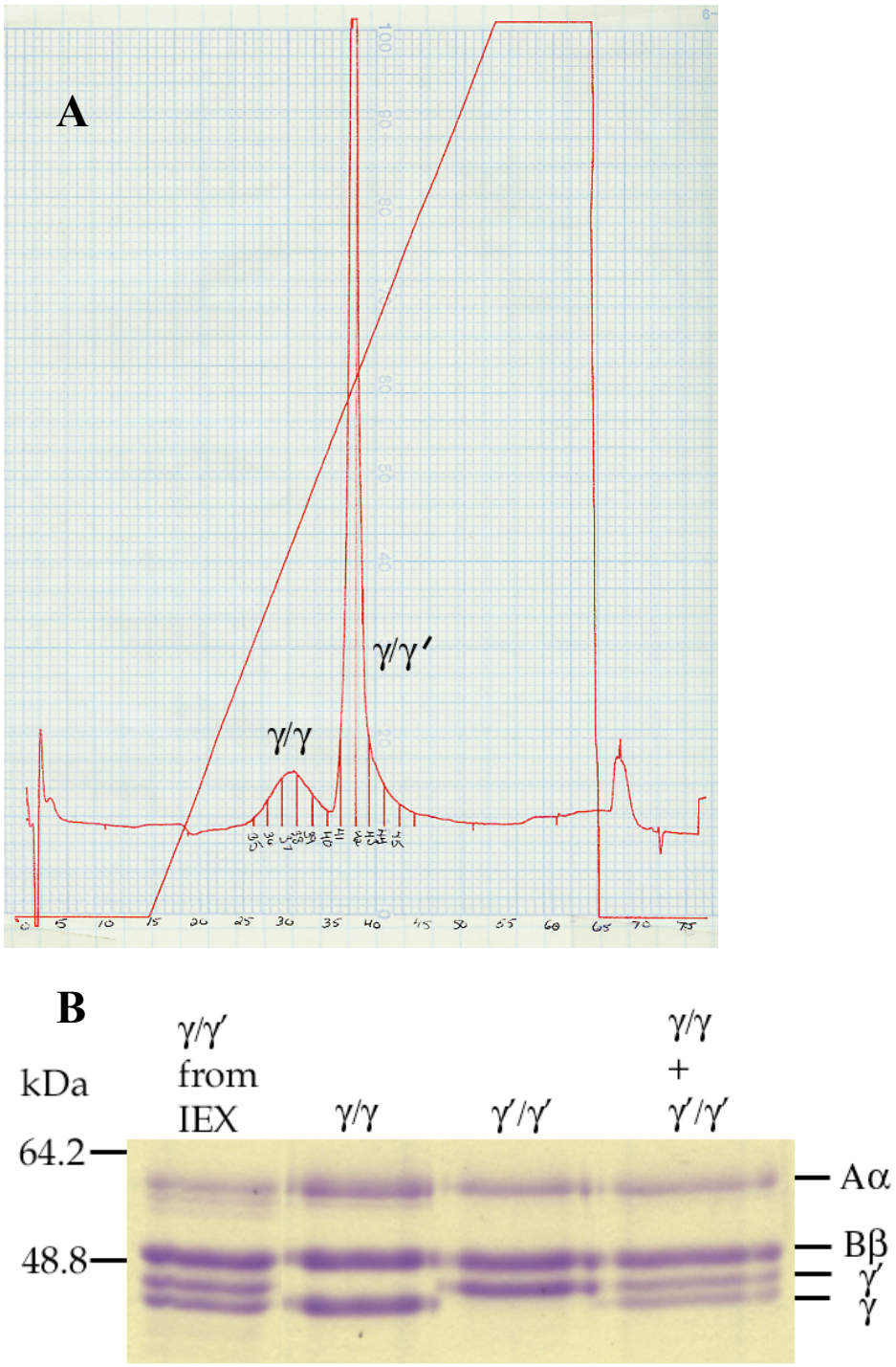


Figure 2.7: A) Samples eluted from the 4A5 column were run on the ion-exchange column to separate γ/γ′ heterodimers from γ/γ homodimers. The diagonal line reflects the gradient of 0-100% buffer B used to elute fibrinogen from the column (see Methods). B) 11% reducing gel with molecular weight indicated on the left and bands identified on the right. γ/γ′ fractions from ion-exchange were pooled, dialyzed, concentrated, reduced, and run on the gel. Standards are recombinant γ/γ, γ′/γ′, and a 50% mixture of γ/γ and γ′/γ′.

Determination of Tyrosine Sulfation

Recombinant γ′/γ′ fibrinogen was digested with trypsin and the fragments analyzed by QTOF and MS/MS fragmentation to determine whether the γ′ chain of CHO-expressed recombinant fibrinogen was sulfated. Analysis of the QTOF results by MASCOT search identified fibrinogen Aα, Bβ, and γ′ chains as significant hits, confirming that this protein sample is indeed γ′/γ′ fibrinogen. Of the γ′ chain hits, four peptides corresponded to the relative molecular mass of the unsulfated γ′ fragment (2502 Da), while nine matched the singly sulfated γ′ relative molecular mass (2582 Da). These results indicate that sulfation did take place during fibrinogen production in CHO cells and that at least one of the tyrosines in the γ′ peptide was sulfated.

MS/MS fragmentation (see Figure 2.8) analysis of the sulfated γ′ peptide peaks showed that tyrosine 422 was sulfated; there is no evidence that 418 was sulfated also. However, because sulfation is a labile post-translational modification, it is possible that this sulfate may have been lost during mass spec manipulations.

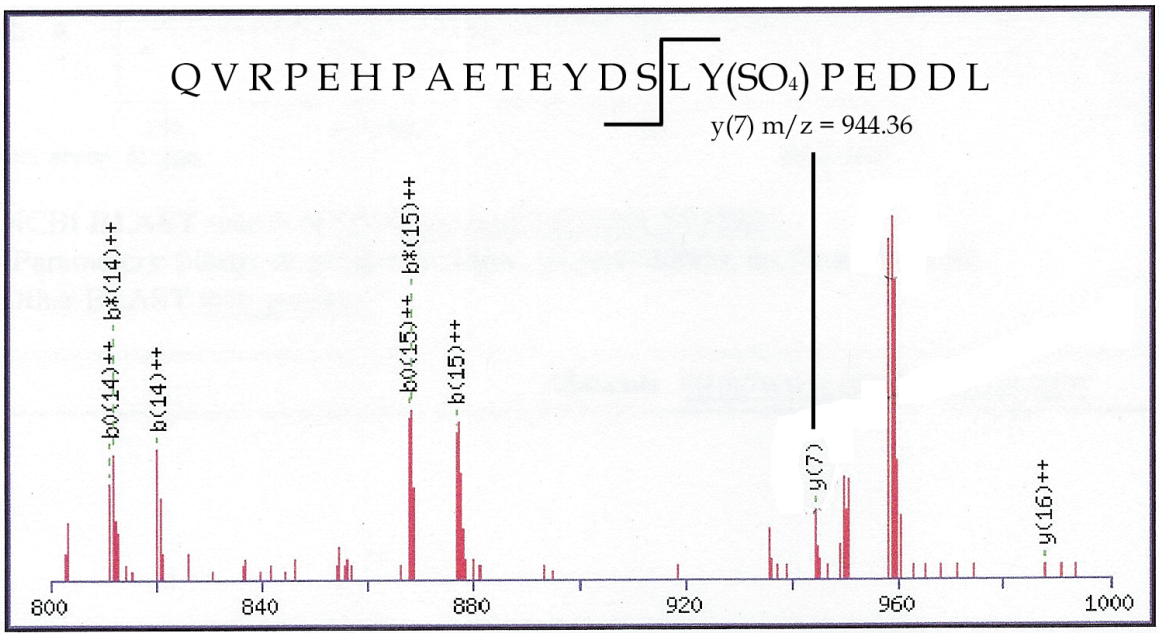


Figure 2.8. MS/MS fragmentation of γ′ peptide. The peptide sequence is shown above the mass spec plot. Fragment y(7) is split between the two tyrosines in the peptide. It is found at m/z 944, indicating that tyrosine 422 is sulfated.

Quantitation of γ/γ′ mRNA

Expression of the γ′ chain in the CHO system was studied by comparing the ratio of γ to γ′ chain RNA inside the cells with the ratio of γ to γ′ chain protein expressed and secreted from the cells. RT-PCR was used to compare the amount of mRNA for γ chains to the amount of mRNA for γ′ chains. As illustrated in Figure 2.9, we designed a common forward primer, a common probe, and two reverse primers, one that would amplify mRNA coding for the γ chain and another that would amplify γ′ mRNA. Sequences, characteristics, and location of the primers and probe are shown in Table 2.1.

For each clone, A-G, RT-PCR experiments were performed in triplicate for each primer set: common forward with γ reverse and common forward with γ′ reverse. Data from the experiment was reported as a C_t, the number of cycles required to reach a threshold fluorescence level above background. This threshold is the same for all samples in a given experiment. The ratio of γ′ to γ mRNA was calculated as follows:

$$\gamma' \text{ mRNA} / \gamma \text{ mRNA} = 2\text{^}(\gamma C_t - \gamma' C_t)$$

The percentage of γ′ mRNA out of total γ + γ′ message in each sample was determined:

$$\%\ \gamma' \text{ mRNA} = 100 - (100 / (1 + \gamma' : \gamma \text{ ratio}))$$ [1]

Table 2.2 shows the results of the RT-PCR experiments.

[1] For example, if we determine that the two C_t values are equal at 60 cycles, then γ′ / γ = 2^(60 – 60) = 2^(0) = 1. Then % γ′ mRNA = 100 – (100 / (1 + 1)) = 50% γ′ mRNA and 50% γ mRNA, just as we would predict from equal C_t values.

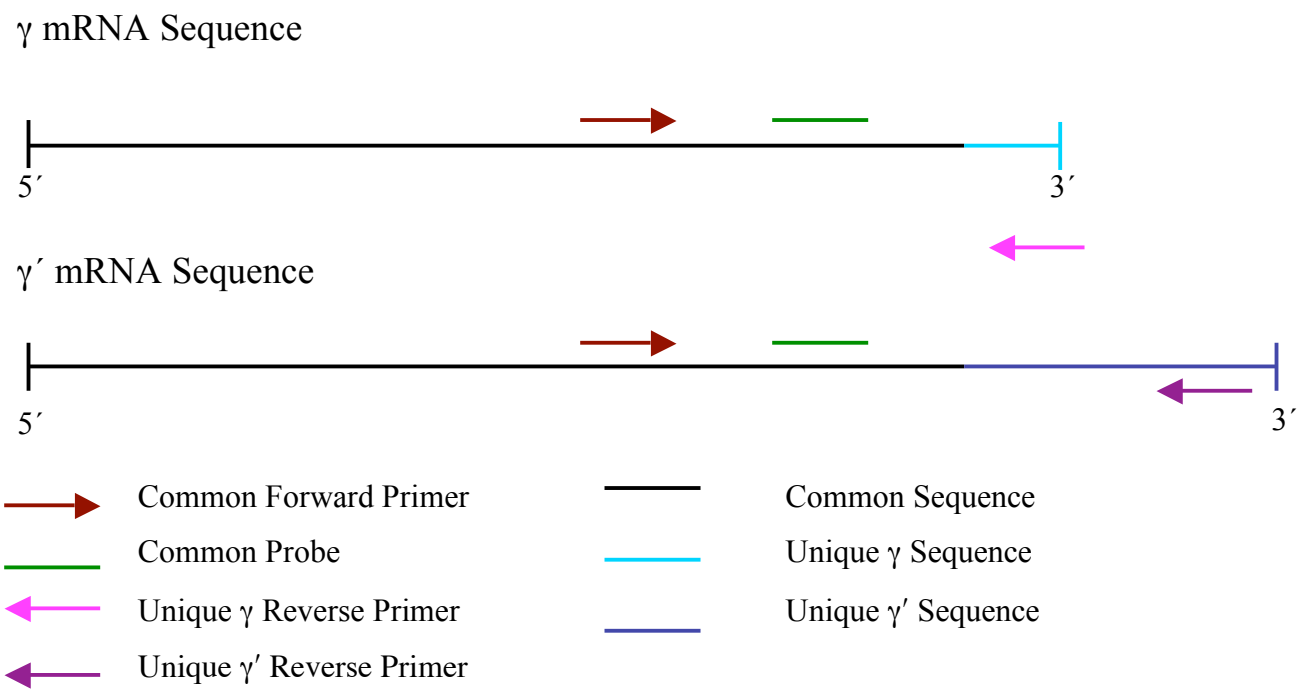


Figure 2.9: Design of primers and probes for RT-PCR.

Primer and Probe Sequences for RT-PCR

Primer or Probe	Sequence	T_m (°C)	%GC	Recognizes
Common Forward	CCACTATGAAGATAATCCCATTC	52	39	γ and γ′ 1223 - 1245
γ Reverse	CGGTCTTTTAAACGTCTCCAGC	55	50	γ 1300 – 1314 and 9 bases of adjacent plasmid
γ′ Reverse	TCATCCTCAGGGTAAAGTGAGTC	55	48	γ′ 1333 – 1355
Common Probe	CCCAGGTGGTGTTGCTGTCCTTCTC	63	60	Reverse complement of γ and γ′ 1262 - 1286

Table 2.1. Sequences, melting temperature (°C), % GC content, and mRNA sequence recognized by each primer or probe. The common probe contains the 5′ reporter dye FAM and the 3′ quencher TAMRA. mRNA of interest is transcribed into a DNA template by Taq DNA polymerase using the forward and reverse primers. The DNA template is then amplified through 40 PCR cycles, during which the probe anneals. Each time the complementary sequence is extended, however, the probe will be hydrolyzed, separating the TAMRA quencher from the FAM reporter. This process releases fluorescence that can be quantified by the ABI Prism 7700 Sequence Detector and is proportional to the number of copies of the mRNA template present.

Clone	**% γ′ mRNA**
A (all γ)	0.000 +/- 0.001
B (all γ′)	99. 9 +/- 0.1
C	67.5 +/- 9.1
D	65.8 +/- 17.8
E	29.3 +/- 11.8
F	98.3 +/- 2.0
G	98.2 +/- 1.7

Table 2.2. % γ′ mRNA for seven clones determined from RT-PCR experiments as described above.

Quantitation of γ/γ′ Protein

I used ELISA to determine the concentrations of γ and γ′ chain protein produced by each cell line with the γ-specific monoclonal antibody 4A5 to determine the concentration of γ chains and the γ′-specific antibody 2G2H9 to measure the concentration of γ′ chains. To determine the ratio of γ′ to γ chains in the expressed protein, I needed a way to relate the concentrations determined by each antibody to one another. I measured the total concentration of γ chains (2 x [fibrinogen]) for clone A (γ chains only) and the total concentration of γ′chains for clone B (γ′ chains only) media using a Cappell polyclonal anti-fibrinogen plate and pure plasma fibrinogen as a standard. The clone A media ([γ] = 5.97 μg/mL) was then used to create a standard curve of γ chains on the 4A5 plate while the clone B media ([γ′] = 1.42 μg/mL) was used to prepare a standard curve of γ′ chains on the 2G2H9 plate. Each of six media samples for each clone was then measured in triplicate on each plate. Results of these experiments are shown in Table 2.3.

By comparing the RT-PCR data with the ELISA data for these six γ′-chain producing clones, we investigated the level of expression for the γ′ chain compared to the γ chain in CHO cells. As shown in Figure 2.10, the percentage of γ′ message RNA in each cell line (red) approximated the percentage of γ′ protein in total fibrinogen (blue). Clones that showed significant differences in mRNA and protein level, as indicated by a Student's T-test are marked with an asterisk. Although there is a slightly greater percentage of γ′ protein than γ′mRNA in clones C and D, this trend does not hold for all five γ/γ′ clones. Thus, in CHO cells, both the γ and γ′ chains appear to be produced at the same level.

Clone	**[γ Chain] (μg/mL)**	**[γ′ Chain] (μg/mL)**	**% γ′ Protein**
A (all γ)	11.0 +/- 3.3	0.0089 +/- 0.0126	0.076 +/- 0.106
B (all γ′)	0.00 +/- 0	3.42 +/- 1.36	100 +/- 0
C	0.349 +/- 0.061	6.26 +/- 2.10	94.2 +/- 2.1
D	0.834 +/- 0.122	5.06 +/- 1.72	84.7 +/- 4.5
E	4.35 +/- 0.73	1.10 +/- 0.42	20.0 +/- 7.2
F	0.116 +/- 0.027	2.22 +/- 0.66	94.6 +/- 2.5
G	0.0486 +/- 0.0161	3.43 +/- 1.46	98.5 +/- 0.5

Table 2.3. % γ′ Chain expressed in seven clones, as determined by ELISA. [γ chain] and [γ′ chain] reported in μg/mL from the average of 7 experiments. % γ′ protein was calculated from the % γ′ protein determined in each experiment and is reported +/- standard deviation.

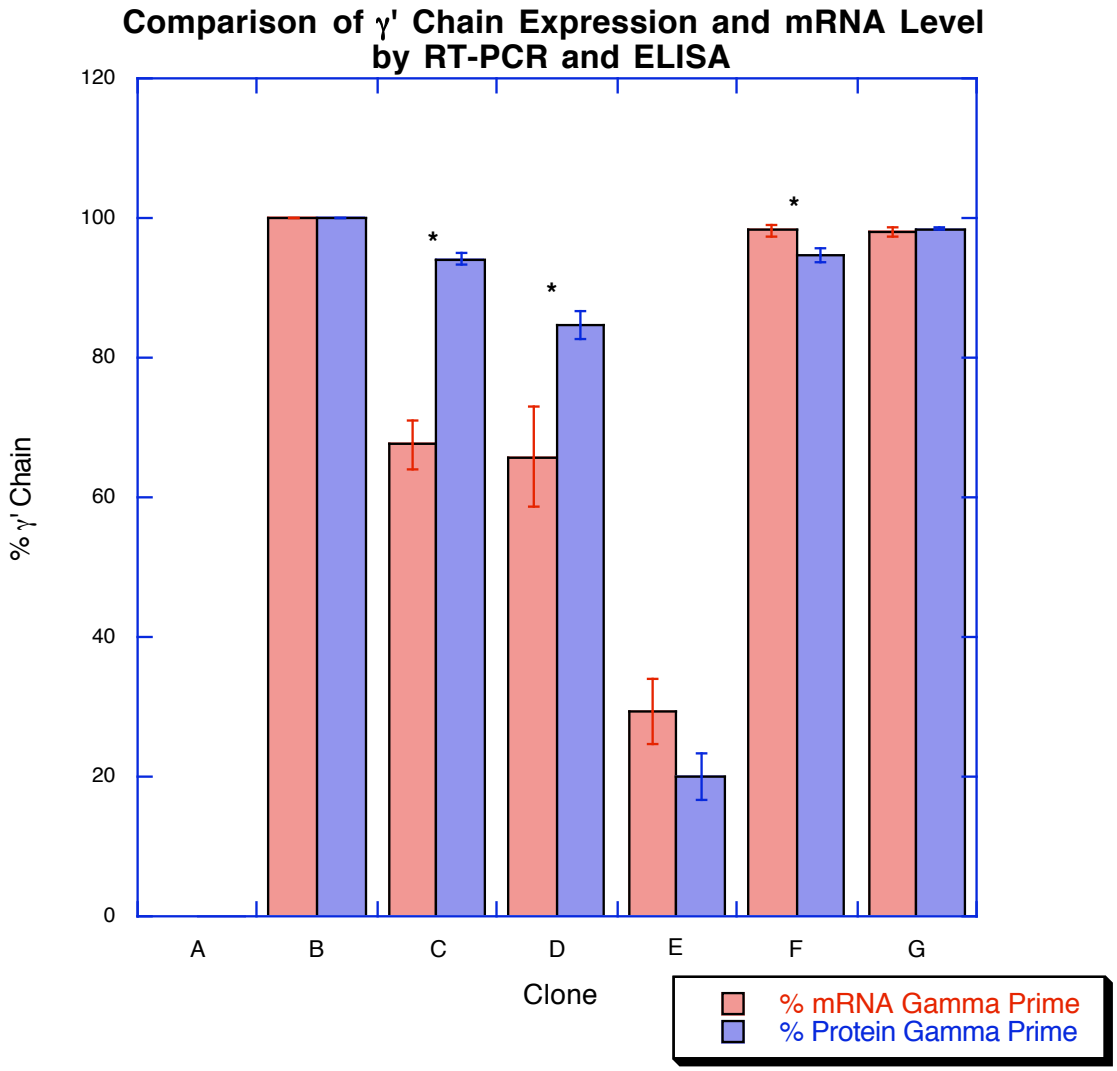


Figure 2.10. Comparison of mRNA and protein levels for γ/γ′ containing CHO cell lines. Standard error is plotted as error bars and p values < 0.05 are shown with an asterisk.

Quantitation of Heterodimer Percentage

I used immunoprecipitation experiments to measure the percentage of heterodimers formed in a sample of fibrinogen expressed by CHO cells that contained a mixture of γ/γ homodimers, γ′/γ′ homodimers, and γ/γ′ heterodimers. I covalently coupled anti-γ chain antibody 4A5 to CNBr-sepharose, and incubated it with a sample of purified, but unfractionated fibrinogen for two hours. After washing, the only fibrinogen still bound to the beads should have been that fibrinogen that contained a γ chain. Control experiments demonstrated that the addition of 10% BSA to the beads as a blocker and the appropriate design of wash buffer prevented nonspecific adsorption of fibrinogen to the beads; as shown in Figure 2.11A, immunoprecipitation of γ′/γ′ fibrinogen with 4A5-sepharase did not pull down any protein.

Assuming a 1:1 ratio of γ:γ′ chains in a particular clone, the ratio of expected products is:

1 (γ/γ) : 2 (γ/γ′) : 1 (γ′/γ′)

In any clone, the expected % heterodimer formed is:

(2 * (%γ) * (%γ′))*100

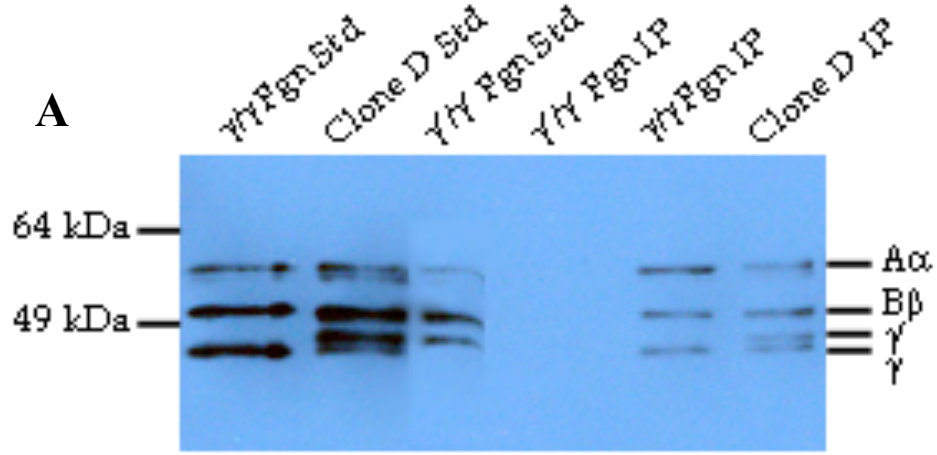
when (%γ) and (%γ′) are expressed as decimal percents of total γ(′) chains.

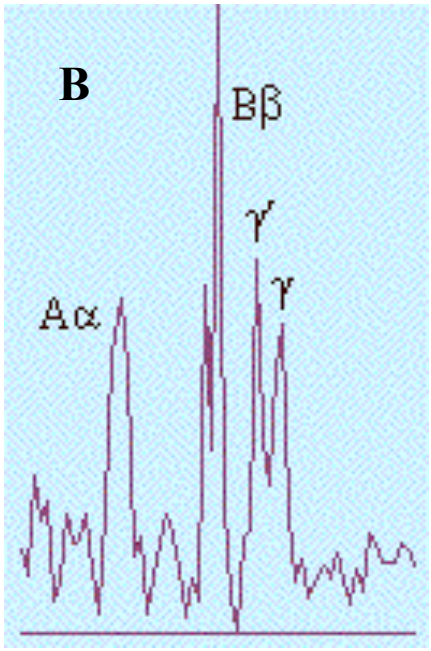
Clone D fibrinogen was chosen for immunoprecipitation experiments because it was expected to produce the greatest proportion of heterodimers. After subcloning the CHO cell line, Western blots of pure protein expressed by clone D in roller bottles indicated that the percentage of γ chain protein in this clone was 37 +/- 5% and the percentage of γ′ chain protein was 63 +/- 5%. Therefore, the expected percentage of γ/γ′ heterodimers in the protein was 46 +/- 3%.

Results of a representative immunoprecipitation experiment are shown in Figure 2.11. γ/γ and γ′/γ′ fibrinogen standards mark the locations of the γ and γ′ chains, respectively, while the clone D standard shows that the unfractionated sample has a greater percentage of γ′ chains than γ chains. Immunoprecipitation of γ′/γ′ fibrinogen shows that no γ′ chains are recognized by 4A5, while immunoprecipitation of γ/γ homodimer has just γ chains. Immunopreciptiation of a 50% mixture of γ/γ and γ′/γ′ leads to just γ chains visible on the blot (data not shown). Immunoprecipitation of clone D results in a mixture of γ and γ′ chains.

Densitometry of the clone D immunoprecipitation is shown in Figure 2.11B. From this calculation, approximately 45% of the total γ or γ′ chains pulled down in the IP were γ′ chains. Since the only γ′ chains pulled down during the immunoprecipitation reaction were those that were part of heterodimers, this experiment showed that 60% of the fibrinogen molecules produced by this clone were heterodimers[2]. The average from all immunoprecipitation experiments showed that the γ/γ′ fibrinogen sample contained 52 +/- 12% heterodimers. Within error, this result was the same as the expected 46 +/- 3% heterodimers, suggesting that no impediment existed to heterodimer formation.

[2] Assume intensity is proportional to the number of chains pulled down by the IP and that 4A5 will bind to any γ chain with equal affinity. 37% of the γ and γ′ chains in clone D are γ chains, so if γ intensity = 525, then this is 37% of the total number of γ or γ′ chains, or 1419 chains. Because there are two γ or γ′ chains per molecule, there are 709 molecules in the sample. The only γ′ chains pulled down in the IP are part of heterodimers, and every heterodimer has a γ′ chain that shows up on the blot. Therefore, the % heterodimers is 426 / 709 = 60%.





Chain	Intensity
γ′	426
γ	525

Figure 2.11. A) Fibrinogen samples immunoprecipitated with 4A5 (anti-γ chain)-conjugated sepharose beads and run on an 11% reducing gel, probed with polyclonal anti-fibrinogen antibody. γ/γ, γ′/γ′, and clone D standards were run directly on the blot without immunoprecipitation. Immunoprecipitated samples were washed with washing buffer and HBS, then boiled in 6X Laemmli with BME. Samples were spun down and the supernatant was loaded. B) Densitometric scan of IP-Western blot with peaks marked and calculated intensities listed in the table below.

Discussion

We have successfully produced milligram quantities of recombinant γ′/γ′ homodimer and γ/γ′ heterodimer fibrinogen. With proper collection technique, Aα-chain degradation can be mostly avoided.

Mass spec experiments show that the γ′ chains produced in CHO cells are, indeed, sulfated. This analysis revealed a combination of unsulfated and singly sulfated γ′ chains. Because sulfation is an inherently unstable modification, especially under the conditions used for electrospray ionization [18], it is not appropriate to conclude that the amount of sulfation detected by mass spec analysis is an exact reflection of what exists in the protein sample. These results simply show that tyrosine sulfation does take place during γ′ chain production in CHO cells, particularly on tyrosine 422. The presence of unsulfated peptides does not necessarily mean that a population of fibrinogen is unsulfated; nor does the absence of doubly sulfated peptides mean that tyrosine 418 cannot be sulfated. The results of this experiment simply show that the tyrosine sulfation modification, which provides the extra negative charge carried by the fibrinogen γ′ chain, takes place in CHO cells during fibrinogen expression.

Sabo *et al*. have recently shown that only one modified tyrosine is absolutely required for thrombin binding, and that tyrosine 422 is more important in this role than is tyrosine 418. They prepared synthetic γ′ peptides with one or both tyrosine residues phosphorylated and used them in NMR studies of the structure of the γ′ / thrombin complex. Phosphorylation is not acid-labile like sulfation, but still provides the same negative charge. They showed that when the doubly phosphorylated peptide was mixed with thrombin, lines in the 1D-NMR spectrum of the mixture broadened compared to the spectrum of the peptide

alone. This broadening suggests that conformational changes have taken place in the peptide upon binding. When the peptide phosphorylated only at tyrosine 422 is added to thrombin, significant line broadening takes place, but very little occurs for the peptide phosphorylated at tyrosine 418 [19]. These findings confirm that even if our γ′ chains are only sulfated at residue 422, they should function in a manner similar to the doubly sulfated γ′ chain.

In 2002, Okumura and coworkers studied expression of truncated γ-chain mutant fibrinogens in CHO cells. While they found equal message levels for fibrinogens that terminated between γ379 and the normal C-terminus γ411, they discovered varying amounts of these fibrinogens in cell lysates and culture medium. Specifically, mutants with γ chains that ended at or before residue 386 were never secreted from the CHO cell, while those with longer chains were secreted, at levels that increased with increasing length of the γ chain. From these studies, they concluded that the C-terminus of the γ chain is essential for normal assembly of the fibrinogen molecule, and therefore its secretion from CHO cells [20].

These results prompted us to consider whether changing the C-terminus of the γ chain by including the γ′ sequence would affect fibrinogen assembly and secretion. We find no suppression in γ′ chain production, and perhaps even some preference for γ′ formation compared to γ, although this trend is only seen in two of the clones studied. Therefore, there is nothing inherent in the γ′ sequence that would slow this chain from being assembled and secreted by CHO cells. This finding suggests that it is only the relative amount of γ and γ′ plasmid present in each cell line that determines the ratio of γ to γ′ protein expressed in our CHO system.

As shown in our immunoprecipitation experiments, fibrinogen γ/γ′ heterodimers form during expression in CHO cells at approximately the frequency expected for random association of chains. There is no preference for either heterodimer or homodimer assembly.

Information about fibrinogen expression in CHO cells may or may not translate to humans; however, the experiments described in this chapter could be carried out in Hep-G2 cells or systems that more closely mimic human fibrinogen production to learn about human γ′ fibrinogen expression.

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CHAPTER THREE

CHARACTERIZING THE POLYMERIZATION OF RECOMBINANT γ′/γ′ AND γ/γ′ FIBRINOGEN VARIANTS

Summary

We characterized the polymerization of recombinant γ′/γ′ and γ/γ′ fibrinogen and compared it to recombinant γ/γ fibrinogen with normally spliced γ chains. Measurement of turbidity during the course of polymerization at three concentrations of calcium showed a diminished rate of polymerization and lower final absorbance for γ′/γ′ fibrinogen compared to γ/γ fibrinogen. γ/γ′ Fibrinogen displayed intermediate V_{max} and final absorbance values compared to γ′/γ′ and γ/γ. At 10 μ*M* $CaCl_2$, γ′/γ′ fibrinogen had a longer lag time than γ/γ fibrinogen, but when the calcium concentration was increased to 2 or 5 m*M*, the lag time for γ′/γ′ fibrinogen normalized to that of γ/γ fibrinogen. Fibrinopeptide release assays monitored by HPLC indicated that FpA release from γ′/γ′ and γ/γ′ fibrinogen was normal while the rate constant for FpB release from γ′/γ′ fibrinogen was increased.

Introduction

Polymerization

Fibrin polymerization is typically observed by changes in turbidity, a technique first explored for fibrin in 1947 by Ferry and Morrison [1]. The order and rate of reactions that take place during fibrin polymerization were quantitated by use of stopped flow light scattering in 1979 by Hantgan and Hermans. Measurements with rate-limiting concentrations of thrombin suggested that polymerization begins prior to FpB release, but experiments with reptilase-catalzyed polymerization showed that leaving FpB intact slows fiber formation. Because light scattering measures the formation of small oligomers, Hantgan and Hermans demonstrated that the lag time observed in turbidity studies is due to the time required for protofibril formation prior to fiber formation. This study espoused the

idea of a two-step mechanism for fiber formation; the polymerization of fibrin monomers into a protofibril followed by lateral aggregation of protofibrils into fibers [2].

The effects of particular variables on clot formation and final structure can be determined by monitoring polymerization by turbidity. For example, Carr and coworkers used turbidity to show that at a given ionic strength, increasing calcium concentration to 5 m*M* over the absence of added calcium increased both the rate of clot formation as well as its final turbidity or fiber thickness [3]. Turbidity measurements can also demonstrate differences in clot formation among variant fibrinogens. Such analysis, for example, showed that clots formed from deglycosylated fibrinogen polymerized faster and with a higher final absorbance compared to normal fibrinogen, a difference that was not detected by clinical clotting assays [4].

Weisel and Nagaswami developed a kinetic model to translate turbidity data into polymerization rate constants to describe fibrinopeptide A cleavage, fibrin monomer aggregation into protofibrils, and protofibril aggregation into fibers [5]. They used electron microscope images of fibrin(ogen) in various states of polymerization in combination with turbidity curves to demonstrate the validity this model. Chemical reactions for each step of polymerization were described with appropriate kinetic equations and rate constants such that their combination would accurately generate the shape of a polymerization curve as measured by turbidity. The lag time observed in typical polymerization curves was modeled by requiring oligomers to assemble up to a minimum length before aggregating into protofibrils. A minimum length of around 10 – 20 monomers generates model polymerization curves that match well with experimental curves determined at physiological conditions. The observation that turbidity levels off with time is modeled by using a rate

constant for protofibril growth that is larger than the rate constant for protofibril initiation, yielding fewer longer protofibrils rather than many short ones. The power of this model lies in its ability to both predict the effect of changing conditions on clot formation, and to explain changes in experimentally-determined polymerization profiles on a mechanistic level [5].

Fibrinopeptide Release

While overall polymerization of a fibrinogen variant is often characterized by monitoring turbidity, the early steps of fibrinopeptide release may be analyzed by HPLC. The kinetic pathway for fibrinopeptide release and fibrin polymerization has been thoroughly characterized [6]; comparing rate constants for a mutant or variant fibrinogen polymerization compared to normal fibrinogen provides evidence of whether its change in structure influences polymerization.

The use of HPLC to quantify fibrinopeptide release was pioneered by Martinelli and Scheraga. They first demonstrated the ability to separate bovine fibrinopeptides A and B by isocratic elution on HPLC [7], and then described an assay to measure kinetics of fibrinopeptide release from bovine fibrinogen using this detection method [8]. Higgins and Shafer refined the experiment to employ gradient elution for better separation of FpA and FpB. They were then able to use this assay to characterize an inherited dysfibrinogenemia (Petoskey) that releases an abnormal FpA upon thrombin-cleavage [9].

Materials and Methods

Materials

Recombinant γ′/γ′ and γ/γ′ fibrinogen were expressed and purified as described in Chapter Two. Normal, recombinant γ/γ fibrinogen was prepared as described [10] and purified as described [11]. Human α-thrombin (lot 2690PR) was purchased from Enzyme Research Laboratories (South Bend, IN). Synthetic fibrinopeptide standards were synthesized at the UNC Peptide Synthesis Facility (Chapel Hill, NC), according to the following sequences:

FpA: ADSGEGDFLAEGGGVR

FpB: QGVNDNEEGFFSAR

All chemicals were purchased from Sigma or Fisher unless otherwise specified.

Polymerization

I performed polymerization reactions in 100 μL in Corning half-area untreated polystyrene microtiter plates under the following conditions:

0.15 mg/mL fibrinogen (recombinant γ/γ, recombinant γ′/γ′, or recombinant γ/γ′)

20 m*M* HEPES pH 7.4

10 μ*M*, 2.0 m*M*, or 5.0 m*M* $CaCl_2$

NaCl to bring ionic strength to 0.15

1.0 n*M* (0.10 U/mL) α-thrombin

10 μL of 10 n*M* α-thrombin in HBS was added to solutions of fibrinogen, HEPES, $CaCl_2$, and NaCl prepared in 90 μL to initiate clot formation. Turbidity was monitored on a SpectraMax 340PC microtiter plate reader from Molecular Devices at 350 nm every 10 seconds for 90 minutes. Data was recorded in SoftmaxPro version 4.3.1 (Molecular Devices,

Sunnyvale, CA). Absorbance was plotted versus time for each polymerization curve. Lag time was measured as the time when the change in OD first reached a non-zero slope. Final absorbance of the clot was measured when the slope returned to zero. V_{max} (maximal rate of change) was determined from the five points on the curve with the highest slope as determined by Softmax.

Fibrinopeptide Release

Recombinant fibrinogen (γ/γ, γ′/γ′, or γ/γ′) was diluted to 0.10 mg/mL in 20 m*M* HEPES pH 7.4, 150 m*M* NaCl, 1.0 m*M* $CaCl_2$ (HBS+Ca). I transferred aliquots (240 μL) of this solution to time = 0 and time = infinity Eppendorf tubes, and placed the remaining 2 mL in a 15-mL conical tube for reaction. α-Thrombin (5000 U/mL) was diluted to 50 U/mL in HBS+Ca+0.1% polyethylene glycol. A secondary dilution was prepared as 2 U/mL in HBS+Ca. I initiated reaction by addition of 10 μL of 2 U/mL thrombin to the fibrinogen for 0.010 U/mL thrombin (0.10 n*M*). Aliquots (240 μL) were quickly transferred to Eppendorf tubes after mixing and were quenched at appropriate times (2, 5, 10, 20, 40, 80, 120 minutes) by boiling for 15 minutes. All manipulations were completed within 90 seconds before the solution became viscous. Thrombin (0.5 μL of 5000 U/mL) was added to the infinity reaction, and it was allowed to incubate for 30 minutes before quenching. This timepoint served as a measure of complete fibrinopeptide release. All samples were centrifuged for 15 minutes at 4°C to spin down any clots that formed, and 200 μL of the supernatant was analyzed by HPLC (Shimazdu) on a Supelco Discovery C18 reverse phase column using the following gradient run at 1 mL/minute:

0 – 1 minute	0% B
1 – 2 minutes	0 – 15% B
2 – 25 minutes	15 – 36% B
25 – 26 minutes	36 – 80% B
26 – 27 minutes	80 – 0% B
27 – 32 minutes	0% B

Buffer A is 25 m*M* NaH_2PO_4/Na_2HPO_4 pH 6.0; buffer B is 50% acetonitrile in 25 m*M* NaH_2PO_4/Na_2HPO_4 pH 6.0 [12]. Elution positions of fibrinopeptide A and fibrinopeptide B were determined based on the elution profile of synthetic FpA and FpB standards. The area under each peak of interest was integrated by Shimadzu's Class-VP v. 5.03 data processing program.

Data is plotted as % fibrinopeptide released (setting 100% release from infinity timepoint) versus time. k_1, the first order rate constant for FpA release, is determined by fitting the FpA release curve as follows:

$$\%FpA = (1 - e^{(-k_1 t)})*100$$

k_2 is the first order rate constant for FpB release after the release of FpA and is determined from the following equation:

$$\%FpB = (1 + [k_2/(k_1 - k_2)]e^{(-k_1 t)} - [k_1/(k_1 - k_2)]e^{(-k_2 t)})*100$$

For each fibrinopeptide, the specificity constant of thrombin for the fibrinopeptide is determined as shown:

$$k_{cat} / K_M = k_{(1 \text{ or } 2)} / [IIa]$$

Results

Polymerization

I measured polymerization by turbidity for 0.15 mg/mL recombinant γ/γ, γ′/γ′, and γ/γ′ fibrinogen at 1.0 n*M* thrombin with 10 μ*M*, 2.0 m*M*, or 5.0 m*M* $CaCl_2$. Each variant was polymerized at each calcium concentration in three separate experiments. As shown in Figure 3.1 A and B, γ′/γ′ fibrinogen displayed a longer lag time compared to γ/γ and γ/γ′ fibrinogen at 10 μ*M* $CaCl_2$. This parameter was normalized at higher concentrations of calcium (Figure 3.1 C-F). Lag times for γ/γ′ fibrinogen were the same as for γ/γ fibrinogen at each concentration of calcium. The V_{max} value, or maximal rate of polymerization, was lower for both γ′/γ′ and γ/γ′ fibrinogen at each concentration of calcium than it was for γ/γ fibrinogen. Similarly, at each calcium concentration, the final absorbance of clots formed from fibrinogens containing a γ′ chain was lower than that of fibrinogens without a γ′ chain. In particular, γ′/γ′ fibrinogen displayed the lowest final absorbance, while γ/γ′ fibrinogen had a final absorbance that was intermediate between γ/γ and γ′/γ′.

Table 3.1 contains the values for lag time, maximal rate of polymerization, and final absorbance measured for each fibrinogen variant at three concentrations of calcium.

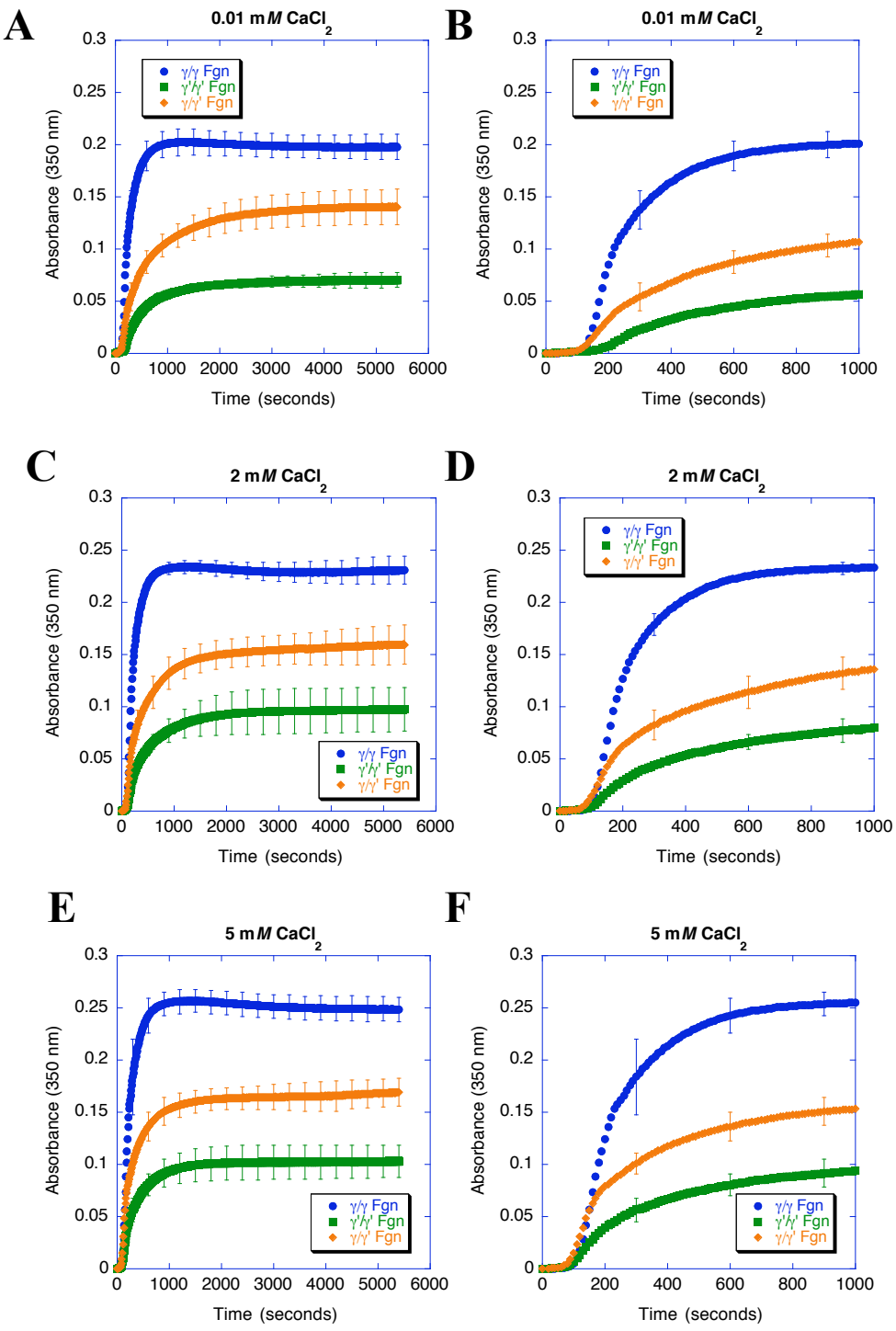

Figure 3.1. Polymerization of recombinant γ/γ (blue), γ′/γ′ (green), and γ/γ′ (orange) fibrinogen at three concentrations of calcium, 0.01 m*M* (A,B), 2.0 m*M* (C,D), and 5.0 m*M* (E,F). Polymerization was initiated by addition of 1.0 nM thrombin to 0.15 mg/mL fibrinogen, and monitored at 350 nm every 10 seconds for 90 minutes. On the left are full polymerization curves (A,C,E); a close-up of the lag time is on the right (B,D,F). Curves are the mean of 3 experiments. Error bars indicate + or – 1 standard deviation.

Polymerization Parameters for γ/γ, γ′/γ′, and γ/γ′ Fibrinogen

Lag Time (seconds)			
	γ/γ Fibrinogen	**γ'/γ' Fibrinogen**	**γ/γ' Fibrinogen**
0.010 m*M* $CaCl_2$	117 +/- 12	175 +/- 7*	120 +/- 30
2.0 m*M* $CaCl_2$	100 +/- 20	97 +/- 12	83 +/- 25
5.0 m*M* $CaCl_2$	103 +/- 25	90 +/- 17	75 +/- 7
Maximal Rate (milliunits/ minute)			
	γ/γ Fibrinogen	**γ'/γ' Fibrinogen**	**γ/γ' Fibrinogen**
0.010 m*M* $CaCl_2$	83 +/- 8	11 +/- 0.2*	26 +/- 6*
2.0 m*M* $CaCl_2$	108 +/- 7	19 +/- 4*	40 +/- 8*
5.0 m*M* $CaCl_2$	108 +/- 17	25 +/- 9*	50 +/- 0.2*
Final Absorbance			
	γ/γ Fibrinogen	**γ'/γ' Fibrinogen**	**γ/γ' Fibrinogen**
0.010 m*M* $CaCl_2$	0.198 +/- 0.012	0.070 +/- 0.007*	0.140 +/- 0.017*
2.0 m*M* $CaCl_2$	0.231 +/- 0.014	0.097 +/- 0.021*	0.160 +/- 0.019*
5.0 m*M* $CaCl_2$	0.248 +/- 0.011	0.103 +/- 0.015*	0.169 +/- 0.013*

Table 3.1. Parameters are listed for lag time, V_{max}, and final absorbance for each fibrinogen at three concentrations of calcium. * Indicates p values less than 0.05, indicating greater than 95% probability of significant variation from γ/γ fibrinogen.

Fibrinopeptide Release

Fibrinopeptide release from fibrinogen γ′ variants was measured at 0.10 mg/mL fibrinogen and 0.10 n*M* (0.01 U/mL) thrombin. Figure 3.2 shows the results of this experiment, and Table 3.2 shows the kinetic constants determined for each fibrinogen. Data was fit to two sequential first-order rate equations as described in Methods. The rate constant for FpA release was k_1, and the specificity constant of thrombin for FpA was k_1/K_M. Both γ′/γ′ and γ/γ′ fibrinogen released FpA at the same rate as did γ/γ fibrinogen. Release of FpB from γ′/γ′, however, was faster compared to γ/γ or γ/γ′ fibrinogen, as shown by a greater rate constant for FpB release (k_2). The specificity constant for the thrombin FpB interaction (k_2/K_M) also increased for γ′/γ′ fibrinogen.

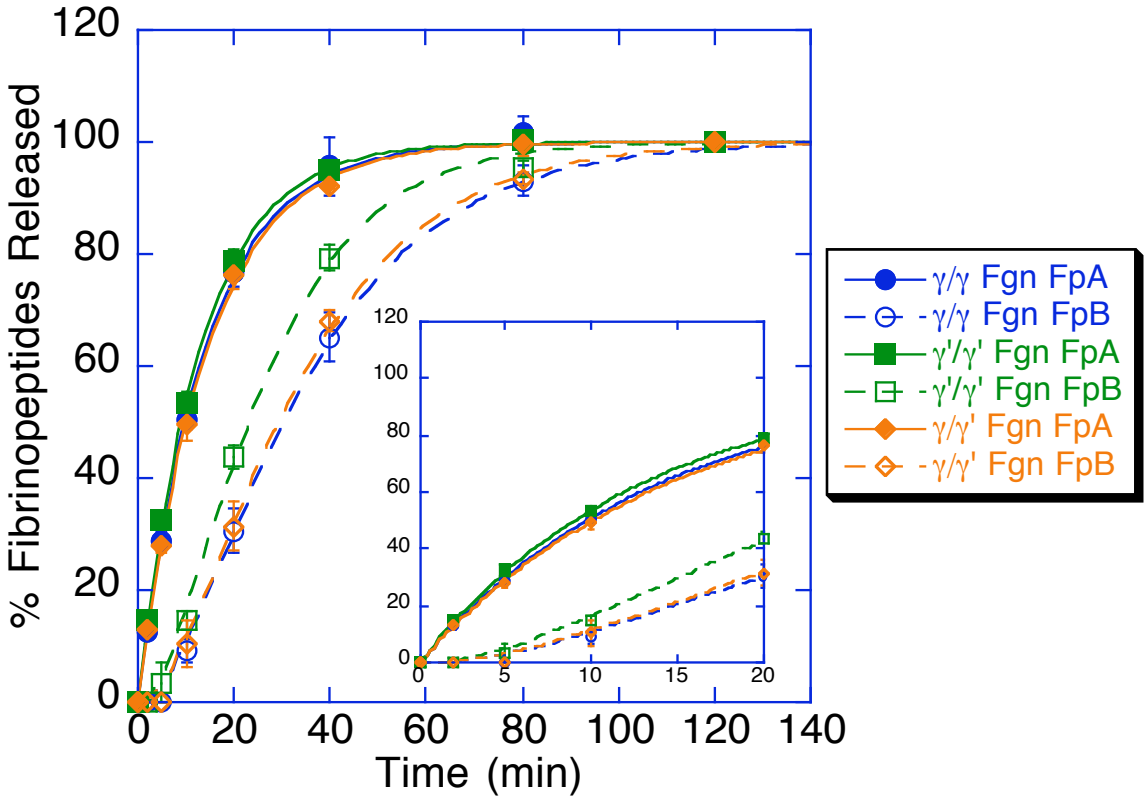

Figure 3.2. Fibrinopeptide release monitored by HPLC from 0.10 mg/mL recombinant fibrinogen by addition of 0.10 n*M* thrombin. FpA release in closed symbols and FpB release in open symbols from normal γ/γ fibrinogen (blue circles), γ′/γ′ fibrinogen (green squares), or γ/γ′ fibrinogen (orange diamonds). The first 20 minutes of the reaction are shown in detail in the inset.

Fibrinopeptide Release Rate Constants

	FpA		**FpB**	
Fibrinogen	k_1 (min^{-1})	k_1/K_M ($s^{-1}M^{-1}$)	k_2 (min^{-1})	k_2/K_M ($s^{-1}M^{-1}$)
γ/γ	0.071 +/- 0.004	$1.2x10^7$ +/- $5.8x10^5$	0.045 +/- 0.003	$7.8x10^6$ +/- $6.9x10^5$
γ'/γ'	0.077 +/- 0.005	$1.3x10^7$ +/- $5.8x10^5$	0.069 +/- 0.006*	$1.2x10^7$ +/- $1.0x10^6$*
γ/γ'	0.069 +/- 0.004	$1.2x10^7$ +/- $5.8x10^5$	0.049 +/- 0.003	$8.6x10^6$ +/- $4.7x10^5$

Table 3.2. k_1 and k_1/K_M are rate constant and specificity constant for FpA release; k_2 and k_2/K_M are rate constant and specificity constant for FpB release. Values are shown +/- standard deviation from 3 experiments. * indicates p values for comparison with normal γ/γ fibrinogen < 0.05, meaning > 95% probability of significant variation from γ/γ fibrinogen.

Discussion

We measured polymerization by turbidity for recombinant γ/γ, γ′/γ′, and γ/γ′ fibrinogen at 10 μ*M*, 2.0 m*M*, and 5.0 m*M* calcium. Lag time comparison indicated that γ′/γ′ fibrinogen formed protofibrils significantly more slowly than γ/γ or γ/γ′ fibrinogen at 10 μM $CaCl_2$. Increasing the concentration of calcium to either 2.0 or 5.0 m*M* resulted in γ′/γ′ fibrinogen displaying a lag time similar to γ/γ fibrinogen. These results suggest that γ′ chain homodimers impede protofibril formation in the absence of calcium, but the addition of calcium abrogates this effect.

At each concentration of calcium investigated, both γ′ variants displayed statistically significant lower V_{max} values compared to γ/γ fibrinogen. These observations demonstrate that the presence of the γ′ chain dramatically slows lateral aggregation during fiber formation.

Similar to the pattern observed for V_{max} values, the final absorbances of clots formed from either γ′/γ′ or γ/γ′ fibrinogens were decreased at each concentration of calcium compared to clots formed from γ/γ fibrinogen. Because the final absorbance of a clot correlates with fiber thickness, we conclude that the presence of a γ′ chain decreases the diameter of fibers formed compared to those formed from γ/γ fibrinogen.

Weisel and Nagaswami's kinetic model, described in the Introduction to this chapter, provides a framework to describe what may be taking place at a molecular level for each fibrinogen variant, and thus highlight the differences in polymerization caused by the presence of the γ′ chain [5]. At 2 m*M* $CaCl_2$, close to physiological concentration of calcium, both γ′/γ′ and γ/γ′ fibrinogen displayed decreased final absorbance and V_{max}, with a similar lag time compared to γ/γ fibrinogen. The polymerization kinetic model suggests that this

pattern of parameter changes is caused by an increase in the fiber initiation rate constant for these fibrinogens, and thus a decrease in the rate of lateral aggregation [5]. Such a decrease in lateral aggregation explains the diminished rate of polymerization as well as the presence of thinner fibers in γ′ clots. The fibrinopeptide release results, however, showed that FpB was released more quickly from γ′/γ′ fibrinogen than from γ/γ fibrinogen. This particular observation is not consistent with our model of decreased lateral aggregation and remains unexplained.

These data are not the first presented about the polymerization of γ′-chain containing fibrinogen. Several reports have been published in recent years characterizing the polymerization behavior of γ/γ′ fibrinogen purified from plasma fibrinogen. Our results may be compared with the previous studies, keeping in mind that we have studied recombinant fibrinogen while most previous work has employed plasma-derived protein. Protein purified from plasma may always contain unidentified polymorphisms, variability in post-translational processing, or significant and variable proteolytic degradation. Recombinant protein, however, provides a population of protein with the exact mutation of interest.

In 2003, Cooper and coworkers published the results of a study of γ/γ′ fibrinogen separated from γ/γ fibrinogen by DE-52 ion-exchange chromatography of plasma fibrinogen [13]. At 10 m*M* $CaCl_2$, they demonstrated a slower rate of polymerization and lower final absorbance for γ/γ′ fibrinogen compared to γ/γ fibrinogen with similar lag times between the two populations. This result is very similar to our observations for γ/γ′ fibrinogen at 5 m*M* $CaCl_2$. From fibrinopeptide release experiments, they showed a similar rate of FpA release between the two populations, but found FpB to be released more slowly from γ/γ′ fibrinogen than from γ/γ fibrinogen [13]. We found no difference in FpB release between γ/γ and γ/γ′

fibrinogen. Notably, these studies were performed at significantly higher concentrations of fibrinogen, thrombin, and calcium than were ours which, along with the fact that they used plasma-derived protein and we studied recombinant fibrinogen, may help to explain the differences in fibrinopeptide release results.

In another study, Siebenlist and coworkers compared fibrinopeptide release for γ/γ′ fibrinogen compared to γ/γ fibrinogen. They prepared γ/γ and γ/γ′ fibrinogen from DE-52 chromatographed plasma fibrinogen and took a further step to remove factor XIII by ammonium-sulfate precipitation of the γ/γ′ fraction. They found from this study that both FpA and FpB release are significantly delayed from γ/γ′ fibrinogen compared to γ/γ [14]. These results are certainly inconsistent with both our results and those of Cooper *et al* [13]. Again, they used significantly higher concentrations of fibrinogen and thrombin in these studies than we used in ours, but with no calcium, an essential cofactor, added to the reaction. It is also of concern that they performed an extra ammonium sulfate precipitation step on the γ/γ′ fibrinogen to remove factor XIII, but this step was not performed on the γ/γ sample. Perhaps a particular population of γ/γ′ fibrinogen was removed during this step leaving something less active in polymerization than would be the entire fraction of γ/γ′ fibrinogen.

Polymerization of recombinant γ′/γ′ fibrinogen has been studied by electron microscopy, but not by turbidity or fibrinopeptide release [15]. Recombinant γ/γ or γ′/γ′ fibrinogen expressed by BHK cells was clotted at 4.4 μ*M* (1.5 mg/mL) with 5 m*M* $CaCl_2$ and 0.9 U/mL thrombin. These clots were then analyzed by laser scanning confocal microscopy and scanning electron microscopy. The structural differences between the two clots were small, with γ′/γ′ fibrinogen displaying 25% less densely packed and 7% thicker fibers [15]. These differences were not statistically significant, however, and their variability from our

fiber thickness results may be explained by experimental differences between the use of turbidity versus electron microscopy for monitoring clot structure.

Comparison of our polymerization and fibrinopeptide release data with the results presented by others [13], [14], [15] suggests that the role of the γ′ chain in polymerization is complicated. In our work with recombinant γ′ fibrinogen variants, we are sure of the presence of pure populations of both γ′/γ′ and γ/γ′ fibrinogen molecules. There are no differences in post-translational modifications among recombinant proteins, nor do we have contamination from any other coagulation proteins. Therefore, our observation that the presence of the γ′ chain in fibrinogen leads to decreased lateral aggregation is the best starting point for analysis of the role of the γ′ chain in polymerization.

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CHAPTER FOUR

FACTOR XIII BINDING TO FIBRINOGEN

Summary

In this chapter I have addressed the question of how factor XIII binds to fibrinogen when it is in both its zymogen form and its active form. I measured binding to recombinant γ/γ fibrinogen, recombinant γ′/γ′ fibrinogen, recombinant γ/γ′ fibrinogen, recombinant Aα251 fibrinogen, and plasma fibrinogen. To quantitate binding, I developed both an ELISA assay with increasing concentrations of factor XIII binding to fibrinogen immobilized on a plate, as well as an immunoprecipitation assay in which I pull down fibrinogen and any factor XIII bound to it in solution. Inhibition experiments show that soluble fibrinogen could prevent binding to immobilized fibrinogen in the ELISA assay. The results of these experiments demonstrate that the γ′ chain plays no part in binding to either factor XIII zymogen or activated factor XIIIa.

Introduction

Although some of the literature addressing the question of factor XIII binding to fibrinogen was discussed in the Introduction chapter of this document, I will present here a more complete overview of previous work. The history of studies measuring factor XIII – fibrinogen interactions is filled with debate and controversy, and some of these unresolved conflicts are described below.

Factor XIII Zymogen Binding to Fibrinogen

An early study of factor XIII binding to fibrinogen was undertaken by Greenberg and Shuman using ^{125}I-labeled plasma or platelet factor XIII and fibrinogen coupled to latex or acrylonitrile beads. Binding assays were performed by incubating the labeled factor XIII with the bead-coupled fibrinogen, and bound material was captured on a 0.4 μm filter.

Radioactivity measured on the beads corresponded to bound factor XIII. From this assay, a K_d for factor XIII zymogen binding to plasma fibrinogen was determined to be 1.4 +/- 0.06 x 10^{-8} *M*, suggesting that factor XIII is bound to fibrinogen in plasma [1]. The concentration of factor XIII in plasma is about 0.15 μ*M* [2], compared to around 7.5 μ*M* fibrinogen [3].

Following Greenberg and Shuman's publication, a plethora of investigations by both the Greenberg and Shafer groups described the enhancement of factor XIII activation when it is bound to fibrin [4], [5], [6], [7], [8]. A kinetic study using HPLC of the release of FpA, FpB, and FXIII activation peptide indicated that AP release from factor XIII is faster when fibrinogen is included in the reaction mixture, and that FpA is likely cleaved before FXIII AP, suggesting that des-A fibrin is the true cofactor for FXIII activation [4]. Greenberg and Miraglia extended these observations by showing that polymerized fibrin was the actual species necessary for promoting FXIIIa formation [5]. They included the polymerization inhibitor GPRP with factor XIII, thrombin, and fibrinogen, and found the fibrinogen-catalyzed rate enhancement for factor XIII activation disappeared [5]. Once polymerized fibrin is cross-linked by factor XIIIa, however, its catalytic effect toward factor XIII zymogen activation is lost. The binding of factor XIII to fibrinogen and fibrinogen's cofactor activity are proposed as a regulatory mechanism for factor XIIIa. By binding to fibrinogen, the enzyme is localized to where it will be needed. It is not activated efficiently until fibrin begins to polymerize, and is no longer activated once it has performed its function and fibrin is cross-linked [6].

Another conclusion from the Greenberg and Shuman binding study is, however, more controversial. In their bead-coupled fibrinogen plus ^{125}I-FXIII binding assays, they demonstrated that unlabeled platelet factor XIII could compete away both platelet and plasma

factor XIII binding to fibrinogen. This observation led them to conclude that the fibrinogen – factor XIII interaction takes place through the A subunits of factor XIII [1]. They confirmed their observations in a later study using platelet-poor plasma. When ^{125}I-labeled platelet factor XIII was added to this plasma sample, 85% of it went into the pellet when fibrinogen was spun down after polymerization by protamine sulfate which causes polymerization in the absence of FpA or FpB cleavage. The authors concluded from this finding that plasma factor XIII also binds to fibrinogen via the A subunits because platelet factor XIII can do so [9]. While I have reservations about whether protamine sulfate polymerized fibrinogen has the same binding behavior as monomeric fibrinogen, I find the competition of platelet factor XIII with plasma factor XIII compelling evidence that there is some binding site available on the A subunits of factor XIII for fibrinogen.

In contrast to this finding, Hornyak and Shafer showed through elegant binding experiments in 1992 that, instead, the B subunits are responsible for binding to fibrinogen. They prepared active site blocked and radiolabeled carbamylmethyl forms of platelet factor XIII (A_2) and factor XIIIa (A_2ai). They measured the binding of these forms of FXIII to fibrin II by adding acid-solubilized fibrin II to a solution of each, incubating for 30 minutes, and then spinning down the clot and measuring the amount of radioactivity incorporated into it. Active-site inhibited FXIIIa (A_2ai) bound fibrin with a K_d of 2.1 μM, while unactivated platelet FXIII (A_2) had a K_d of 14 μM for fibrin. Binding of factor XIII zymogen (A_2B_2) to fibrin, however, was much stronger than either of these interactions with a K_d of 0.20 μM. In addition, factor XIII zymogen did not disrupt binding of FXIIIa to fibrin [10]. In contrast with Greenberg and Shuman's conclusion that plasma and platelet factor XIII binding both occur through the A subunits and with the same dissociation constant, these findings show

that the B subunits of plasma factor XIII provide a high-affinity binding site for fibrin. There are significant differences between these two studies such as the use of fibrinogen versus fibrin and the method used to detect binding, which may explain some of their differing conclusions.

Based on Hornyak and Shafer's rigorous study, however, it has become generally accepted that factor XIII A subunits bind to fibrinogen at a different site than do factor XIII B subunits. Procyk and coworkers localized the binding site for active factor XIIIa (A_2a) to the C-terminal part of the Aα chain of fibrinogen using recombinant factor XIII which is composed of just A subunits. They radiolabeled this factor XIII and measured the amount of buffer required for it to permeate through a fibrin clot formed in a small column. These permeation experiments showed that unactivated rFXIII (A_2) quickly percolated through the clot while rFXIIIa (A_2a) took longer, suggesting that only the active form of the enzyme binds when B subunits are absent. Treatment of the clot with an antibody to the C-terminal region of the Aα-chain blocked rFXIIIa binding to the clot, as did percolation of a fibrinogen fragment corresponding to Aα241-476 along with the rFXIIIa. These results suggest that activated A subunits of factor XIII bind the αC domains of fibrinogen [11].

Localization of the binding site on fibrinogen for factor XIII zymogen began with an immunoblotting study in 1987 in which intact fibrinogen or its fragments (X, D_1, D_2, D_3, D-dimer, and E) were run on reduced or non-reduced SDS-PAGE gels and transferred to nitrocellulose membranes. These membranes were then incubated with factor XIII zymogen, washed, and probed with an anti-FXIII antibody to determine which fragments or chains of fibrinogen bound to factor XIII. Results indicated that X, D_1, D_2, D_3, and D-dimer, but not E fragment bound FXIII while Aα and Bβ chains, but not γ chain also bound FXIII [12]. The

blots shown in this paper are not very clear, and the results of this investigation have been mostly disregarded.

A more widely accepted study was published in 1996 by Siebenlist and coworkers. They observed that factor XIII co-purifies with the γ′ chain of fibrinogen, rather than with the γ chain, and suggested that the unique sequence at the C-terminus of this chain may provide a binding site for factor XIII zymogen. When a mixture of γ/γ fibrinogen and factor XIII are separated by ion-exchange chromatography, they elute as two separate peaks with fibrinogen in the early peak and factor XIII in the later peak, as shown by a factor XIII activity assay of each fraction. However, when γ/γ′ fibrinogen and factor XIII are mixed, they elute as a single peak much later than the peaks described above, with factor XIII activity found throughout the peak. Size-exclusion chromatography gave similar results; individual peaks are observed with a mixture of γ/γ fibrinogen and factor XIII zymogen, while the factor XIII peak shifts up to the position of fibrinogen when mixed with γ/γ′ fibrinogen. These studies suggest that the γ′ chain does, indeed, provide a binding site for factor XIII zymogen [13].

To further characterize this binding site, Moaddel *et al*. measured the dissociation constant for FXIII-γ/γ fibrinogen complexes and FXIII-γ/γ′ fibrinogen complexes by analytical ultracentrifugation. Both types of fibrinogen were purified from plasma. They found an association constant for γ/γ fibrinogen + FXIII zymogen of $1.3 \times 10^{8} M^{-2}$ and a 20-fold stronger association constant for γ/γ′ fibrinogen + FXIII zymogen of $3.6 \times 10^{9} M^{-2}$. Inclusion of a peptide mimicking the γ′ sequence in ultracentrifugation experiments confirmed that this region is responsible for the increased affinity [14]. Recently, however, the conclusions of this study have been called into question. Because it has been shown that factor XIII zymogen can cross-link fibrinogen in the presence of physiological levels of

$CaCl_2$, it is almost certain that such cross-linking took place during the extensive incubation of fibrinogen and factor XIII with 1m*M* $CaCl_2$ in these ultracentrifugation experiments. Siebenlist and coworkers suggest that the noncovalent fibrinogen + FXIII complexes Moaddel's group thought they were measuring in the analytical ultracentrifuge were, in fact, cross-linked fibrinogen molecules [15].

I will contribute to this ongoing discussion about the role of the γ′ chain in factor XIII binding to fibrinogen by using recombinant fibrinogen variants. These variants allow me to isolate the role of the γ′ chain with respect to factor XIII binding. I have compared factor XIII binding to fibrinogen with two γ′ chains, one γ and one γ′, or two γ chains. These three variants only differ in the presence of the γ′ chain; they will not display the variability present in any sample of fibrinogen isolated from a collection of human plasma. Use of an ELISA to measure binding will remove from consideration the problem of fibrinogen cross-linking by factor XIII zymogen. Instead of a two day incubation as was required in Moaddel's ultracentrifuge experiments [14], the two proteins must only be incubated for an hour during an ELISA. In addition, the fibrinogen is immobilized on the plate, thereby limiting the extent of cross-linking that may take place to only those molecules immobilized in direct contact with one another. This format has allowed me to draw clear conclusions about the role of the γ′ chain in binding to factor XIII.

Materials and Methods

Materials.

Human plasma fibrinogen depleted of plasminogen, fibronectin, and factor XIII (lots 030411 and 050518) was purchased from American Diagnostica (Stamford, CT). Human

factor XIII (lots 850A and 3040P) and human α-thrombin (lot 2690PR) were purchased from Enzyme Research Laboratories (South Bend, IN). Recombinant factor XIII was a kind gift from Dr. Rasmus Rojkjaer at Novo Nordisk (Princeton, NJ). Hirudin (lot B68947) was obtained from Calbiochem (San Diego, CA). Bovine serum albumin (lot 103161376) was purchased from Sigma-Aldrich (St. Louis, MO). Antibodies 4A5, 2G2H9, Y-18, polyclonal anti-human fibrinogen, and HRP-conjugated goat anti-rabbit and anti-mouse were described in Chapter Two. A monoclonal antibody to fibrinogen Bβ chain was a kind gift from Dr. John Shainoff (Cleveland State University, Cleveland, OH). Sheep anti-factor XIII A-chain antibody (lotAP155-BR5) and HRP-conjugated donkey anti-sheep IgG (lot HAP1270-B121) were purchased from Affinity Biologicals (Ancaster, Ontario). SureBlue peroxidase substrate was obtained from KPL (Gaithersburg, MD). All chemicals were purchased from Fisher.

Protein Preparation

Recombinant γ′/γ′ and γ/γ′ fibrinogen were expressed and purified as described in Chapter Two. Normal, recombinant γ/γ fibrinogen and recombinant Aα251 fibrinogen in which the C-terminus of the Aα chain is truncated at residue 251 were prepared as described [16], [17] and purified as described [18]. Both fibrinogen (plasma and recombinant) and factor XIII (plasma and recombinant) were dialyzed extensively against 20 m*M* HEPES pH 7.4, 150 m*M* NaCl (HBS).

ELISA Binding Assay

Wells of a Corning half-area untreated microtiter plate were coated with 50 μL of 0.029 μ*M* fibrinogen diluted in HBS + 1 m*M* $CaCl_2$ (HBS+Ca). After one hour, this solution was removed and the plate was washed three times with 200 μL per well of HBS+Ca + 0.5%

Tween-20 (HBST+Ca). All washes were performed in this manner. In some assays, fibrinogen was converted to fibrin by addition of 50 μL of 1 U/mL human α-thrombin for 1 hour, followed by washing, addition of 50 μL of 10 U/mL hirudin for 20 minutes, and another wash. In all assays, fibrin(ogen) was blocked with 100 μL of 0.1% BSA in HBS+Ca (BSA/HBS+Ca) for 1 hour, followed by washing.

Active-site inhibited recombinant factor XIII (rFXIIIai) was prepared for some assays by diluting rFXIII to 20 μ*M* in HBS + 10 m*M* $CaCl_2$. Human α-thrombin diluted in HBS+Ca was added to a final concentration of 32 U/mL, and the solution was incubated for 45 minutes at 37°C to allow for factor XIII activation. After activation, hirudin was added to 320 U/mL to inhibit thrombin, and a final concentration of 20 m*M* iodoacetic acid was added to block the active site of recombinant factor XIII.

Serial dilutions of factor XIII (FXIII), unactivated recombinant factor XIII (rFXIII) or recombinant, activated, active-site inhibited factor XIII (rFXIIIai) were prepared in 50 μL in HBS+Ca ranging from 0.40 μ*M* to 0.00040 μ*M* FXIII, 5.0 – 0.050 μ*M* rFXIII, or 0.80 to 0.00080 μ*M* rFXIIIai. These serial dilutions were added to wells 1 – 11 of the fibrinogen-coated microtiter plate, and 50 μL of HBS+Ca was added to well 12. After 1 hour of incubation, the factor XIII was removed, and the plate was washed. 50 μL of anti-factor XIII antibody diluted 1:3000 in BSA/HBS+Ca was added to each well for one hour and washed. HRP-conjugated donkey anti-sheep antibody was diluted 1:10000 in BSA/HBS+Ca and 50 μL was added to each well for 1 hour followed by removal and three washes. The plate was developed with 50 μL of Sure Blue peroxidase substrate and fixed with 50 μL of 1 *M* phosphoric acid. Absorbance was measured at 450 nm on a SpectraMax 340PC microtiter plate reader.

γ and γ′ Chain Accessibility ELISA

Serial dilutions of recombinant γ/γ or γ′/γ′ fibrinogen were prepared in 50 μL of HBS+Ca from 100 μg/mL to 0.098 μg/mL and placed in wells 1 – 11 of a microtiter plate with 50 μL of HBS+Ca in well 12. After 1 hour of incubation, the plate was washed three times with HBST+Ca and blocked with BSA/HBS+Ca as described above. It was probed with 1:5000 4A5 or 1:1000 2G2H9, followed by 1:5000 HRP-conjugated goat anti-mouse secondary antibody, all diluted in BSA/HBS+Ca. The plate was developed as described above.

Inhibition by Soluble Fibrinogen

Microtiter plates were coated with plasma or recombinant fibrinogen and blocked as described above. Thirty minutes before addition to the plate, 0.10 μ*M* FXIII or rFXIIIai was mixed with an equal volume of plasma fibrinogen serial dilutions in 0.1% BSA in HBS+Ca. The final concentration of (r)FXIII(ai) was 0.050 μ*M* and fibrinogen inhibitor concentrations ranged from 9.0 to 0.56 μ*M*. One well contained 0.050 μ*M* (r)FXIII(ai) with no inhibitor; another well contained buffer only. These mixtures were incubated on the microtiter plate for 1 hour, and the plate was exposed to antibody and developed as described above.

Immunoprecipitation Binding Assay

Plasma, recombinant γ/γ, or recombinant γ′/γ′ fibrinogen (0.29 μ*M*) and factor XIII (serial dilutions from 0.80 – 0.025 μ*M*) were incubated in 25 μL for one hour at room temperature. Y-18 sepharose beads were prepared according to the protocol described in Chapter Two for 4A5 beads. 20 μL of these beads were added to each reaction mixture and rotated for 2 hours at 4°C. Unbound factor XIII was removed by four 1-mL washes with 50 m*M* Tris pH 7.4, 300 m*M* NaCl, 0.1% TritonX-100, 0.05% Tween-20. The beads were

washed once more with HBS, and then all supernatant was removed. Beads were redissolved in 15 μL HBS + 4 μL 6X Laemmli buffer + 2 μL BME. These samples were run in duplicate on 11% gels and transferred to nitrocellulose. Along with them I ran a standard curve of each fibrinogen and a standard curve of factor XIII. One blot of each set was developed with a mixture of 1:5000 anti-Bβ chain and 1:4000 anti-FXIII A chain followed by 1:5000 HRP anti-mouse and 1:5000 HRP anti-sheep; the other was developed with 1:5000 DAKO polyclonal anti-fibrinogen and 1:5000 HRP anti-rabbit. Western blots were scanned and analyzed by densitometry. The concentrations of fibrinogen Bβ chain and factor XIII in each sample were determined by comparison to the fibrinogen and factor XIII standard curves. A binding curve was constructed by plotting the ratio of FXIII/fibrinogen (the concentration of FXIII brought down in each sample divided by the concentration of fibrinogen in that sample, as determined from the standard curve) versus the concentration of FXIII added to the sample.

Results

Measuring Factor XIII Binding to Fibrinogen

I developed an ELISA-type assay to determine K_d values for factor XIII binding to fibrinogen. In this assay, I immobilized fibrinogen variants on a microtiter plate, blocked with BSA, and added a range of concentrations of factor XIII, in the presence of 1 m*M* $CaCl_2$. These proteins incubated for an hour, and then I washed the plate and probed for factor XIII that remained bound to the fibrinogen with a primary antibody for factor XIII followed by an HRP-conjugated secondary antibody. I constructed binding curves by plotting the absorbance at 450 nm for each well versus the concentration of factor XIII

originally added to that well. Fitting the data to the binding equation shown below generated K_d values:

$$y = [(\text{Binding Max}) * x] / [x + K_d]$$

This equation also provides the absorbance that corresponds to maximal binding. However, the actual absorbance of any well depends upon not only the concentration of factor XIII present in that well, but also the precision with which antibody was diluted on that particular day, how fresh the chromogenic substrate was and therefore how quickly color developed, and at what stage of color development I quenched the reaction. Because the maximal absorbance did not relate to any single physical quantity, I chose to divide the absorbance measurements that composed each binding curve by the maximal absorbance determined in the equation above. Doing so generated a binding ratio, a measurement of the amount of factor XIII bound at any point divided by the maximal amount of factor XIII that could bind. No binding corresponded to 0, while maximal binding equaled 1. By converting absorbance data into this binding ratio, I was able to plot binding curves measured on different days on the same scale. This manipulation did not affect the K_d determined for each binding curve.

Factor XIII zymogen, a heterotetramer of two A and two B subunits, has been proposed to bind to the γ′ chain of fibrinogen [13]. If this hypothesis is true, then factor XIII should have a stronger K_d for binding recombinant γ′/γ′ and γ/γ′ fibrinogen than it does for recombinant γ/γ fibrinogen which doesn't have a γ′ chain. To ensure that recombinant fibrinogen behaves similarly to plasma fibrinogen, I have included plasma fibrinogen in each binding experiment. Because it has been suggested that factor XIIIa (A subunits only) binds to the αC domains of fibrinogen [11], I have also studied the binding of factor XIII zymogen to recombinant Aα251 fibrinogen. This fibrinogen is truncated at residue 251 of the Aα

chain and lacks αC domains. It was used as a control to show that αC domains are not involved in the binding of factor XIII zymogen to fibrinogen.

The binding curves for factor XIII zymogen binding to plasma, recombinant γ/γ, recombinant Aα251, recombinant γ′/γ′, and recombinant γ/γ′ fibrinogens as well as bovine serum albumin are shown in Figure 4.1. Very little difference existed between the curves, either at the low concentration points as shown in the inset, or at higher FXIII concentrations, as shown in the main figure. As shown in Table 4.1, the K_d values were, within error, the same for each fibrinogen variant, and around 0.04 μ*M*. The black X data points in Figure 4.1 correspond to factor XIII incubated with BSA immobilized on the microtiter plate. Because no significant binding occurred to the BSA, we concluded that the binding curves shown are fibrinogen specific.

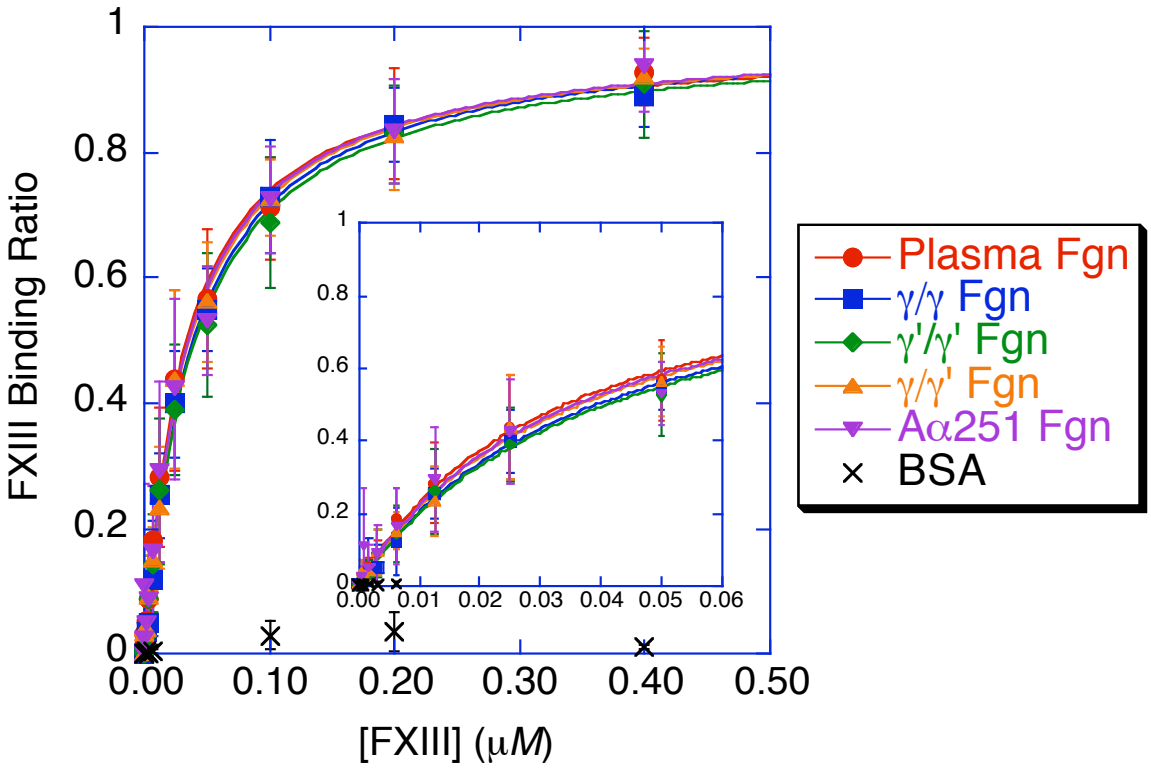


Figure 4.1. Binding of factor XIII zymogen to fibrinogen variants as measured by ELISA. Fibrinogen variants (0.029 μ*M*) or BSA were immobilized on a microtiter plate and blocked with BSA. A range of factor XIII concentrations were added (0.0040 – 0.40 μ*M*), incubated for 1 hour, and washed off. Factor XIII that remained bound to the immobilized fibrinogen was quantitated by ELISA. Data is plotted as binding ratio (absorbance at a particular point divided by maximal absorbance of the curve) versus concentration of FXIII added. Fibrinogens immobilized are shown in the legend. Inset shows detail of the lower concentration region of the graph, from 0 – 0.050 μ*M* factor XIII.

Factor XIII Binding to Fibrin(ogen)

Fibrin(ogen)	FXIII	Kd (u*M*)	Standard Deviation	P Value		
				(compared to γ/γ)		
plasma fgn	FXIII	0.043	0.016	0.42		
γ/γ fgn	FXIII	0.041	0.013			
γ'/γ' fgn	FXIII	0.047	0.027	0.33		
γ/γ' fgn	FXIII	0.040	0.020	0.45		
Aα251 fgn	FXIII	0.040	0.017	0.43		
Fibrin(ogen)	**FXIII**	**Kd (u*M*)**	**Standard Deviation**	**P Value**	**P Value**	
				(compared to γ/γ)	(compared to fgn + FXIII)	
plasma Fn	FXIII	0.025	0.008	0.12	< 0.05	
γ/γ Fn	FXIII	0.018	0.003		< 0.05	
γ'/γ' Fn	FXIII	0.021	0.002	0.10	0.08	
γ/γ' Fn	FXIII	0.021	0.002	0.20	0.13	
Aα251 Fn	FXIII	0.034	0.020	0.12	0.32	
Fibrin(ogen)	**FXIII**	**Kd (u*M*)**	**Standard Deviation**	**P Value**	**P Value**	
				(compared to γ/γ)	(compared to fgn + FXIII)	
plasma Fgn	rFXIII	1.1	0.4	0.33	< 0.05	
γ/γ Fgn	rFXIII	1.2	0.4		< 0.05	
γ'/γ' Fgn	rFXIII	1.0	0.3	0.26	< 0.05	
γ/γ' Fgn	rFXIII	1.2	0.7	0.48	< 0.05	
Aα251 Fgn	rFXIII	1.2	0.3	0.47	< 0.05	
Fibrin(ogen)	**FXIII**	**Kd (u*M*)**	**Standard Deviation**	**P Value**	**P Value**	
				(compared to γ/γ)	(compared to fgn + FXIII)	
plasma Fgn	rFXIIIai	0.032	0.018	0.38	0.14	
γ/γ Fgn	rFXIIIai	0.030	0.009		0.05	
γ'/γ' Fgn	rFXIIIai	0.029	0.007	0.48	0.16	
γ/γ' Fgn	rFXIIIai	0.029	0.007	0.48	0.21	
Aα251 Fgn	rFXIIIai	0.035	0.020	0.31	0.35	
Fibrin(ogen)	**FXIII**	**Kd (u*M*)**	**Standard Deviation**	**P Value**	**P Value**	**P Value**
				(compared to γ/γ)	(compared to fn + FXIII)	(compared to fgn + rFXIIIai)
plasma Fn	rFXIIIai	0.012	0.006	0.34	< 0.05	< 0.05
γ/γ Fn	rFXIIIai	0.014	0.006		0.22	< 0.05
γ'/γ' Fn	rFXIIIai	0.011	0.004	0.23	< 0.05	< 0.05
γ/γ' Fn	rFXIIIai	0.018				
Aα251 Fn	rFXIIIai	0.020	0.013	0.33	0.17	0.37

Table 4.1. K_d values for factor XIII binding to fibrinogen and fibrin. Student's t-tests are used to determine p values for comparison among variants or from one set of reactants to another. P values less than 0.05 are highlighted in red, indicating a greater than 95% probability that the difference is significant.

To show whether the γ′ chain was accessible to factor XIII when fibrinogen was immobilized on an ELISA plate, I performed an ELISA assay with 2G2H9, an anti-γ′ C-terminus antibody, as well as a control experiment with 4A5, an anti-γ chain C-terminus antibody. I prepared serial dilutions of recombinant γ′/γ′ or γ/γ fibrinogen and used each to coat two rows of an ELISA plate. After blocking with BSA, I developed one row of each fibrinogen with each antibody. Figure 4.2a shows that probing with the anti-γ chain antibody, 4A5, generated a dose-dependent curve up to the point of saturation for γ/γ fibrinogen, but that there was very little reaction with γ′/γ′ fibrinogen. Similarly, as shown in Figure 4.2b, when anti-γ′ antibody (2G2H9) was used, it recognized γ′/γ′ fibrinogen in a dose-dependent manner, but did not react with γ/γ fibrinogen. These results show that when fibrinogen is immobilized, both the γ and the γ′ chain C-termini are accessible. Thus, the C-terminus of the γ′ chain, the proposed factor XIII zymogen binding site, is available for binding to factor XIII in the ELISA assay.

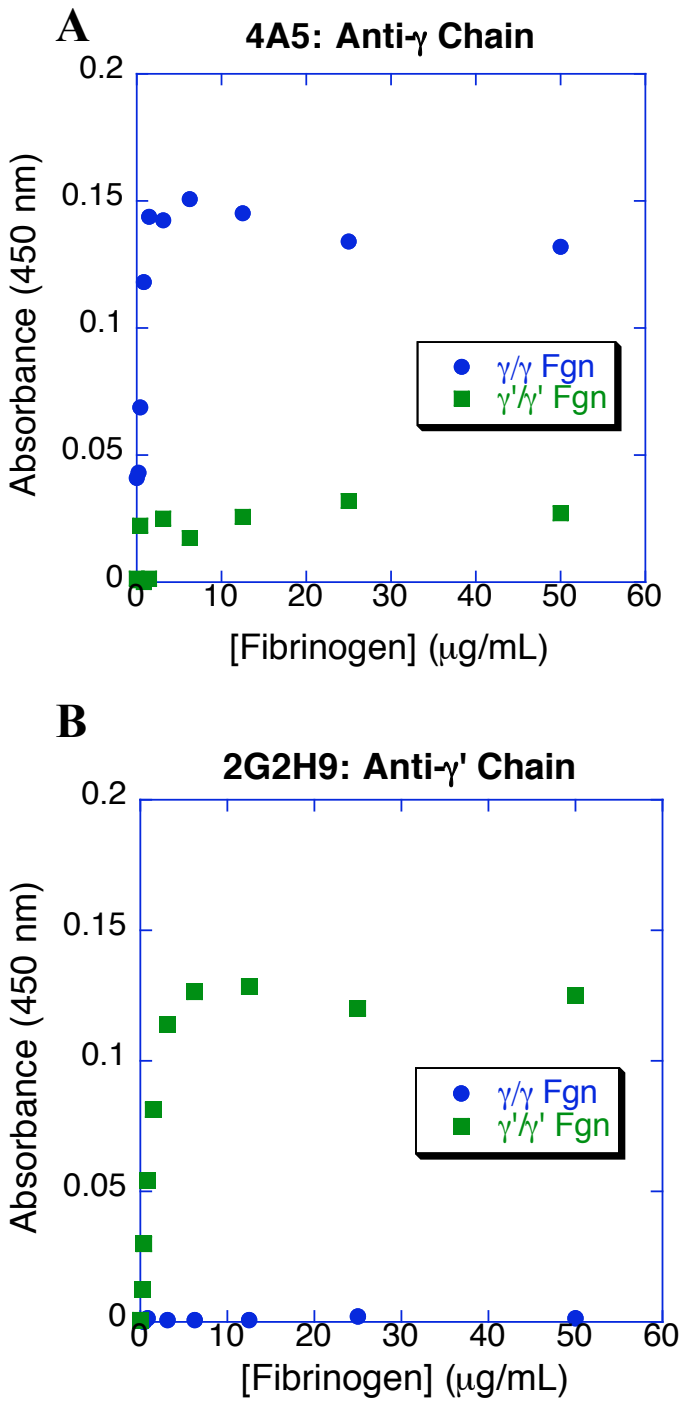


Figure 4.2. A) Fibrinogen (γ/γ or γ′/γ′) was immobilized on an ELISA plate and probed with 4A5, an anti-γ chain C-terminus antibody. The γ/γ fibrinogen is recognized in a dose-dependent manner, while very little antibody binds to γ′/γ′ fibrinogen. B) The same fibrinogens are immobilized, but probed with 2G2H9, an anti-γ′ chain antibody. In this situation, the γ′/γ′ fibrinogen is recognized in a dose-dependent fashion while virtually no antibody binds to γ/γ fibrinogen.

I performed inhibition experiments with soluble plasma fibrinogen to confirm the results of my ELISA binding experiments. I coated an ELISA plate with variant fibrinogens and blocked as described, but then incubated 0.050 μ*M* factor XIII with varying concentrations of plasma fibrinogen in 0.1% BSA for 30 minutes before addition to the ELISA plate, then probed with an anti-factor XIII antibody. To determine the ratio of factor XIII bound at each concentration of inhibiting fibrinogen, I divided the absorbance at that point by the total absorbance for 0.050 μ*M* factor XIII binding when no inhibitor is present. This calculation left me with the percentage of total factor XII bound at each point, expressed as a decimal. I plotted this value versus the concentration of fibrinogen added as inhibitor, and fit the data to the following equation for exponential decay:

$$y = a + b*\exp(-c*x)$$

where a = the percentage of factor XIII binding that corresponds to maximum inhibition

b = the span of inhibition that can take place

c = EC_{50} value for the inhibitor

The results of the inhibition experiments for factor XIII zymogen binding to fibrinogen variants are shown in Figure 4.3 and Table 4.2. Factor XIII was inhibited from binding to each of the fibrinogen variants by approximately the same concentration of plasma fibrinogen, as expected from the similar K_d values for each variant fibrinogen. Notably, none of the variants displayed complete inhibition of factor XIII binding, even at 9 μ*M* fibrinogen inhibitor. One possible explanation for this phenomenon was that the large concentration of fibrinogen present in the fibrinogen + factor XIII mixture bound to the immobilized fibrinogen and kept any factor XIII bound to it present in the well, even though it was not directly interacting with the original immobilized layer.

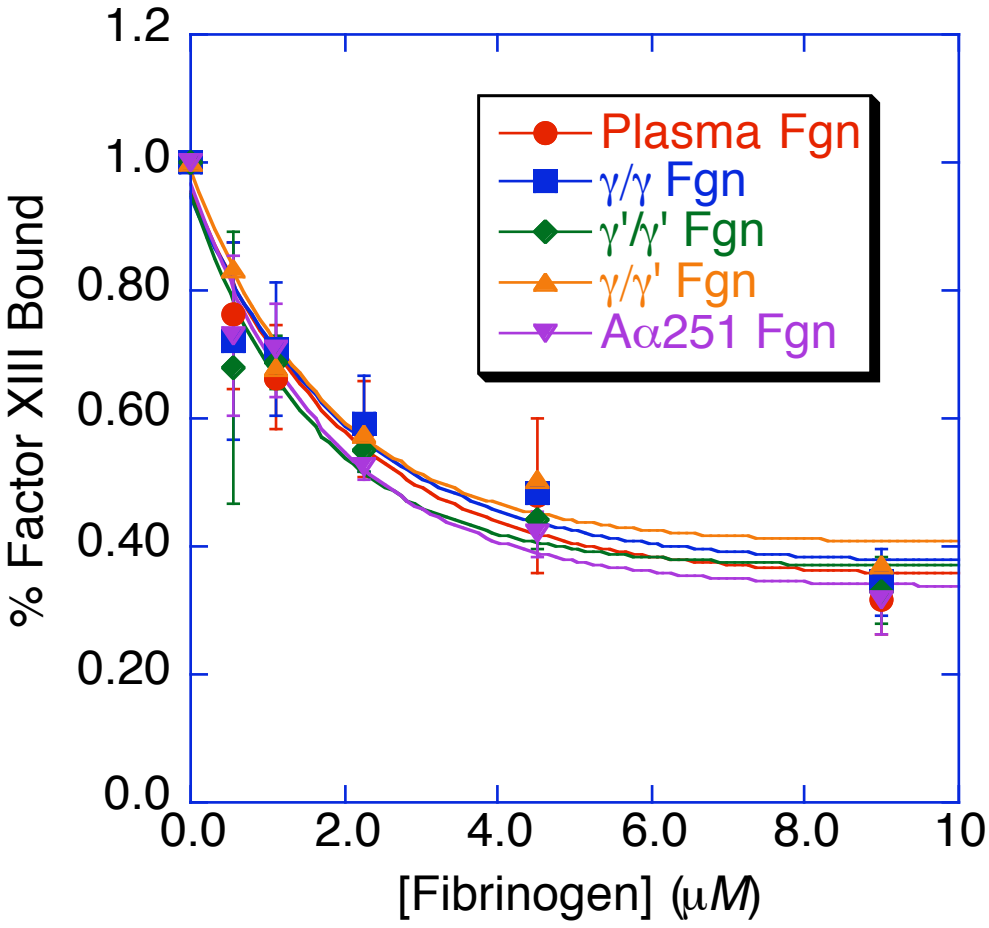


Figure 4.3. Inhibition of factor XIII binding to immobilized fibrinogen variants by soluble plasma fibrinogen. ELISA plates were coated with the fibrinogen variants listed in the legend, blocked with BSA, then incubated with 0.050 μ*M* FXIII mixed with 0.56 – 9.0 μ*M* plasma fibrinogen, washed, and probed for bound factor XIII. Absorbance at each point was divided by absorbance in the absence of inhibitor to generate % factor XIII bound, expressed as a decimal.

Inhibition of Factor XIII Binding to Immobilized Fibrinogen by Soluble Plasma Fibrinogen

Immobilized Fibrinogen	IC_{50} (μM)	Standard Deviation	P Value
			(compared to γ/γ)
plasma	0.46	0.18	0.48
γ/γ	0.47	0.16	
γ'/γ'	0.53	0.07	0.30
γ/γ'	0.56		
Aα251	0.52	0.08	0.34

Table 4.2. IC_{50} values for plasma fibrinogen inhibition of factor XIII binding to the immobilized fibrinogen variants listed in the table. IC_{50} values were determined from the equation y = a + b*exp(-c*x); where a = the percentage of factor XIII binding that corresponds to maximum inhibition, b = the span of inhibition that can take place, and c = EC_{50} value for the inhibitor. Because it displayed similar results to γ′/γ′ fibrinogen, experiments were performed only once for γ/γ′ fibrinogen, and therefore have no standard deviation or p values.

To further confirm the ELISA assay data, I developed another technique to use for measuring binding of factor XIII to fibrinogen. This technique made it possible to measure binding in solution, rather than to immobilized fibrinogen. I incubated 0.29 μ*M* fibrinogen (plasma, recombinant γ/γ, or recombinant γ′/γ′) with 0.025 – 0.80 μ*M* factor XIII zymogen in a series of Eppendorf tubes for one hour. This concentration of fibrinogen was determined by control experiments so it would not be a limiting factor in the reaction. I added Y-18 conjugated sepharose beads and rotated for two hours to immunoprecipitate fibrinogen through binding of fibrinogen E regions to Y-18. I washed the beads to remove unbound protein and then boiled them in reducing buffer and ran the samples out on an 11% gel. I transferred to nitrocellulose and probed with a mixture of fibrinogen anti-Bβ chain antibody and anti-factor XIII A chain antibody. The ratio of factor XIII to fibrinogen in each sample was determined by densitometry and a standard curve composed of known concentrations of fibrinogen or factor XIII run on the gel. I then plotted this ratio versus the concentration of factor XIII added to the sample to generate a binding curve.

Figure 4.4 A shows a representative Western blot used for quantification of FXIII versus fibrinogen Bβ chain for each fibrinogen variant. The lane on the left of each variant's samples is the highest concentration of factor XIII, with concentrations decreasing to the right. Figure 4.4 B shows the samples used to construct standard curves for fibrinogen and factor XIII. This data was then translated into binding curves as shown in Figure 4.5. Fitting these curves to the binding equation yielded different maximal binding ratios, but a similar K_d for each variant. The similar K_d for plasma, γ/γ, and γ′/γ′ fibrinogen was consistent with the results of the ELISA experiments.

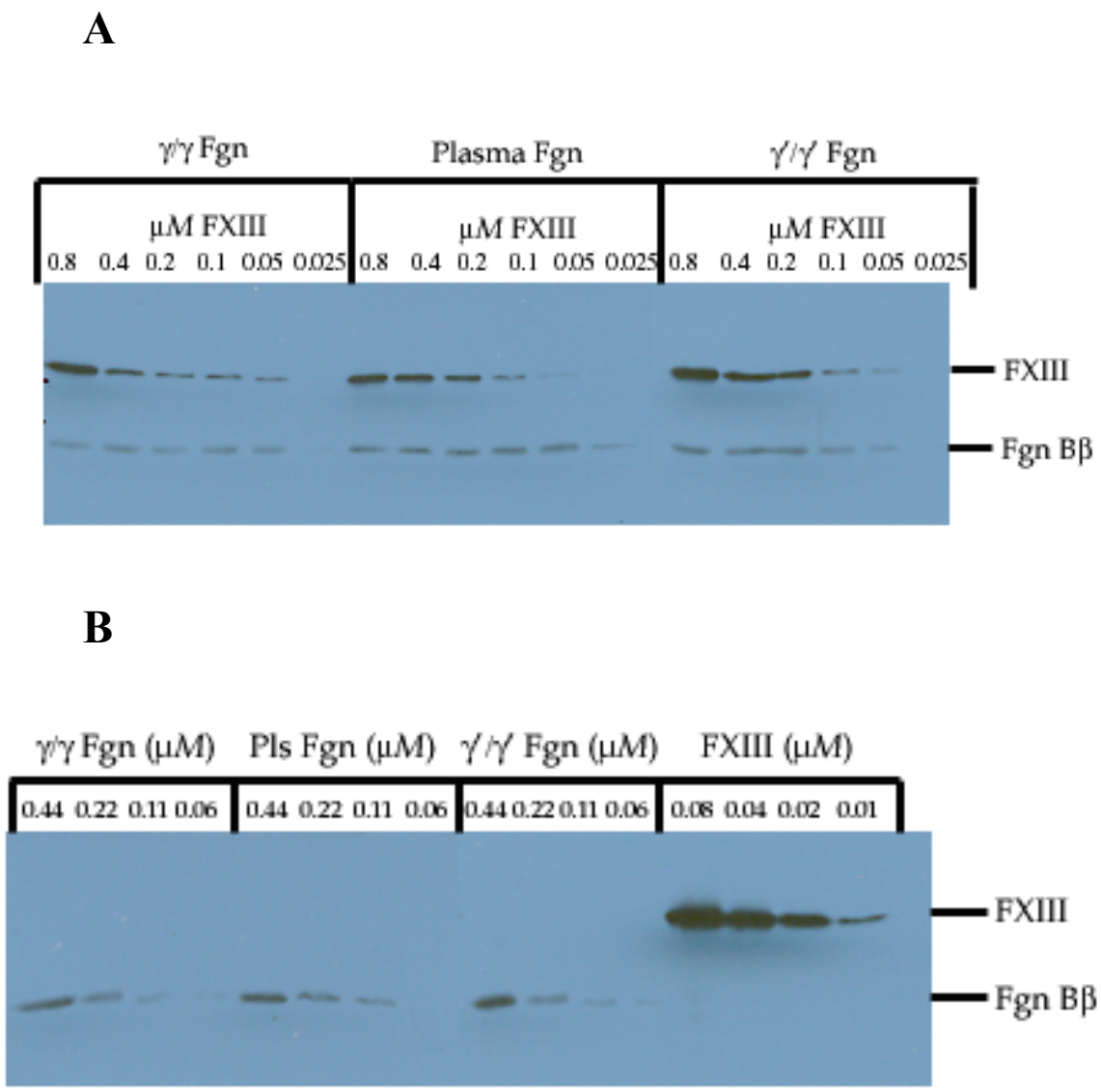
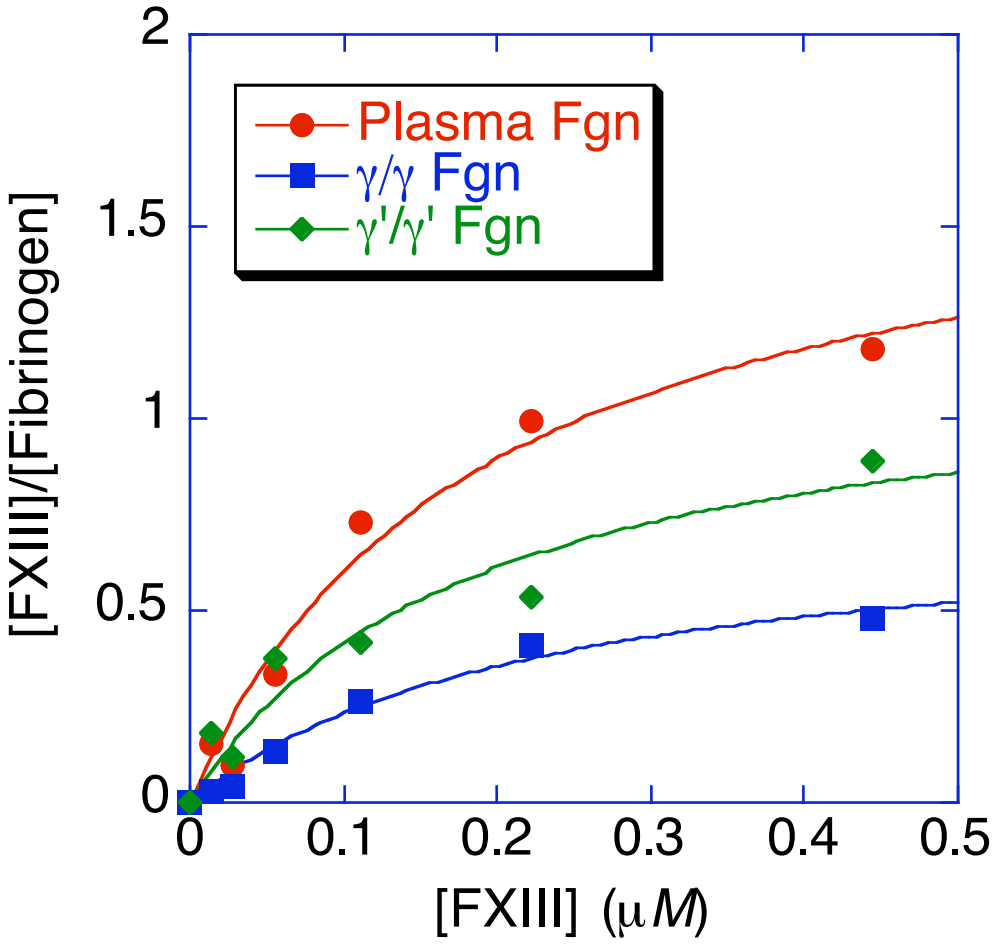


Figure 4.4. A) Western blot of factor XIII + fibrinogen immunoprecipitation reactions, developed with anti-fibrinogen Bβ chain and anti-factor XIII antibodies. Locations of factor XIII and fibrinogen Bβ chain are marked on the right. Equal concentrations of each fibrinogen are mixed with a range of concentrations of factor XIII zymogen prior to immunoprecipitation. The concentrations of factor XIII originally added to the samples are listed above each well. B) Western blot of fibrinogen and factor XIII standard curves developed with the same antibody solutions used in A. Concentrations of fibrinogen and factor XIII are listed above each well. Densitometry was used to measure the intensity of each band, and standard curves were prepared. The concentration of fibrinogen and factor XIII in each sample well was determined from these curves.



Fibrinogen	K_d (uM)
plasma	0.19
γ/γ	0.23
γ′/γ′	0.18

Figure 4.5. Binding curves constructed from an immunoprecipitation binding assay. Densitometry of Western blots and correlation with a standard curve for each protein were used to determine the ratio of factor XIII (μ*M*) to fibrinogen (μ*M*) pulled down in each sample. This ratio was plotted versus the concentration of factor XIII present during the IP. K_d values are listed for a representative experiment.

The K_d values calculated from Figure 4.5 are about five times weaker than those calculated from ELISA binding curves. While this decrease may be due to differences in binding to fibrinogen in solution versus fibrinogen immobilized on a plate, it may also have its origins in the construction of the immunoprecipitation experiment. It was difficult to determine whether Y-18 sepharose beads were able to pull down the large fibrinogen + factor XIII complex with the same ease as they would unbound fibrinogen. This assay contained significant variability, inherent in the nature of Western blotting, development by chemiluminescence, and densitometry. These variables led to the calculation of different maximum binding ratios each time the experiment was performed. However, the general trend is clear. In solution there was no difference in the affinity of factor XIII for fibrinogen that contains γ′ chains and fibrinogen that does not. The consistency of this experiment with the ELISA results for the same proteins showed that the ELISA format provided a relevant approximation of binding in solution.

Measuring Factor XIII Binding to Fibrin

I repeated the ELISA binding assay with fibrin to compare its binding to factor XIII with that of fibrinogen. After coating the plate with fibrinogen, I washed off excess fibrinogen and incubated with 1.0 U/mL thrombin for an hour, followed by 10 U/mL hirudin to inactivate the thrombin, then washed and blocked with BSA. I carried out the rest of the assay as described for fibrinogen. Binding curves for factor XIII binding to fibrin variants are shown in Figure 4.6a and K_d values are listed in Table 4.1. As discovered for factor XIII binding to fibrinogen, there was no significant difference among the K_ds for factor XIII binding to any of the fibrin variants.

Figure 4.6b is an overlay of the factor XIII binding curves for fibrinogen and fibrin, with only γ/γ, γ′/γ′, and Aα251 fibrinogen shown for clarity. This plot shows that factor XIII binding to γ/γ or γ′/γ′ fibrin was stronger than factor XIII binding to γ/γ fibrinogen ($p < 0.05$) or γ′/γ′ fibrinogen ($p = 0.05$). Although not statistically significant for γ/γ′ or Aα251 fibrin, a similar trend existed for each fibrinogen variant (see Table 4.1).

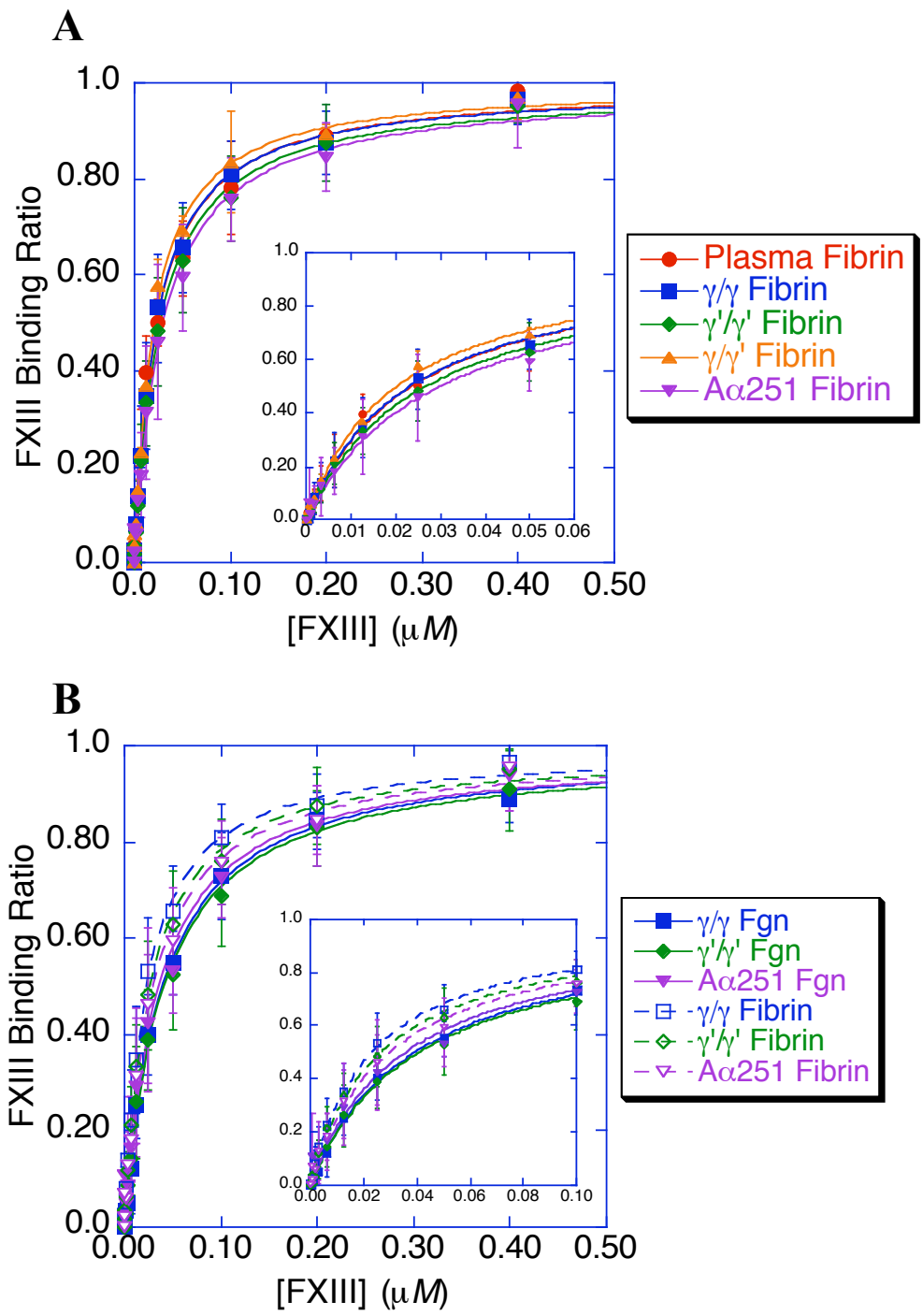


Figure 4.6. A) Factor XIII binding to fibrin variants. An ELISA assay was performed as described above, after converting immobilized fibrinogen to fibrin with 1.0 U/mL thrombin followed by 10 U/mL hirudin. The inset shows the low concentration points on the binding curve. B) Overlay of factor XIII binding to γ/γ, γ′/γ′, and Aα251 fibrinogen with factor XIII binding to γ/γ, γ′/γ′, Aα251 fibrin. Fibrin binding curves are shown with open symbols and a broken curve fit while fibrinogen binding curves are shown with closed symbols and a solid fit. The inset shows the low concentration points on the binding curve.

Recombinant Factor XIII Binding to Fibrinogen

Next, I performed ELISA binding experiments using recombinant factor XIII which is composed of only the A subunits. These experiments were set up using the same protocols as those for factor XIII zymogen; the only difference was that concentrations up to 5.0 μ*M* rFXIII were used to generate binding curves. These curves are shown in Figure 4.7 for plasma fibrinogen, each of the recombinant fibrinogens, and BSA as a control. Once again, since little or no binding occured on wells coated with BSA, binding to these fibrinogen variants was specific. Like factor XIII zymogen, recombinant factor XIII bound with equal affinity to each recombinant fibrinogen, suggesting that neither the γ′ chain nor the αC domain is responsible for binding to the unactivated A subunits of factor XIII. K_ds for rFXIII binding to fibrinogen are listed in Table 4.1. These values were all significantly weaker than the K_ds for factor XIII zymogen binding to fibrinogen.

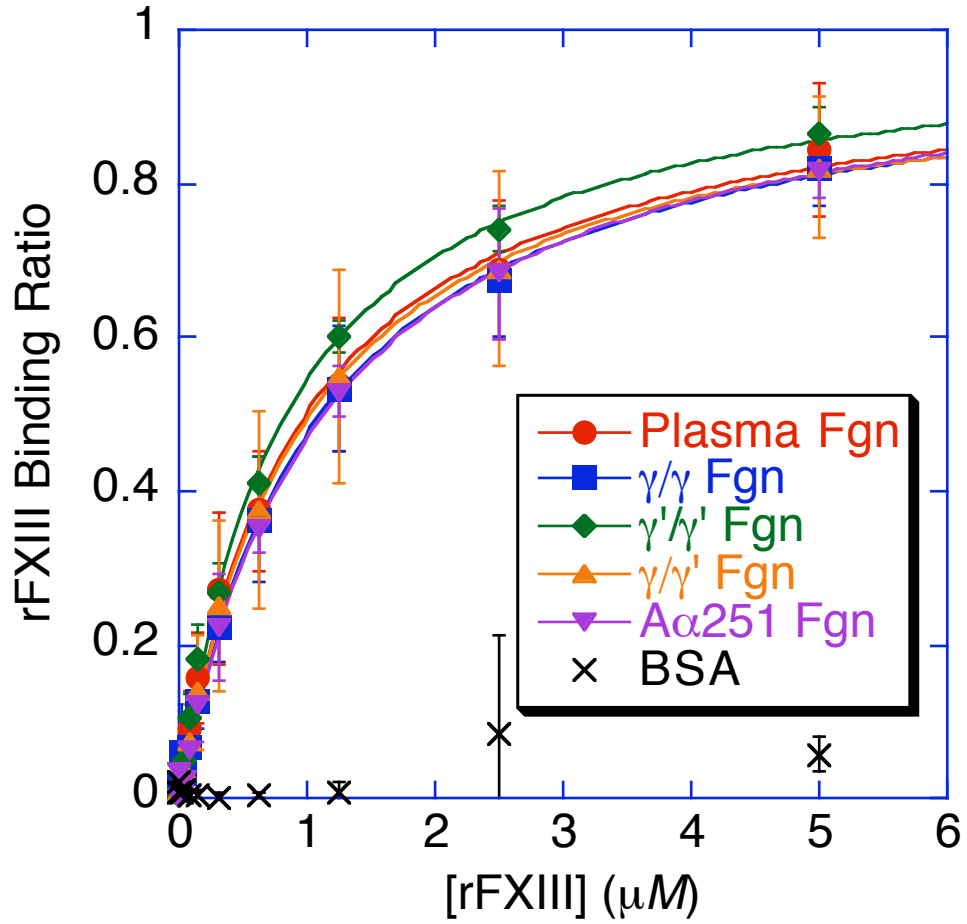


Figure 4.7. Recombinant factor XIII (rFXIII) binding to fibrinogen variants listed in the legend. ELISA plates were coated with each fibrinogen, blocked with BSA, incubated with recombinant factor XIII, and probed for rFXIII that remained bound after washing. Absorbance data was analyzed as described for Figure 4.1.

Recombinant, Activated Factor XIII Binding to Fibrinogen

In order to compare the binding sites on fibrinogen for factor XII in its active form to its zymogen state, I activated recombinant factor XIII using 32 U/mL thrombin and incubating at 37°C for 45 minutes. After the rFXIII had become active, I quenched the thrombin with 320 U/mL hirudin and blocked the active site of rFXIIIa with 20 m*M* iodoacetic acid. The iodoacetic acid forms a bond with the active site sulfhydryl group, effectively blocking rFXIIIa activity. Figure 4.8 is an SDS-PAGE gel of recombinant factor XIII in various stages of activation incubated with recombinant γ/γ fibrinogen for 1 hour. Lane 4 of this gel shows that active-site inhibited, activated recombinant factor XIIIa (rFXIIIai) did not cross-link fibrinogen to any extent while rFXIIIa did so extensively. Thus, the rFXIIIai used in the ELISA binding experiments was fully inhibited.

Binding experiments with rFXIIIai binding to fibrinogen were performed much as described for factor XIII zymogen and rFXIII, except that the highest concentration of rFXIIIai used was 0.80 μ*M*. Binding curves for rFXIIIai binding to fibrinogen variants are shown in Figure 4.9 with the inset displaying an enlarged view of the low rFXIIIai concentration points. This plot shows that neither the presence of the γ′ chain nor the loss of the αC domains had an effect on activated factor XIII binding to fibrinogen. K_d values are listed in Table 4.1.

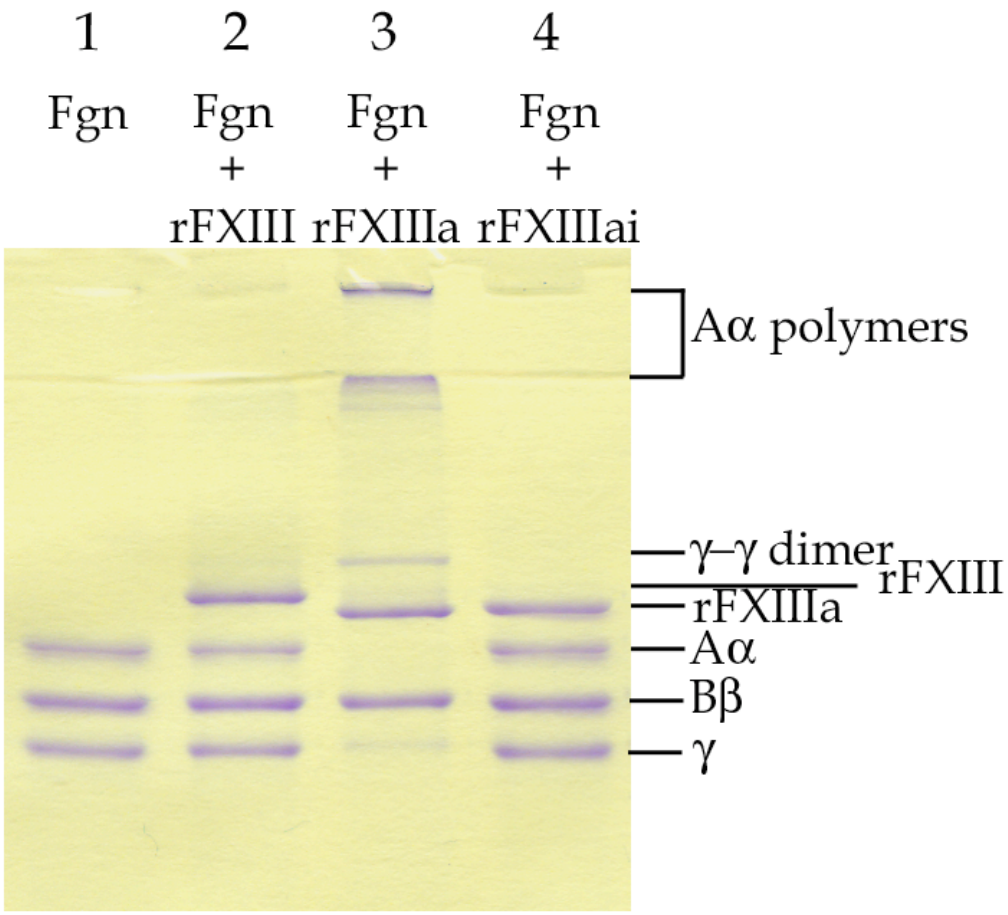


Figure 4.8. Coomassie-stained 11% SDS-PAGE of γ/γ fibrinogen (lane 1), γ/γ fibrinogen incubated with rFXIII for 1 hour (lane 2), γ/γ fibrinogen incubated with rFXIIIa for 1 hour (lane 3), and γ/γ fibrinogen incubated with rFXIIIai for 1 hour (lane 4). Samples were prepared from 0.30 mg/mL fibrinogen and 0.80 μ*M* rFXIII(a)(i), and were quenched with Laemmli buffer and reduced with BME.

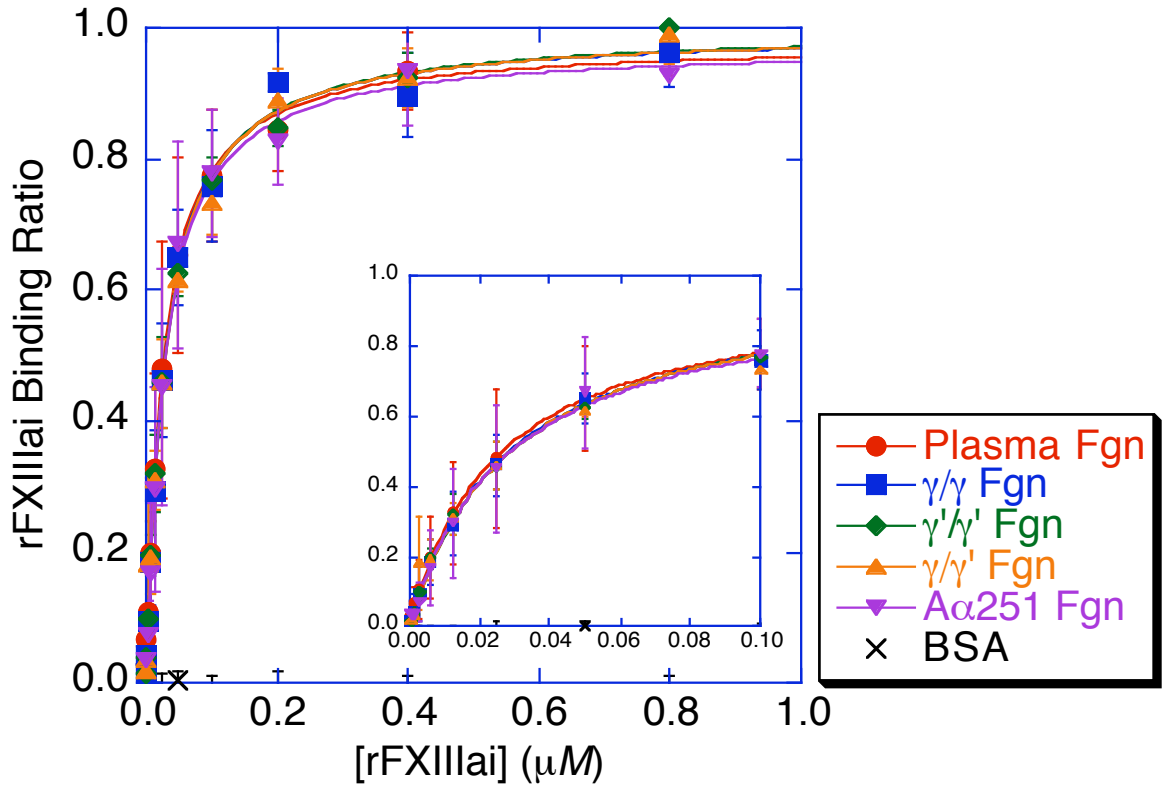


Figure 4.9. Binding curves for ELISA binding assay of rFXIIIai binding to fibrinogen variants. Experiments were performed and data processed as described in the legend for Figure 4.1. Inset shows detail of the rFXIIIai concentrations from 0 – 0.10 μ*M*.

To confirm that there was no difference in rFXIIIai binding to any of the fibrinogen variants, I performed inhibition experiments as described for factor XIII zymogen. Briefly, I incubated 0.050 μ*M* rFXIIIai for 30 minutes with varying concentrations of plasma fibrinogen before addition to fibrinogen-coated ELISA wells. Results of these inhibition experiments are shown in Figure 4.10. Plasma fibrinogen inhibited rFXIIIai binding to each of the fibrinogen variants to relatively the same extent, and each of the variants retained approximately 50% binding, even at high concentrations of fibrinogen inhibitor. Table 4.3 lists the IC_{50} values for plasma fibrinogen inhibiting binding to each fibrinogen variant and shows that there is no statistical difference in ease of inhibition among variants.

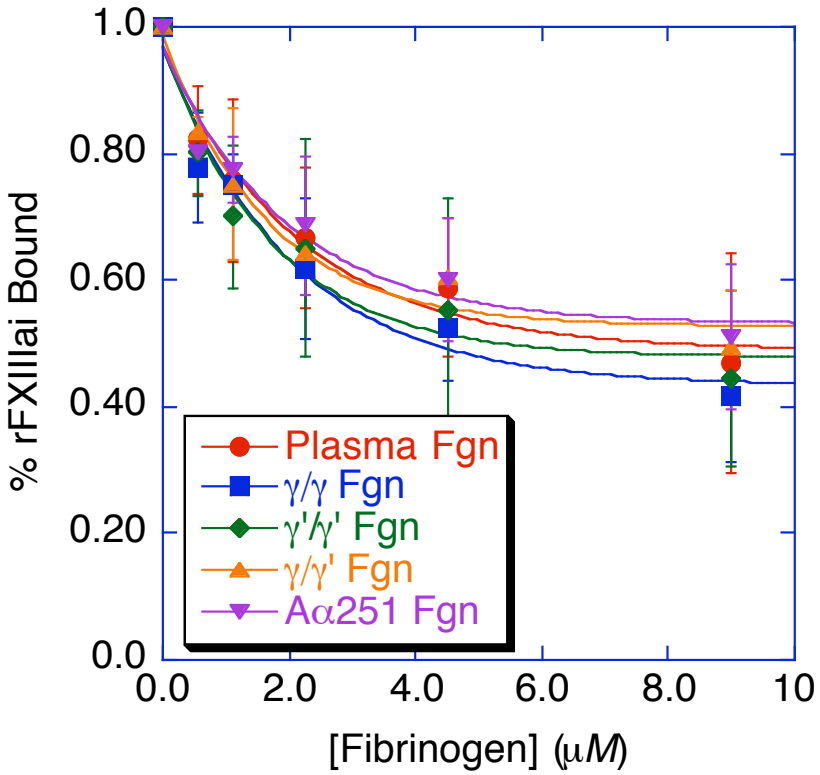


Figure 4.10. Inhibition of rFXIIIai binding to immobilized fibrinogen variants by plasma fibrinogen in solution. Experiments were performed and data analyzed as described in the legend of figure 4.3, except that rFXIIIai was used, rather than FXIII zymogen.

Inhibition of rFXIIIai Binding to Fibrinogen Variants by Plasma Fibrinogen in Solution

Immobilized Fibrinogen	IC_{50} (μM)	Standard Deviation	P Value
			(compared to γ/γ)
plasma	0.47	0.11	0.43
γ/γ	0.48	0.09	
γ'/γ'	0.52	0.34	0.43
γ/γ'	0.62	0.09	0.08
Aα251	0.51	0.07	0.33

Table 4.3. Inhibition data was fit to the equation y = a + b*exp(-c*x), as described in the legend of Table 4.2. P values were determined by a Student's t-test.

Recombinant, Activated Factor XIII Binding to Fibrin

I converted the fibrinogen layer coating an ELISA plate to fibrin using 1.0 U/mL thrombin incubation for 1 hour, followed by treatment with 10 U/mL hirudin for 10 minutes, and then added rFXIIIai as described for fibrinogen binding assays. Binding curves are shown in Figure 4.11 A and B. rFXIIIai bound to each fibrin variant, within error, with the same affinity (Figure 4.11 A). Table 4.1 shows that rFXIIIai generally bound fibrin more tightly than factor XIII zymogen bound fibrin; this trend was significant for plasma and γ′/γ′ fibrinogen. Figure 4.11 B indicates that for γ/γ, γ′/γ′, and Aα251 fibrinogen, the binding of rFXIIIai to fibrin was significantly tighter than rFXIIIai binding to fibrinogen. A similar trend holds true for plasma and γ/γ′ fibrinogen, not shown in the figure for clarity.

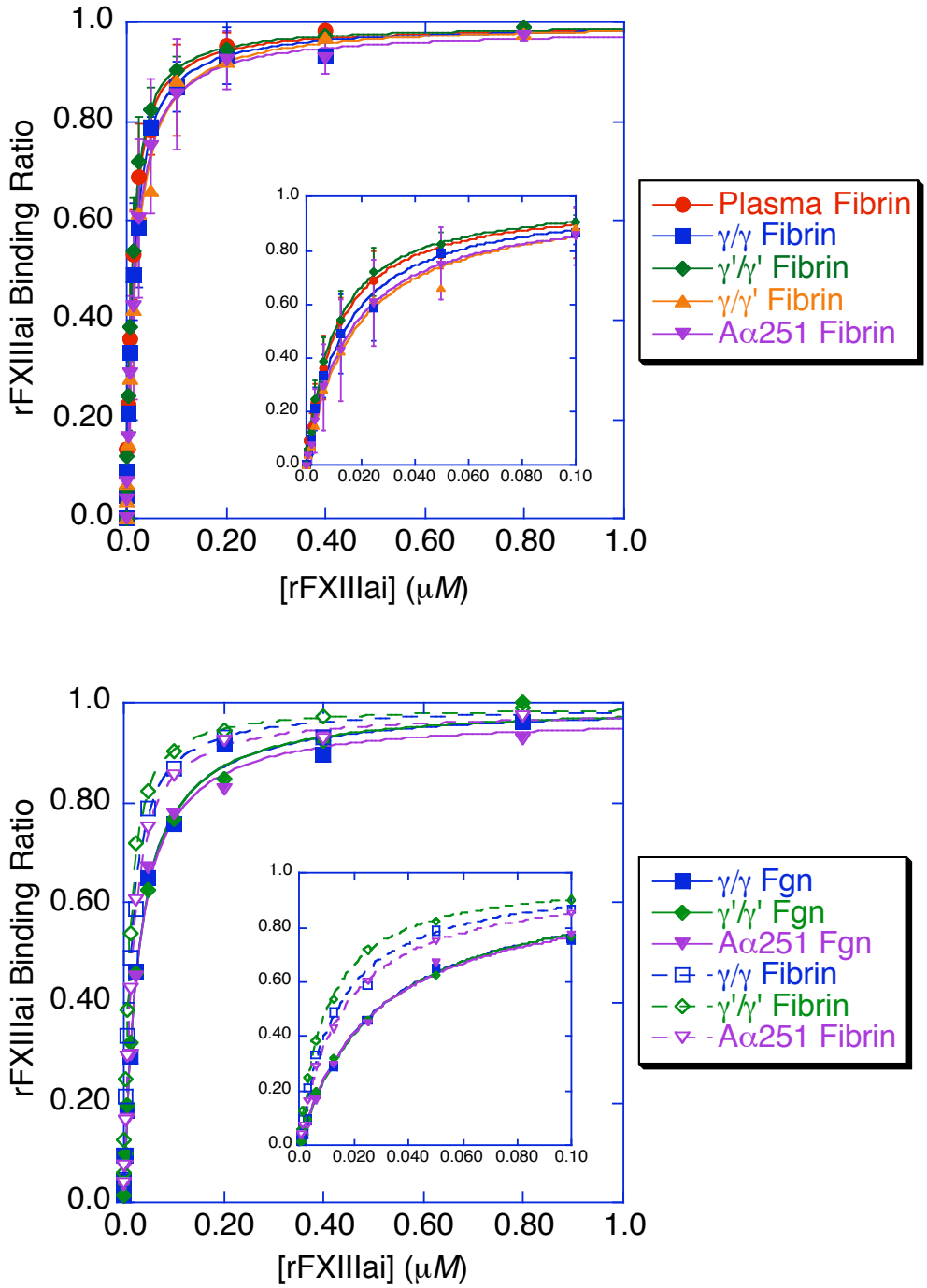


Figure 4.11. A) Binding curves for rFXIIIai binding to fibrin variants. Inset shows binding to low concentrations of rFXIIIai. B) Comparison of binding curves for rFXIIIai binding to fibrinogen (filled symbols and solid lines) versus fibrin (open symbols and dashed lines). Inset shows low concentrations of rFXIIIai. For simplicity, only γ/γ, γ′/γ′, and Aα251 fibrinogens are shown. Other variants display the same pattern.

Discussion

The experiments presented in this work show, unequivocally, that the γ′ chain of fibrinogen is not involved in binding to factor XIII zymogen. Antibody recognition experiments showed that the C-terminus of the γ′ chain was accessible when γ′/γ′ fibrinogen was immobilized on a microtiter plate. However, when measured by ELISA, the binding curve for factor XIII zymogen binding to immobilized γ/γ fibrinogen overlapped the binding curves for γ′/γ′ fibrinogen, γ/γ′ fibrinogen, and even plasma fibrinogen which contains ~90% γ/γ molecules and ~10% γ/γ′ molecules. Thus, there ias no enhancement for factor XIII binding to a fibrinogen variant with a γ′ chain present compared to binding to γ/γ fibrinogen. The K_d determined for plasma fibrinogen binding to factor XIII zymogen was 0.043 +/- 0.016 μM, on the same order as the 0.01 μ*M* K_d reported by Greenberg and Shuman [1].

The results of the ELISA binding experiments are supported by inhibition experiments in which binding of factor XIII zymogen to fibrinogen on the ELISA plate is inhibited by fibrinogen in solution. Within error, the same concentration of fibrinogen in solution was required to achieve 50% inhibition of factor XIII binding to each of the fibrinogen variants, whether or not they contained a γ′ chain. This finding that the γ′ chains are not implicated in factor XIII binding is not just an artifact of the ELISA system. K_d values determined by mixing fibrinogen and factor XIII in solution followed by immunoprecipitation of fibrinogen and any bound factor XIII demonstrated the same pattern; that the presence of the γ′ chain did not influence the K_d of factor XIII zymogen binding to fibrinogen.

This conclusion is in direct conflict with the work of Siebenlist, Meh, and Mosesson, as well as with Moaddel, Farrell, Daugherty, and Fried [13], [14]. Moaddel and coworkers

performed analytical ultracentrifugation experiments to compare the K_a for factor XIII zymogen binding to plasma γ/γ fibrinogen with that of plasma γ/γ′ fibrinogen [14]. While they found an enhanced association between γ/γ′ fibrinogen and factor XIII compared to γ/γ fibrinogen, these results have recently been called into question by the finding that factor XIII zymogen is capable of cross-linking fibrinogen prior to activation by thrombin, particularly over the long incubation period required for the analytical ultracentrifuge experiments [15]. In support of their assertions, however, Moaddel *et al*. also performed an ultracentrifuge experiment in which they included a 20-amino acid polypeptide of the γ′ sequence and found that this peptide was capable of inhibiting fibrinogen binding to factor XIII [14]. While this experiment lends more credence to the hypothesis that γ′ binds factor XIII since the peptide was not subject to cross-linking by factor XIII, it is by no means proof of a high affinity γ′ site. Rather high concentrations of peptide were required for inhibition (approximately 100 μ*M*), and control experiments mixing this peptide with γ/γ fibrinogen to check for nonspecific inhibition were never reported.

Siebenlist *et al*. demonstrated factor XIII binding to plasma γ/γ′ fibrinogen rather than γ/γ fibrinogen by ion-exchange and size-exclusion chromatography [13]. Using an identical column set-up, I was unable to replicate their size-exclusion chromatography results (data not shown). One step that I omitted which they performed for both these experiments and the ion-exchange studies was to dialyze the fibrinogen and factor XIII after mixing them together. Because I was concerned with zymogen-catalyzed cross-linking, I simply dialyzed the two proteins into the same buffer individually, then incubated them for 15 minutes, rather than a (presumably) overnight dialysis. Both my observations described in the Appendix to this document as well as the authors' own later publication [15] indicate that such a dialysis

period would lead to a significant population of cross-linked fibrinogen molecules. In fact, a distinct shoulder is seen in the ion-exchange profile of Siebenlist *et al.*'s γ/γ′ fibrinogen dialyzed with factor XIII suggesting two populations of molecules may be present. Cross-linking of either or both γ/γ or γ/γ′ fibrinogen would complicate analysis of factor XIII zymogen binding since the enzyme could be actively cross-linking its substrate during the experiment and thus lead to false interpretations of binding data. Another problem with these authors' experimental set-up is that they performed an ammonium sulfate precipitation on γ/γ′ fibrinogen to remove factor XIII, but did not perform this same step on the γ/γ fibrinogen, perhaps somehow altering the γ/γ′ fibrinogen compared to the γ/γ fibrinogen. Perhaps one of these experimental oversights led to the erroneous conclusion that factor XIII zymogen binds to the γ′ chain of fibrinogen.

The investigation described in this chapter also showed that the γ′ chain does not provide a binding site for the A subunits of factor XIII, either in their unactivated form (rFXIII) or their activated form (rFXIIIai). As with factor XIII zymogen, ELISA binding curves and resultant K_ds were identical for unactivated recombinant factor XIII binding to γ/γ, γ′/γ′, or γ/γ′ fibrinogen. Activating recombinant factor XIII did not cause it to bind γ′ preferentially either; the K_ds for rFXIIIai binding to γ/γ, γ′/γ′, and γ/γ′ fibrinogen, as determined by ELISA, were identical. Figure 4.7 shows that rFXIIIai binding to each of these variants was inhibited by a similar amount of plasma fibrinogen, suggesting that the rFXIIIai plus variant fibrinogen interaction is the same strength for each γ or γ′ variant.

Although never hypothesized as such, these experiments demonstrated that the αC domains of fibrinogen are not binding sites for factor XIII zymogen. ELISA experiments showed that the binding curve for factor XIII zymogen binding to Aα251 fibrinogen,

truncated at Aα residue 251, were identical to that of γ/γ fibrinogen, the normal fibrinogen molecule with intact Aα chains. Inhibition experiments with plasma fibrinogen in solution showed that the same amount of plasma fibrinogen was required to inhibit factor XIII binding to Aα251 fibrinogen as was needed for normal fibrinogen.

Also, αC domains may not be required for optimal binding of the A subunits of factor XIII to fibrinogen. ELISA binding experiments with recombinant factor XIII showed that binding to Aα251 fibrinogen took place with the same affinity as binding to fibrinogen that contained the αC domains. A similar result was obtained for activated, active-site inhibited recombinant factor XIII binding to Aα251 fibrinogen or fibrin. The αC domains have been suggested by Procyk *et al*. as a binding site for the A subunits of factor XIII, but this site was only investigated for the active form of the molecule (rFXIIIa), not unactivated recombinant factor XIII [11]. Not blocking the active site of rFXIIIa would lead to significant cross-linking of the fibrin clot through which rFXIIIa was percolated, retarding its flow. Addition of a fibrinogen fragment or antibody to the αC domain would inhibit this cross-linking, thus increasing the rate at which rFXIIIa passed through the clot. Such an increase might appear as loss of a binding site.

My determination of K_d for plasma fibrin binding to factor XIII zymogen was 0.023 +/- 0.008 μ*M*, which is in good agreement with Naski *et al*.'s 1991 determination of 0.065 +/- 0.015 μ*M* [8]. For each fibrin(ogen) in my ELISA experiments, binding of factor XIII zymogen to fibrin produced a lower K_d than binding to fibrinogen (regardless of γ′ content). Given Janus *et al*.'s assertion that des-A fibrin is the true activator of factor XIII to active factor XIIIa [4], the increased affinity of factor XIII zymogen for fibrin versus fibrinogen

makes sense. Once fibrinogen is converted to fibrin at the site of a wound, it recruits factor XIII to the site of activation.

Similarly, rFXIIIai bound more strongly to fibrin than to fibrinogen for all variants. This increase in affinity of rFXIIIai for fibrin compared to fibrinogen is a new observation. *In vivo*, it would be impossible to generate active factor XIII in the presence of fibrinogen since thrombin-catalyzed FpA cleavage takes place before thrombin-catalyzed activation peptide cleavage [4].

Comparison of ELISA binding studies of factor XIII zymogen (A_2B_2), unactivated recombinant factor XIII (A_2), and activated, active-site inhibited recombinant factor XIII (A_2a) provides me with a framework for discussing the role of the A and B subunits of factor XIII in binding to fibrinogen. Because we have learned that the γ′ chains do not play a role in binding to any form of factor XIII, I will focus this discussion on factor XIII binding to normal, recombinant γ/γ fibrinogen. As shown in Table 4.1, the K_d for factor XIII zymogen binding to γ/γ fibrinogen was 0.041 +/- 0.013 μ*M*; the K_d for rFXIII binding to γ/γ fibrinogen was 1.2 +/- 0.4 μ*M* ($p < 0.05$ compared to FXIII); and the K_d for rFXIIIai binding to γ/γ fibrinogen was 0.030 +/- 0.009 μ*M* ($p = 0.05$ compared to γ/γ fibrinogen). Thus, loss of the B subunits from unactivated factor XIII dramatically lowered its affinity for fibrinogen, but activation of this protein returned its affinity to a similar, and possibly even stronger, value. Binding was not, however, fully abrogated by loss of the B subunits; rFXIII still bound to fibrinogen with a K_d of around 1 μ*M*. This suggests that the B subunits of factor XIII zymogen provide a high affinity site for binding to fibrinogen. When they are lost, there is still a weak site on the A subunits that is responsible for some binding affinity and becomes a stronger site once factor XIII is activated.

This model of a high affinity binding site on the B subunits that does not negate a low affinity binding site present on unactivated A subunits helps to explain how platelet factor XIII with only A subunits could compete away plasma factor XIII from fibrinogen in Greenberg and Shuman's initial study of factor XIII binding to fibrinogen. It does not, however, explain their similar K_d determinations of around 0.01 μM for both forms of factor XIII [1]. This model is also consistent with Hornyak and Shafer's claims that the binding sites on factor XIII zymogen and active factor XIIIa are separate. They found that factor XIII zymogen did not inhibit binding of factor XIIIa to fibrin and thus suggested separate binding sites on factor XIII for when it is in its zymogen form or its active state [10].

I have also shown that active factor XIIIa bound more strongly to fibrin than did factor XIII zymogen. This makes sense physiologically for localization of active enzyme to an appropriate substrate. We can conclude that the A subunit binding site has a higher affinity for fibrin than does the B subunit binding site, while, as discussed above, the reverse is true for binding to fibrinogen.

In summary, I have strong evidence to suggest that the γ′ chain of fibrinogen does not play a role in binding to any form of factor XIII. Similarly, the αC domains of fibrinogen are not required for optimal factor XIII binding. Factor XIII is able to bind to fibrinogen through both its A and B subunits, though it must be activated before A subunit binding takes place with high affinity.

Generally, sites of functional importance, such as binding sites, are conserved among species. The γ′ chain, on the other hand, differs in human fibrinogen compared to other species. The human γ′ sequence (VRPEHPAETEYDSLYPEDDL) contains six negatively-charged amino acids and two sulfated tyrosines [19]. Rat fibrinogen has a γ′ sequence

(VSVEHEVDVEYP) that is much shorter than that of human γ′ with only four negatively-charged amino acids and one tyrosine available for sulfation [20]. Bovine fibrinogen has a γ′ sequence similar to that of rats [21]. Such an abbreviated γ′ sequence removes a significant fraction of the negative charge that was suggested as a part of the factor XIII binding site.

In addition, the amount of alternatively spliced γ′ chains differ among species. Human γ′ chains constitute around 10% of fibrinogen γ chains [22]. In rats, the γ′ chain is found in 30% of γ chains [23]. Chicken fibrinogen contains up to 50% γ′ chains, possibly one on every fibrinogen molecule [24]. Such variability in the frequency of the γ′ chain also suggests against it being a binding site for factor XIII since some species would have a plethora of these binding sites while others would have very few. Future experiments must be performed to identify the true factor XIII zymogen binding site on fibrinogen.

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CHAPTER FIVE

THE γ′ CHAIN OF FIBRINOGEN AS SUBSTRATE FOR FACTOR XIII

Summary

We investigated the role of the γ′ chain as a substrate for factor XIII-catalyzed cross-linking into γ′-γ′ or γ-γ′ dimers. By comparing polymerization of fibrinogen in the absence and presence of factor XIII zymogen, we learned that addition of factor XIII to recombinant γ/γ fibrinogen had no effect on the basic polymerization parameters: lag time, maximal rate, and final absorbance. This was also the case for both γ′/γ′ fibrinogen and a mixture of γ/γ with γ/γ′ fibrinogen, suggesting that the γ′ chain does not influence polymerization in the presence of factor XIII. We also monitored factor XIII-catalyzed cross-linking of γ′ fibrin variants and found that the γ′ chain slows the initial rate of γ(′)-γ(′) dimer formation.

Introduction

In the last chapter of this document, I demonstrated that the γ′ chain is not a binding site for factor XIII zymogen. While provision of this binding site is how fibrinogen acts as a cofactor for factor XIII activation, I have not yet explored the effect of the γ′ chain on fibrinogen's role as substrate for factor XIII. In this chapter, I will investigate how the presence of the γ′ chain influences cross-linking of fibrinogen by factor XIII.

Polymerization of Fibrinogen in the Presence of Factor XIII

Polymerization of fibrinogen in the presence of factor XIII was first studied by Carr, Gabriel, and McDonagh in 1987 [1]. They monitored polymerization for just ten minutes and then analyzed mass-to-length ratios after 24 hours of reaction. The authors claim the following: 1) the addition of factor XIII into a polymerization reaction does not cause a change in lag time for the reaction, 2) the rate of turbidity increase is unchanged by addition of factor XIII, 3) mass-to-length ratios of fibers did not change upon addition of factor XIII,

and 4) fiber density increased with factor XIII present during polymerization [1]. It is difficult, however, to interpret these results because the authors included 5 m*M* $CaCl_2$ in any reaction mixture without factor XIII and 10 m*M* $CaCl_2$ when factor XIII was present. Because calcium has such a clear effect on polymerization [2], it may mask the effect of factor XIII in these reaction mixtures.

Ryan *et al*. performed electron microscopy studies of plasma-derived fibrin clots polymerized in the presence of endogenous factor XIII as well as with 1 m*M* factor XIII inhibitor to prevent cross-linking [3]. Visual inspection of the clots revealed no clear differences between fibrin clots formed in the presence of active factor XIII and those formed in its absence. Rigorous mathematical analysis showed a small increase in fiber length and concomitant decrease in fiber diameter when factor XIII was present. There was no change in fiber or branch-point density [3]. Lim and coworkers presented an abstract of similar findings from scanning electron microscopy of fibrin clots formed in the presence of factor XIII [4]. They also demonstrated a slower rate of lateral aggregation and lower final absorbance when monitoring polymerization in a spectrophotometer. Because they observed these same results upon polymerization of recombinant Aα251 fibrinogen with factor XIII, they hypothesized that factor XIII's effect on fibrin clot structure occurs through cross-linking of γ chains [4]. My recombinant γ′ chain variants provide a way to further these studies.

Factor XIIIa-Catalyzed Cross-Linking of Fibrin

In 1995, Taubenfeld and coworkers demonstrated that the C-terminus of the γ chain is necessary for factor XIIIa-catalyzed cross-linking of fibrin [5]. They showed that addition of increasing concentrations of 4A5, a monoclonal antibody to C-terminal γ chain residues 402-

411, could inhibit cross-linking. The authors suggested two modes of action for this inhibition. One suggestion was that the antibody sterically blocks factor XIII, preventing it from gaining access to the γ-chain cross-linking sites. Another method of action was that it may prevent fibrin from assembling into the proper orientation for cross-linking to take place [5]. In either case, these observations point out that changes to the C-terminus of the γ chain, such as removal of the four C-terminal amino acids and addition of a large, mobile, and highly-charged region such as the γ′ chain, may influence the ability of factor XIII to cross-link its fibrin substrate.

How the γ′ chain affects the rate and extent of factor XIII-catalyzed cross-linking of fibrin has been a subject of debate [6],[7],[8]. Moaddel, Falls, and Farrell published a study in 2000 investigating the role of plasma-derived γ/γ′ fibrinogen in factor XIII activation [6]. They incubated a fixed amount of factor XIII (43 n*M*) with increasing concentrations of fibrinogen in the presence of 1 m*M* $CaCl_2$ and 1 unit/mL thrombin for 6 minutes, then quenched the reaction and ran it on a reducing gel. They determined the concentration of γ-γ(′) dimers in each sample by dividing the optical density of the γ-γ(′) band by that of the β chain band. Results of this experiment showed that more cross-links form in γ/γ′ fibrinogen compared to γ/γ fibrinogen. A similar experiment quantifying α-chain multimer formation demonstrated a large excess of α-multimers in γ/γ′ fibrinogen compared to γ/γ fibrinogen. They also showed that γ/γ′ fibrinogen is a more effective cofactor for factor XIII activation by incubating factor XIII with thrombin and γ/γ or γ/γ′ fibrinogen and then measuring 5-(biotinamido)pentylamine incorporation into N,N′-dimethlycasein [6].

Siebenlist *et al.*'s results are virtually opposite those of Moaddel *et al*. Instead of measuring cross-linking versus concentration of fibrinogen, Siebenlist and coworkers looked

at cross-linking of plasma γ/γ versus γ/γ′ fibrinogen over time [7]. They showed that at early timepoints, γ/γ′ fibrinogen cross-linking is delayed with respect to γ/γ fibrinogen, but they quickly achieve the same rate of cross-linking and plateau of percent cross-linked chains. These experiments were performed with 0.124 μ*M* factor XIII, 5 m*M* $CaCl_2$, and 0.5 U/mL thrombin. They also performed experiments to measure thrombin cleavage of activation peptide from factor XIII in the presence of fibrinogen and found that γ/γ fibrinogen is a better cofactor for this reaction than is γ/γ′ fibrinogen [7].

The two investigations into factor XIII-catalyzed cross-linking of γ/γ′ fibrinogen compared to γ/γ fibrinogen were performed differently at different conditions; not surprisingly their conclusions also differ. In addition to these differences, Siebenlist also pointed out that Moaddel's plasma fibrinogen samples were not free of co-purifying factor XIII which would certainly affect cross-linking rates [7]. In a letter to the editor, Farrell provides data suggesting that his fibrinogen samples are not contaminated with factor XIII and claims that Siebenlist's method of measuring factor XIII activation by activation peptide release is flawed. Siebenlist refutes both of these assertions in a rebuttal [8]. Once again, my recombinant fibrinogen γ′ variants provide a way to consider the role of the γ′ chain as substrate for factor XIII, without having to worry about purification from plasma, removal of factor XIII, or any other complications of plasma-derived proteins.

Materials and Methods

Materials

Human plasma fibrinogen depleted of plasminogen, fibronectin, and factor XIII (lots 030411 and 050518) was purchased from American Diagnostica (Stamford, CT). Human

factor XIII (lots 850A and 3040P) and human α-thrombin (lot 2690PR) were purchased from Enzyme Research Laboratories (South Bend, IN). Recombinant γ′/γ′ and γ/γ′ fibrinogen were expressed and purified as described in Chapter Two. Normal, recombinant γ/γ fibrinogen was prepared as described [9] and purified as described [10]. Both fibrinogen (plasma and recombinant) and factor XIII (plasma and recombinant) were dialyzed extensively against 20 m*M* HEPES pH 7.4, 150 m*M* NaCl (HBS).

Polymerization of Fibrinogen in the Presence of Factor XIII

Polymerization reactions were performed in 100 μL in Corning half-area untreated polystyrene microtiter plates under the following conditions:

0.15 mg/mL fibrinogen (plasma, recombinant γ/γ, recombinant γ′/γ′, or a mixture of 90% recombinant γ/γ + 10% recombinant γ/γ′)

20 m*M* HEPES pH 7.4

5.0 m*M* $CaCl_2$

NaCl to bring ionic strength to 0.15

1.0 n*M* (0.10 U/mL) α-thrombin

with or without 15 μg/mL factor XIII zymogen

Reactions were prepared without factor XIII or thrombin and incubated at 4°C for 30 minutes followed by 30 minutes at room temperature. Factor XIII was added to the appropriate wells followed by 10 μL of 10 n*M* α-thrombin in HBS to initiate clot formation. Turbidity was monitored on a SpectraMax 340PC microtiter plate reader from Molecular Devices at 350 nm every 10 seconds for 90 minutes. Data was recorded in SoftmaxPro version 4.3.1. Absorbance was plotted versus time for each polymerization curve. Lag time and final absorbance of the clot were determined by inspection, while V_{max} (maximal rate of change)

was determined from the five points on the curve with the highest slope as determined by Softmax.

Factor XIII-Catalyzed Cross-Linking of Fibrinogen

Cross-linking reactions were performed in HBS + 5.0 m*M* $CaCl_2$ + 0.50 m*M* DTT. Recombinant γ/γ, γ′/γ′, or γ/γ′ fibrinogen (0.30 mg/mL) was mixed with factor XIII zymogen (0.50 μg/mL). We added 0.20 U/mL thrombin to start the reaction, quickly mixed the sample by pipetting, and transferred 10 μL to each of 7 tubes, all in the first 60 seconds after thrombin addition before the solution became viscous. The reaction was quenched at designated timepoints (0, 1, 2, 5, 10, 30, and 60 minutes) by addition of 10 μL of 2X Laemmli buffer (30mM Tris, pH 8.5, 4% SDS, 0.02% bromophenol blue, 4% glycerol) and 12% BME. The Laemmli/BME solution was placed in the 0 timepoint tube prior to the start of the reaction for immediate quenching. Complete solubilization was achieved by incubation of the quenched reactions at 37°C overnight prior to boiling. Samples were run on an 11% gel with an extra tall stacking gel at 60 – 80V to increase separation of γ and γ′ chains, then stained with Coomassie R-250. The formation of γ-γ dimers was quantitated by densitometry using ImageJ (NIH, Bethesda, MD). The intensity of the γ-γ dimer band was divided by the total intensity of γ monomers + γ-γ dimers to calculate the ratio of dimers formed. The γ′-γ′ dimer band was measured for γ′/γ′ fibrinogen, while the sum of γ-γ, γ-γ′, and γ′-γ′ bands was determined for γ/γ′ fibrinogen.

We also compared cross-linking of each fibrinogen variant using active factor XIIIa. Factor XIII (1.67 μg/mL) in HBS + 5 m*M* $CaCl_2$ + 0.5 m*M* DTT was incubated for 30 minutes at 37°C with 0.67 U/mL thrombin. This activation was set up so that when the thrombin / factor XIIIa mixture was added to fibrinogen, the concentration of factor XIII

would be 0.50 μg/mL and the concentration of thrombin would be 0.20 U/mL. Reactions were quenched and run on 11% gels as described above.

Results

Polymerization of Fibrinogen in the Presence of Factor XIII

To determine whether the addition of factor XIII has a different effect on polymerization of fibrinogens with γ′ chains than it does on those without, I performed polymerization experiments, much as described in Chapter Three of this document, in the presence and absence of 15 μg/mL factor XIII zymogen. The fibrinogens I studied were plasma fibrinogen with ~90% γ/γ molecules and ~10% γ/γ′ molecules, recombinant γ/γ, recombinant γ′/γ′, and a mixture of 90% recombinant γ/γ + 10% recombinant γ/γ′. This recombinant mixture was designed to mimic the γ′ content of plasma fibrinogen to determine whether differences between plasma and recombinant fibrinogen were due to the presence of γ′ chains.

I mixed 0.15 mg/mL fibrinogen with 5.0 m*M* $CaCl_2$, 20 m*M* HEPES, and NaCl to bring ionic strength to 0.15. I incubated this mixture at 4°C for 30 minutes and then at room temperature for 30 minutes to make sure all components were at equilibrium. Just before addition of thrombin, I added 15 μg/mL factor XIII to selected wells, and then started polymerization by addition of 0.10 U/mL thrombin. Results of these polymerizations are shown in Figure 5.1; lag time, V_{max}, and final absorbance are listed in Table 5.1.

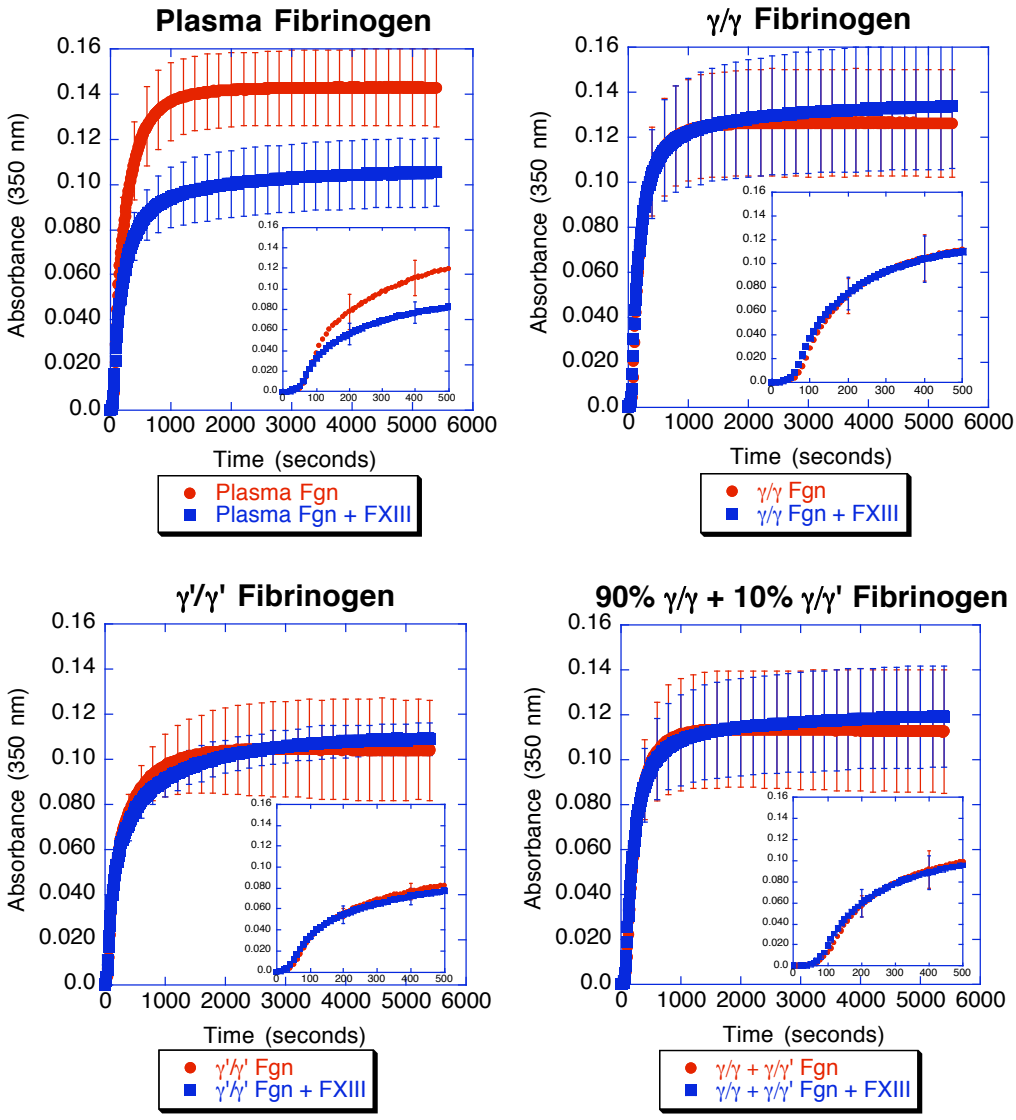

Figure 5.1. Polymerization curves for 0.15 mg/mL plasma fibrinogen, recombinant γ/γ, recombinant γ′/γ′, and 90% recombinant γ/γ + 10% recombinant γ/γ′ fibrinogen in the absence (red) and presence (blue) of 15 μg/mL factor XIII zymogen. Absorbance was read at 350 nm every 10 seconds for 5400 seconds. Insets contain detail of the first 500 seconds of polymerization reaction.

Polymerization Parameters With and Without Factor XIII

	Plasma Fgn		
Condition	Lag Time	Vmax	Final OD
	(sec)	(munits/min)	
Fibrinogen Only			
Mean	43	47.3	0.14
Standard Deviation	5	10.4	0.02
Fibrinogen + FXIII			
Mean	42	34.2	0.11
Standard Deviation	4	9.9	0.02
P Values	0.27	< 0.05	< 0.05
	γ/γ Fgn		
	Lag Time	Vmax	Final OD
	(sec)	(munits/min)	
Fibrinogen Only			
Mean	53	43.9	0.13
Standard Deviation	14	5.3	0.02
Fibrinogen + FXIII			
Mean	47	44.3	0.13
Standard Deviation	10	7.9	0.03
P Values	0.18	0.46	0.33
	γ'/γ' Fgn		
	Lag Time	Vmax	Final OD
	(sec)	(munits/min)	
Fibrinogen Only			
Mean	27	32.1	0.11
Standard Deviation	6	4.9	0.02
Fibrinogen + FXIII			
Mean	23	31.8	0.11
Standard Deviation	12	10.2	0.01
P Values	0.34	0.48	0.38
	γ/γ + γ/γ' Fgn		
	Lag Time	Vmax	Final OD
	(sec)	(munits/min)	
Fibrinogen Only			
Mean	73	36.9	0.11
Standard Deviation	16	5.7	0.03
Fibrinogen + FXIII			
Mean	60	39.2	0.12
Standard Deviation	15	8.8	0.02
P Values	0.09	0.30	0.34

Table 5.1. Lag time, V_{max}, and final absorbance are reported for each fibrinogen variant or mixture with and without factor XIII. P values < 0.05 are highlighted in red, indicating a > 95% chance that the difference between polymerization with and without factor XIII is significant.

Polymerization of plasma fibrinogen in the presence of factor XIII led to a similar lag time, but both a decreased maximal velocity and lower final absorbance compared to polymerization without factor XIII, suggesting the presence of thinner fibers. As shown in Table 5.1, these differences were significant. Neither recombinant γ/γ nor γ′/γ′ fibrinogen displayed these changes upon addition of factor XIII. To approximate the γ′ content of plasma fibrinogen, 90% recombinant γ/γ fibrinogen was mixed with 10% recombinant γ/γ′ fibrinogen heterodimer. Like the other recombinant fibrinogens, this mixture did not display a change in polymerization parameters upon addition of factor XIII zymogen. These results suggest that something inherent in plasma fibrinogen, other than the presence of γ′ chains, is responsible for the thinner fibers that form in the presence of factor XIII.

Factor XIIIa-Catalyzed Cross-Linking of Fibrin Variants

We studied the rate of cross-linking of γ/γ, γ′/γ′, and γ/γ′ fibrinogen in the presence of 0.20 U/mL thrombin and 0.50 μg/mL factor XIII zymogen. To determine the percent γ(′)-γ(′) dimer formed, we divided the intensity of γ-γ dimer bands by the total intensity of γ monomer + γ-γ dimer bands present in each sample. This ratio was plotted versus time to determine the rate of cross-linking. A representative cross-linking gel is shown for each of the three fibrinogen variants in Figure 5.2.

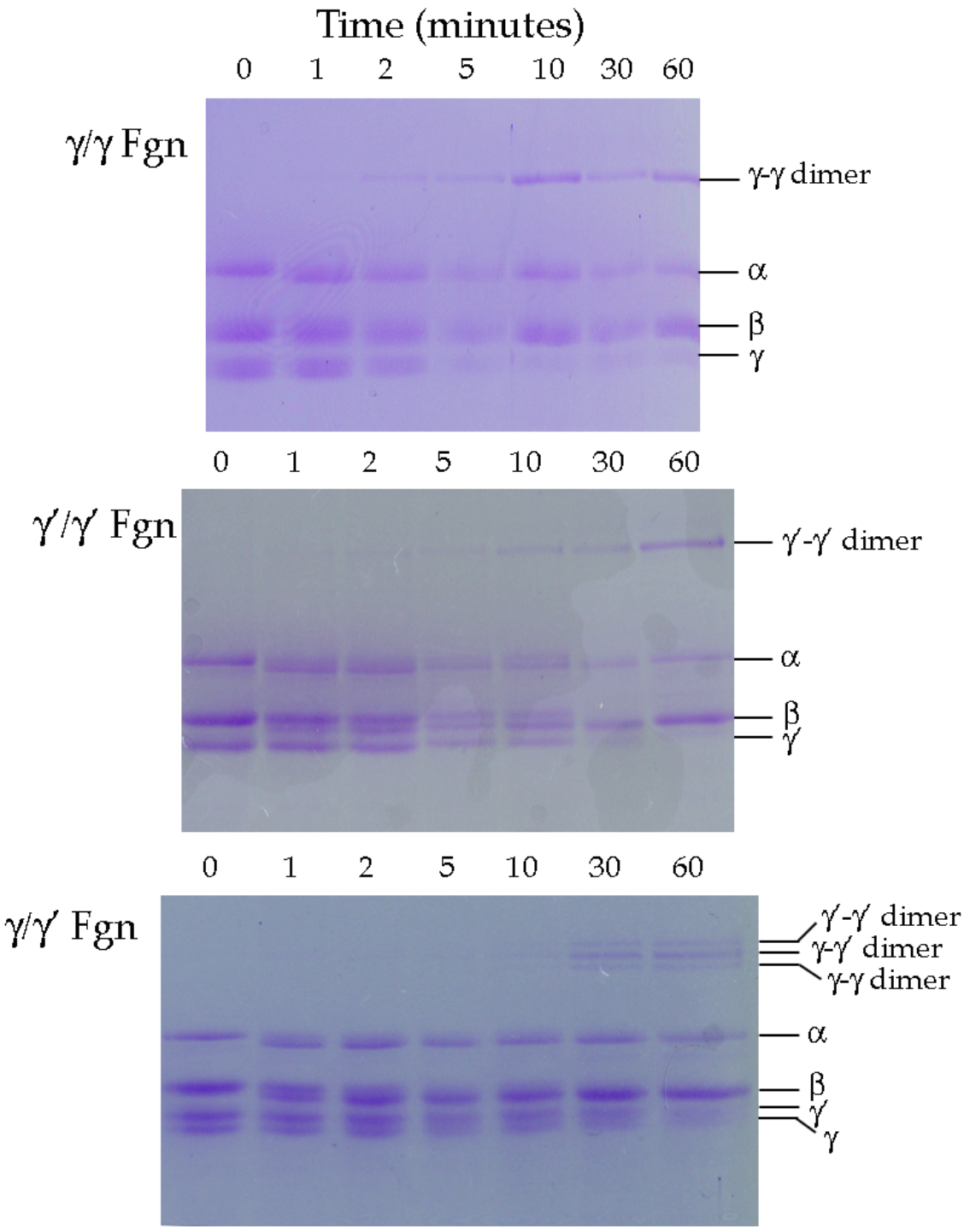

Figure 5.2. Representative 11% reducing gels of cross-linking reactions for γ/γ, γ′/γ′, and γ/γ′ fibrinogen. Recombinant fibrinogen (0.30 mg/mL) and factor XIII zymogen (0.50 μg/mL) were mixed with thrombin (0.20 U/mL) and incubated for the time periods shown above each gel. Samples were quenched with Laemmli buffer and BME, incubated at 37°C overnight, boiled, run on an 11% gel, and stained with Coomassie.

Figure 5.3 shows a representative cross-linking profile for factor XIII-catalyzed cross-linking of γ/γ, γ′/γ′, and γ/γ′ fibrinogen. A smooth curve was fit to the data for ease of comparison. γ/γ Fibrinogen appeared to have a faster initial rate of cross-linking than the γ′ variants, but γ′/γ′ fibrinogen had approximately the same extent of γ-γ dimer formation by 60 minutes, while γ/γ′ never moved past approximately 20% dimers.

It was possible to obtain a quantitative comparison of cross-linking rates by looking at the initial rate of cross-linking, before γ-γ dimerization slows and the dimer % in each sample becomes constant. We determined the cross-linking rate, in % dimers / minute, between 0 and 10 minutes of reaction time. Figure 5.4 shows this average rate for each fibrinogen upon factor XIII-catalyzed cross-linking.

The initial rates, standard deviations, and Student's t-test p values for factor XIII-catalyzed cross-linking are listed in Table 5.2. These initial rates were slower for variants with a γ′ chain compared to γ/γ fibrinogen.

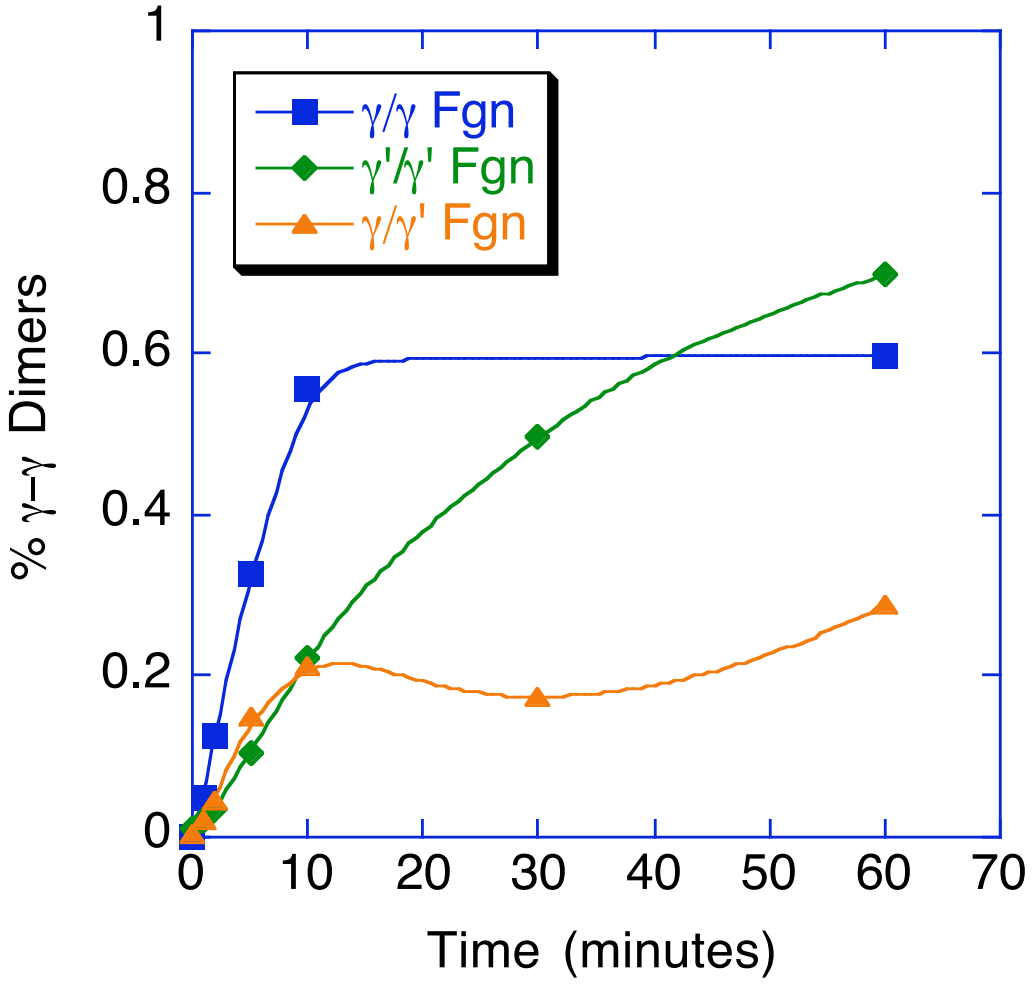

Figure 5.3. Representative cross-linking curves for γ/γ, γ′/γ′, and γ/γ′ fibrinogen (0.30 mg/mL) catalyzed by 0.50 μg/mL factor XIII and 0.20 U/mL thrombin. % γ-γ dimers are determined by dividing the intensity of the γ-γ dimer band (γ′-γ′ for γ′/γ′ fgn or γ-γ + γ′-γ′ + γ-γ′ for γ/γ′ fgn) by the sum of the γ monomer band + the γ-γ dimer band.

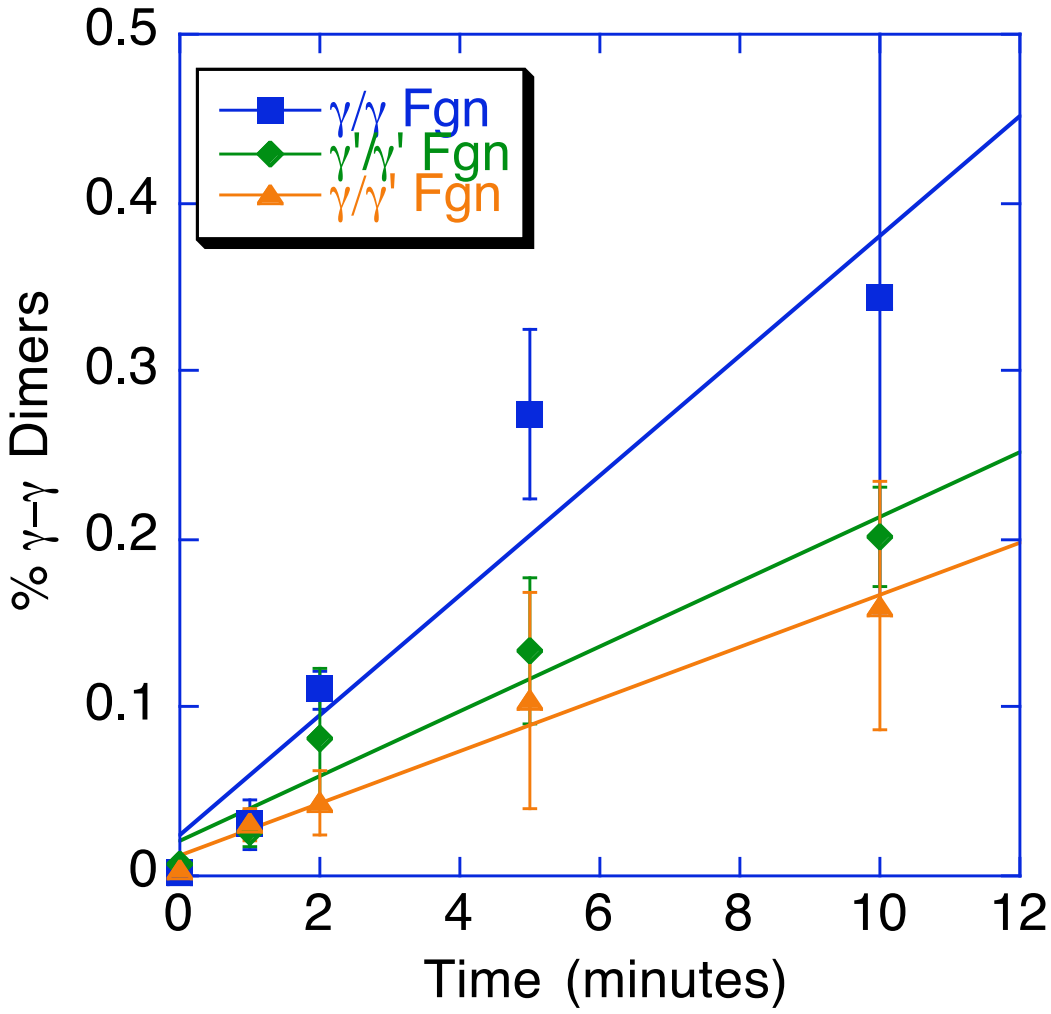

Figure 5.4. Initial rate of factor XIII-catalyzed cross-linking for the average of 3-4 reactions of γ/γ, γ′/γ′, or γ/γ′ fibrinogen. Data points between 0 and 10 minutes were fit to a line, the slope of which (% γ-γ dimers / minute) describes cross-linking rate.

γ(′)-γ(′) Dimer Formation By Factor XIII

	Fibrinogen		
	γ/γ Fgn	γ'/γ' Fgn	γ/γ' Fgn
Rate (% dimers/min)	0.053	0.036	0.020
Standard Deviation	0.005	0.016	0.01
P (compared to γ/γ)		0.08	< 0.05

Table 5.2. Initial rate (% γ-γ dimers/min) and standard deviation determined for each fibrinogen variant cross-linked by factor XIII zymogen. P values were determined by Student's t-test to compare variant fibrinogens.

Discussion

Close observation of Figure 5.1 indicates a small difference in the shape of the polymerization curve measured in the presence of factor XIII compared to those without. This was the case for each fibrinogen studied, both with or without γ′ chains. The absorbance at later timepoints did not quite level off the way it did without factor XIII. This slow, continued fiber growth was also observed by Carr et al [1], and suggests continued fiber cross-linking and rearrangement even an hour after addition of thrombin. In all other respects, however, recombinant fibrinogens in the presence of factor XIII polymerized in much the same manner as they did in the absence of factor XIII. This finding is consistent with the observations made by Ryan and coworkers of only very subtle differences in clot architecture in the presence or absence of active factor XIII [3].

There was a striking contrast between the muted response of recombinant fibrinogen to factor XIII and the dramatic change in polymerization of plasma fibrinogen when factor XIII is added. While lag time remained unchanged, addition of factor XIII to plasma resulted in a significant decrease in both V_{max} (reflecting rate of lateral aggregation) and final OD (reflecting fiber thickness). These two observations are much more similar to Lim's assertions than Ryan's publication [4],[3]. Clearly, there was some element of the plasma fibrinogen that caused addition of factor XIII to have such a striking effect compared to recombinant fibrinogen. This element may be the presence of a contaminating protein or a polymorphism that is not present in recombinant fibrinogen. Further experiments would be needed to determine the origin of these differences.

Factor XIII zymogen-catalyzed cross-linking began with an initial rate that was faster for γ/γ fibrinogen than for γ′/γ′ or γ/γ′ fibrinogen. After this initial burst, however, the cross-

linking of γ′/γ′ fibrinogen increased in speed so that by 60 minutes of incubation, it had formed approximately the same amount of γ′-γ′ dimers as γ/γ fibrinogen had formed γ-γ dimers. γ/γ′ Fibrinogen, on the other hand, continued to be cross-linked at a slow speed and plateaued at a lower dimer percentage. These results suggest that either 1) the presence of the γ′ chain diminishes fibrinogen's role as cofactor for factor XIII activation, or 2) the presence of γ′ chains inhibits γ(′)-γ(′) dimer formation at early timepoints. For some reason, this inhibitory activity is no longer present at later timepoints for γ′/γ′ fibrinogen, but remains in place for γ/γ′ fibrinogen.

We attempted to distinguish between these two options by using active factor XIIIa in our cross-linking experiments. We activated 1.67 μg/mL factor XIII with 0.67 U/mL thrombin so that upon addition to fibrinogen, the concentration of factor XIII would be 0.50 μg/mL and the concentration of thrombin would be 0.20 U/mL, just as for the factor XIII zymogen experiments. We did not include fibrinogen as a cofactor in this mixture because it is the variable in question. Upon addition of this AP-cleaved factor XIII to fibrinogen, the initial rate of dimer formation for each fibrinogen variant was less than half the rate for factor XIII zymogen-catalyzed cross-linking, not greater as we expected for pre-activated factor XIIIa (data not shown). This finding indicated that without including fibrinogen as a cofactor during factor XIII activation, AP-cleaved factor XIII is not in an active form, and therefore will not help us determine whether the γ′ chain has an effect on factor XIII activation.

Although we cannot fully explain the reason why the γ′ chain slows factor XIII-catalyzed dimer formation, our results are quite similar to those of Siebenlist and coworkers [7]. They also found a delayed initial rate of γ(′)-γ(′) dimer formation for γ/γ′ fibrinogen

compared to γ/γ fibrinogen, although their results do not appear to be as statistically significant as are ours. Because we did not perform our experiments at variable concentrations of fibrinogen, we cannot directly compare our results with those of Moaddel et al [6]. However, they claim a small enhancement in γ-γ′ dimer formation compared to γ-γ formation, and we do not reach such a conclusion.

In summary, we find addition of factor XIII during polymerization has little effect on the basic polymerization parameters, and the presence of the γ′ chain does not change this finding. Cross-linking of the γ′ chain by factor XIII, however, is slower upon the start of the reaction, but may rebound to the rate of γ/γ fibrinogen by later timepoints.

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CHAPTER SIX

CONCLUSION

Expression of γ′/γ′ and γ/γ′ fibrinogen in CHO cells was a significant portion of the work described in this document. From characterization of this expression system, I learned that secretion of γ′ chains occurs with the same efficiency as secretion of γ chains. The amount of γ′ protein produced is simply determined by the amount of message for this chain introduced into the cells; there is no impediment to translation of the mRNA message into γ′ protein or secretion of this protein from the cell. In addition, it appears that each γ or γ′ chain has an equal chance of assembling into a heterodimer or a homodimer, and the ratio of these products is determined by the ratio of γ to γ′ chains present inside the cell.

The next step in this kind of study would be to translate what we have learned about γ′ expression in CHO cells into humans. To my knowledge, no one has ever compared γ′ fibrinogen message levels from human hepatocyte cells with γ′ protein levels in blood samples from the same donor. Such an experiment would demonstrate whether, as we have shown in CHO cells, expression of γ′ protein in human plasma occurs with the same efficiency as expression of the γ chain. If there is no difference in the level of γ′ protein compared to γ′ mRNA, then we can conclude that the actual determinant of γ′ fibrinogen levels in human plasma is the frequency of alternative splicing into this sequence. Lovely and coworkers demonstrated the existence of elevated concentrations of γ/γ′ fibrinogen in coronary artery disease patients [1]. Inclusion of such patients could provide even more information for the study described.

Next I characterized the polymerization of γ′/γ′ and γ/γ′ fibrinogen compared to normal γ/γ fibrinogen. At 10 μ*M* calcium, the lag time of polymerization was significantly diminished for γ′/γ′ fibrinogen compared to γ/γ or γ/γ′ fibrinogen. As the concentration of calcium increased, however, this parameter returned to a normal value. Perhaps the high

concentration of extended γ′ chains in the γ′/γ′ homodimer causes some steric or electrostatic repulsion that prevents proper alignment of the fibrin monomers into a protofibril. Then when calcium is added to the reaction mixture, it interacts with the negatively charged γ′ sequence to mitigate the repulsion between molecules so that, in turn, polymerization returns to a normal rate.

Polymerization experiments also demonstrated that the presence of the γ′ chain, whether in heterodimer or homodimer form, led to a diminished rate of polymerization and a decrease in the final absorbance of the clot. Both of these observations may be explained by an increase in the rate constant for fiber initiation, or a decrease in the rate of lateral aggregation. Because this effect was seen at each concentration of calcium studied, I do not believe that the decrease is due to electrostatic repulsions since the addition of higher concentrations of calcium should counterbalance the negative charge of the γ′ chain. The γ′ region, however, is highly flexible, and may inhibit the formation of fibers by steric hindrance.

I studied the initial rate of factor XIIIa-catalyzed cross-linking of recombinant fibrin because at this point the γ(′)-γ(′) dimer formation rate is linear, and it best reflects the early steps necessary to make γ or γ′ fibrinogen a substrate for factor XIIIa. I found that the initial rate of γ/γ cross-linking is significantly faster than that of either γ′/γ′ or γ/γ′ fibrinogen. As I discussed in Chapter Five, there are two possibilities for this inhibition of cross-linking by the γ′ chain. One possible reason is that γ′/γ′ and γ/γ′ fibrinogen are not as efficient cofactors for factor XIII activation as is γ/γ fibrinogen. Moaddel and coworkers' experiments with γ/γ and γ/γ′ fibrinogen purified from plasma suggest that this is not the case. In fact, they demonstrated enhanced factor XIII activation in the presence of γ/γ′ fibrinogen [2]. This

finding suggests that a slower activation of factor XIII in the presence of γ′/γ′ or γ/γ′ fibrinogen does not occur.

The more likely possibility for a slower rate of cross-linking for γ′ fibrinogen is that the presence of the γ′ chain presents some structural inhibition to dimerization. Just as we observed for polymerization of γ′ fibrinogen, in cross-linking reactions the γ′ chain may block access to the cross-linking sites in the D region at Gln 398 and 399 or Lys 402. No crystal structure yet exists for the γ′ chain or the γ chain cross-linking sites because both are such flexible regions [3]. This flexibility suggests motion that may be impacted by the presence of the γ′ chain.

Mass spectrometry experiments have confirmed that tyrosine 422 of the γ′ chain in my recombinant γ′/γ′ fibrinogen is sulfated. We have no evidence that would suggest tyrosine 418 is also sulfated, although we also have no evidence that it is not. The fact that Tyr 422 is sulfated means that the extra negative charge of the sulfate group is present on our γ′ chain, just as it is in plasma fibrinogen. Sabo and coworkers have shown that in the binding of γ′ to thrombin, only one tyrosine must be sulfated and it is tyrosine 422 that is more important for this interaction than tyrosine 418 [4]. Taken together, these two findings suggest that if the γ′ chain of plasma fibrinogen really were a binding site for factor XIII, so should be our recombinant γ′ chain. However, we found no evidence of enhanced binding of factor XIII zymogen to the γ′ chain of fibrinogen. This means that unlike previous assertions [5], the γ′ chain is not a binding site for factor XIII zymogen.

From the experiments described in this document, I am unable to postulate a more likely binding site. Addition of the γ′ chain does not increase factor XIII binding compared to normal fibrinogen; removal of the αC domains does not decrease factor XIII binding

compared to normal fibrinogen. Thus, it seems that the binding site is somewhere other than these two regions.

My binding experiments, do, however provide information for the presentation of a unified model of factor XIII A and B subunit binding to fibrinogen and fibrin. In the interaction of factor XIII zymogen and fibrinogen, the B subunits provide a higher affinity binding site than do the A subunits. Activation of factor XIII strengthens the binding of the A subunits to fibrinogen. This effect may be due to a conformational change in the A subunits that better allows fibrinogen to interact with factor XIIIa's binding site or to the removal of B subunits which block a high affinity site.

Both factor XIII zymogen and active factor XIIIa bind more strongly to fibrin than to fibrinogen. Since fibrin is the substrate of factor XIIIa, this increased affinity may help localize factor XIII(a) to molecules that are ready to be cross-linked. Loss of the αC domains from fibrin renders factor XIII zymogen binding to Aα251 fibrin weaker than the zymogen's binding to other fibrin variants. This finding suggests that it is the mobilization of the αC domains that creates the higher affinity binding of factor XIII zymogen to fibrin.

The next step in these experiments would be to determine where the binding site on fibrinogen is for factor XIII zymogen, since it is not the γ′ chain. Clearly, there is some interaction between these two proteins since factor XIII co-purifies with fibrinogen [6]. My ELISA binding system could be used to screen fibrinogen fragments from different parts of the molecule. Those that led to weaker factor XIII binding could then be further characterized to determine the location of this binding site.

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APPENDIX

THROMBIN-INDEPENDENT ACTIVATION OF FACTOR XIII

Summary

In addition to the well-known thrombin-catalyzed pathway for activation of factor XIII, another method of generating active factor XIIIa exists that does not require thrombin-catalyzed activation peptide cleavage [1]. Because such a pathway will likely lead to activation of factor XIII zymogen during binding experiments, we performed these experiments to characterize this activation method. It was shown qualitatively that thrombin-independent activation of factor XIII takes place in the presence of calcium and fibrinogen after no more than an hour of incubation. A quantitative measure of factor XIII activity, the dansylcadaverine incorporation experiment, demonstrated that factor XIII activity increased with increasing concentration of calcium and that fibrinogen was a required cofactor at low, physiological levels of calcium. Native electrophoresis indicated that activation by calcium and fibrinogen leads to factor XIII that has a different structure from thrombin-activated factor XIIIa.

Introduction

The "traditional" activation process for factor XIII conversion to factor XIIIa is catalyzed by thrombin and was described in the Introduction to this document. In short, thrombin catalyzes cleavage of the activation peptide blocking the active site of one of the two A subunits in the molecule [2], [3]. Next, B subunits dissociate from the cleaved A subunits, and the A_2 dimer changes conformation to reveal the active site [4]. In addition to this well-known thrombin-catalyzed pathway for activation of factor XIII, a thrombin-independent pathway for activation has also been observed. In 1978, Credo and coworkers first described this pathway. At calcium concentrations of 50 m*M* or above, they observed

incorporation of radiolabeled iodoacetamide into the active site of the enzyme. This activity was not affected by the presence of fibrinogen, a cofactor of thrombin-catalyzed activation of factor XIII [1]. In the presence of EDTA activation was reversed [5].

Thrombin-independent activity at high concentrations of calcium is neither physiologically relevant, nor a factor in the binding experiments described in chapter 4, but recently such an activation pathway was described at lower concentrations of calcium. Siebenlist *et al*. demonstrated by SDS-PAGE the formation of γ-γ dimers at concentrations of calcium as low as 50 μ*M*, and the appearance of Aα polymers at slightly higher concentrations, in the m*M* range, when 3 mg/mL fibrinogen and 100 Loewy units/mL factor XIII were incubated for one hour without thrombin and with various concentrations of calcium. This phenomenon is also seen with platelet factor XIII (A_2). It lacks the several minute lag time before generation of activity that plasma factor XIII displays. γ/γ Fibrinogen was shown to be a better substrate for thrombin-independent activation of factor XIII than γ/γ′ fibrinogen by measuring the percent insoluble fibrinogen generated over time. Thrombin-independent activity is highly substrate-dependent. When the nonphysiogical substrate cadaverine was incorporated into N,N'-dimethylcasein, factor XIII displayed significantly less activity than it did with fibrinogen as substrate. The authors of this study concluded from these observations that fibrinogen may bind near the active site of factor XIII, thereby inducing a conformational change that leads to activation. Thrombin-independent activation is faster for platelet factor XIII which lacks B subunits to block the active site. The binding is less favorable for γ/γ′ fibrinogen. They claim that this effect is due to the B subunits of factor XIII binding to the γ′ chain and preventing active site binding

[6], but based on the results described earlier in this document, this particular interpretation may be erroneous.

Whether or not thrombin-independent activation is relevant *in vivo* remains in question. A recent challenge to the above mentioned study suggested that if thrombin-independent activation of factor XIII took place as described, then fibrinogen dimers and multimers should be found in circulation. Since virtually none of these multimers are detected with a detection limit of < 0.5%, this activation is likely an *in vitro* phenomenon [7]. The authors of the original study dispute the assertion that cross-linked chains are not found in circulation based on their previous work demonstrating the existence of intramolecularly cross-linked Aα and γ chains [8]. They claim that *in vitro* effects are not responsible for the activity of factor XIII zymogen and more attention should be paid to the underlying mechanism for this activity [9]. Debate continues as to the nature and existence of thrombin-independent activation at physiological concentrations of calcium.

Consideration of this thrombin-independent activation at low concentrations of calcium explains the recent criticism of Moaddel's analytical ultracentrifugation study of factor XIII binding to fibrinogen [10], [6]. Sedimentation equilibrium studies are run at a slow speed that requires long experimental runs. Incubation of fibrinogen and factor XIII at 1 m*M* $CaCl_2$ for hours to days would certainly lead to some degree of factor XIII activation and formation of fibrinogen dimers. These dimers complicate the analysis of sedimentation equilibrium experiments by forming covalent attachments between the fibrinogen monomers whose noncovalent associations are being measured.

The goal of this investigation is to further characterize the process that takes place during ultracentrifugation and other experiments that involve lengthy incubations of

fibrinogen and factor XIII in the presence of $CaCl_2$. Siebenlist's work extensively outlined the ways in which thrombin-independent activation of factor XIII could be accelerated; I wished to determine just which cofactors were necessary for minimal activation as well as what form was taken by this alternatively-activated factor XIII.

Materials and Methods

Materials

All reagents came from Fisher or Sigma, unless otherwise noted. Human fibrinogen that was plasminogen, fibronectin, and factor XIII depleted came from American Diagnostica (Stamford, CT) (lot 030411). Human factor XIII (lot 850A) and human α-thrombin (lot 2690PR) came from Enzyme Research Laboratories (South Bend, IN). Hirudin (lot B68947) and HRP-conjugated polyclonal goat anti-mouse IgG were purchased from Calbiochem (San Diego, CA). Rabbit monoclonal anti-fibrinogen γ chain (NC-15) custom antisera was purchased from Hazleton Research Products (Princeton, NJ); antibody Y-18 was from Dr. Willem Nieuwenhuizen at the Foundation of Liver Cell and Endothelial Cell Technology, Netherlands. HRP-conjugated polyclonal goat anti-rabbit IgG was purchased from Oncogene. 96-well microtiter EIA/RIA medium-binding plates were purchased from Corning.

Monitoring Cross-Link Formation by Coomassie Gel and Western Blot

Fibrinogen (3.0 mg/mL) and factor XIII (0.030 mg/mL) were incubated at room temperature for up to two days. At appropriate timepoints, tubes containing the reaction mixture were quenched by reducing Laemmli buffer. Samples were run on an 11% SDS gel stained with Coomassie or immunoblotted according to the protocol described in Chapter 2.

To visualize γ chain bands, membranes were probed with anti-γ chain antibody NC-15 + HRP-conjugated anti-mouse IgG. To probe for Aα chain bands, membranes were developed with anti-Aα chain antibody Y-18 + HRP-conjugated anti-mouse IgG.

Cofactor Requirement Experiments

To probe the requirement for calcium as a cofactor in thrombin-independent activation of factor XIII, fibrinogen (8.8 μ*M*) and factor XIII (8.8 μ*M*) were incubated in HBS + 1.0 m*M* $CaCl_2$ or 1.0 m*M* EDTA for 2 days. One EDTA sample was spiked with 3.0 m*M* $CaCl_2$ for 5 minutes after the 2 day incubation. All samples were reduced and run on an 11% gel as described above. To determine if fibrinogen was required, I performed a similar experiment incubating factor XIII (8.8 μ*M*) and $CaCl_2$ (1.0 m*M*) with or without fibrinogen (8.8 μ*M*) for 2 days. I added 8.8 μ*M* fibrinogen back to the fibrinogen-free reaction mixtures for 5 minutes and then the samples were reduced and run on an 11% gel.

Dansylcadaverine Incorporation

Factor XIII (0.30 mg/mL) was thrombin-independently activated in 10 μL overnight. To generate this activation, factor XIII was incubated with the appropriate concentration of $CaCl_2$ (1.0 m*M* unless otherwise noted) with or without 0.30 mg/mL fibrinogen (plasma or recombinant) in TBS. Thrombin-activated factor XIII was prepared by incubating 0.50 mg/mL factor XIII with 2.5 U/mL thrombin in proteolytic preactivation buffer (10m*M* DTT + 20 m*M* $CaCl_2$ in TBS) for 20 minutes at 37°C. At the end of this incubation, thrombin was inactivated by the addition of 5.0 U/mL hirudin. Reactions were run in a 96-well plate. $CaCl_2$ concentration was adjusted to 1.0 m*M* for the incorporation reaction, N,N′-dimethylcasein was added (20 mg/mL), and volume was brought to 90 μL with incorporation buffer (5.0 m*M* DTT in TBS). The incorporation reaction was initiated with the addition of

2.5 m*M* dansylcadaverine. The reaction was followed by fluorescence with excitation at 355 nm and emission at 495 nm monitored for 2 hours. Incorporation was quantitated by calculating the rate of fluorescence increase over time.

Native Gels

Factor XIII (3.0 μg) was mixed with fibrinogen (3.0 μg) and variable concentrations of $CaCl_2$ and incubated overnight at room temperature. Another sample of factor XIII (3.0 μg) was mixed with 10 U/mL thrombin, 3.0 μg fibrinogen, and 1.0 m*M* $CaCl_2$ and incubated at 37°C for 20 minutes. Samples were mixed with native sample buffer (40% glycerol + 0.01% bromophenol blue in 62.5 m*M* Tris, pH 6.8) and loaded onto a 4-15% precast Tris-glycine gel. The gel was run with native running buffer (25 m*M* Tris-HCl, pH 8.3 + 192 m*M* glycine) and stained with Coomassie.

Results

Factor XIII Zymogen Catalyzes Cross-linking of Fibrinogen at 1 mM $CaCl_2$

Factor XIII (0.030 mg/mL), factor XIII-free plasma fibrinogen (3.0 mg/mL), and calcium (1.0 m*M*) were incubated at room temperature for various timepoints up to 2 days, then reduced and run on an 11% gel. Starting at 1 hour, a band corresponding to γ-γ dimers can be seen, along with a decrease in intensity of the γ chain relative to the Bβ chain (Figure A.1). A Western blot of this reaction indicates that this newly appearing band is indeed γ-γ (Figure A.2). These data clearly show that at 1.0 m*M* $CaCl_2$ factor XIII zymogen was capable of cross-linking fibrinogen into γ-γ dimers. The addition of hirudin to these samples does not abrogate cross-link formation, indicating that contamination by thrombin was not responsible for this activity (data not shown).

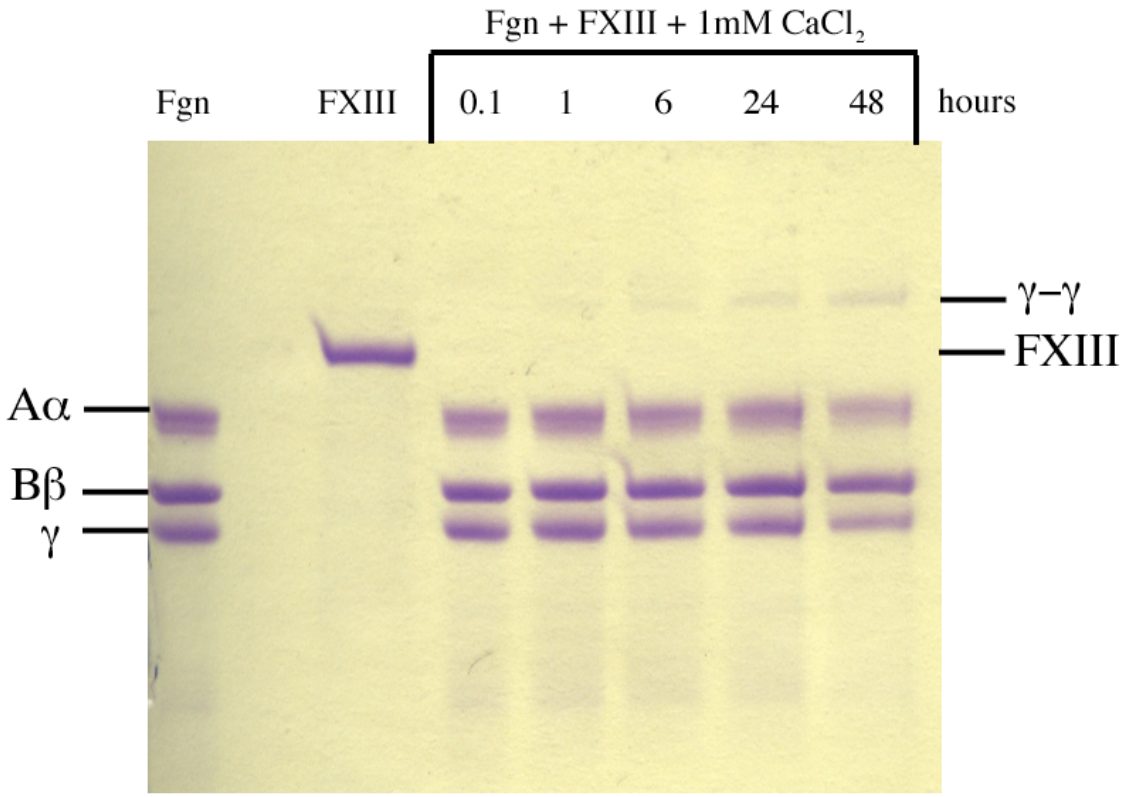

Figure A.1 Factor XIII (0.030 mg/mL) and fibrinogen (3.0 mg/mL) incubated with 1.0 m*M* $CaCl_2$ in HBS for the time periods shown, then reduced and run on an 11% gel. Location of γ-γ dimers is marked.

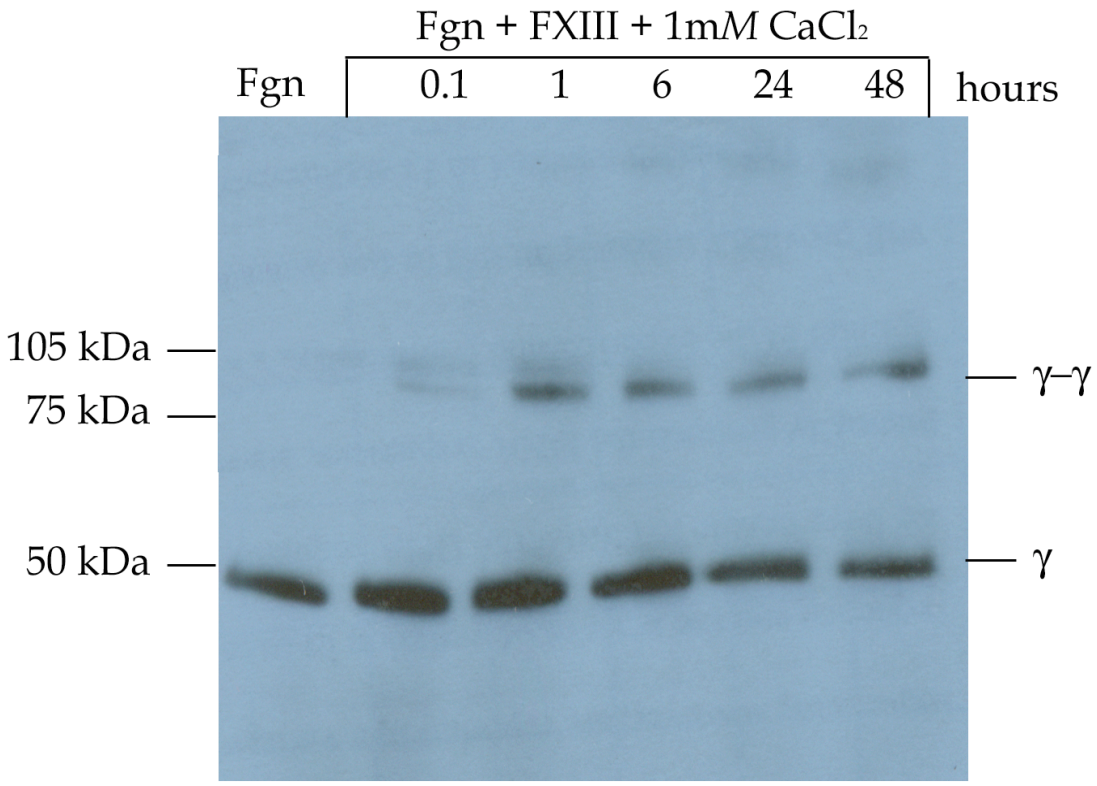

Figure A.2 Western blot of fibrinogen + factor XIII zymogen incubation reactions in the presence of 1.0 m*M* $CaCl_2$ as described in the legend to Figure A.1. The blot was developed with NC-15, a γ-chain specific antibody.

Cofactor Requirements: Calcium

To determine whether calcium was a required cofactor for thrombin-independent activation, it was omitted from the activation mixture using the assay described above. However, the indicator of factor XIII activation in this experiment is cross-linking of fibrinogen, a calcium-dependent process. Figure A.1 shows that 5 minutes was not long enough to thrombin independently activate factor XIII and form significant cross-links, but control experiments show that activated factor XIIIa can catalyze cross-links in this amount of time (data not shown). Adding calcium back to the reaction mixture for 5 minutes will provide a chance for fibrinogen to be cross-linked if factor XIII has already been activated, but will not be long enough for measurable new activation of the enzyme.

Figure A.3 shows the results of this experiment. Fibrinogen and factor XIII standards are shown on the left. An equimolar mixture of fibrinogen and factor XIII with 1.0 m*M* $CaCl_2$ was incubated for 2 days at room temperature during which factor XIII was thrombin-independently activated. Both γ-γ dimers (~90 kDa) and Aα polymers (at the base of sample well) formed. In a mixture of fibrinogen, factor XIII, and 1.0 m*M* EDTA incubated for 2 days, neither γ-γ dimers nor Aα polymers formed. The last lane on the gel shows the same incubation mixture with 3.0 m*M* $CaCl_2$ added during the last five minutes of incubation. This concentration of calcium balanced out the effect of the EDTA and returned 1.0 m*M* $CaCl_2$ to the reactants for the 5-minute cross-linking period. Because no cross-links formed between fibrinogen molecules when EDTA was present during activation even when $CaCl_2$ is returned during cross-linking, $CaCl_2$ is a required cofactor for thrombin-independent activation.

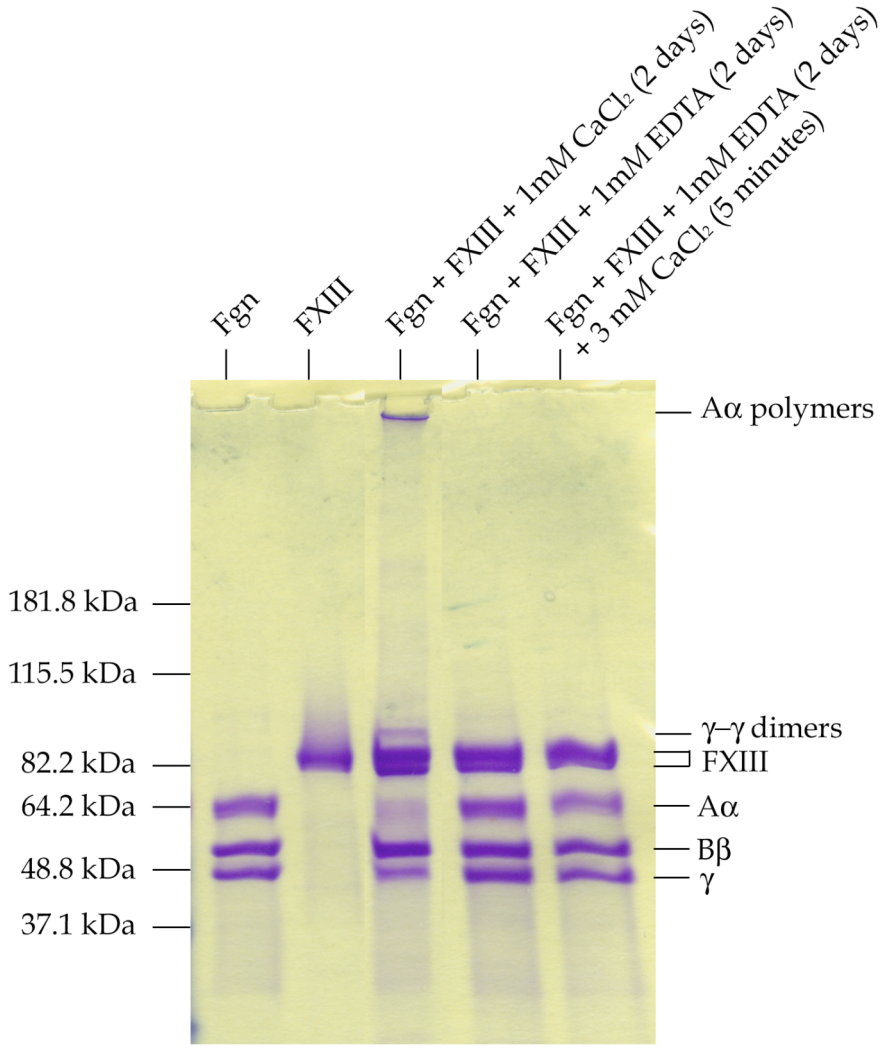

Figure A.3. Calcium is a required cofactor for thrombin-independent factor XIII activation. 8.8 μ*M* fibrinogen standard and 8.8 μ*M* factor XIII standard are in the first two wells. For the other samples, 8.8 μ*M* each of fibrinogen and factor XIII are incubated for 2 days with 1.0 m*M* $CaCl_2$, 1.0 m*M* EDTA, or 1.0 m*M* EDTA plus 3.0 m*M* $CaCl_2$ for the last 5 minutes. The locations of γ-γ dimer and Aα-polymer bands are shown.

Cofactor Requirements: Fibrinogen

In a system similar to the one described above for evaluating the contribution of calcium to thrombin-independent activation of factor XIII, the requirement for fibrinogen as a cofactor was also investigated. Fibrinogen was omitted during the original 2 day factor XIII activation period and added back for the 5 minute cross-linking period. Figure A.4 shows that under these conditions cross-links did not form; along with calcium, fibrinogen is a necessary cofactor for thrombin-independent activation of factor XIII.

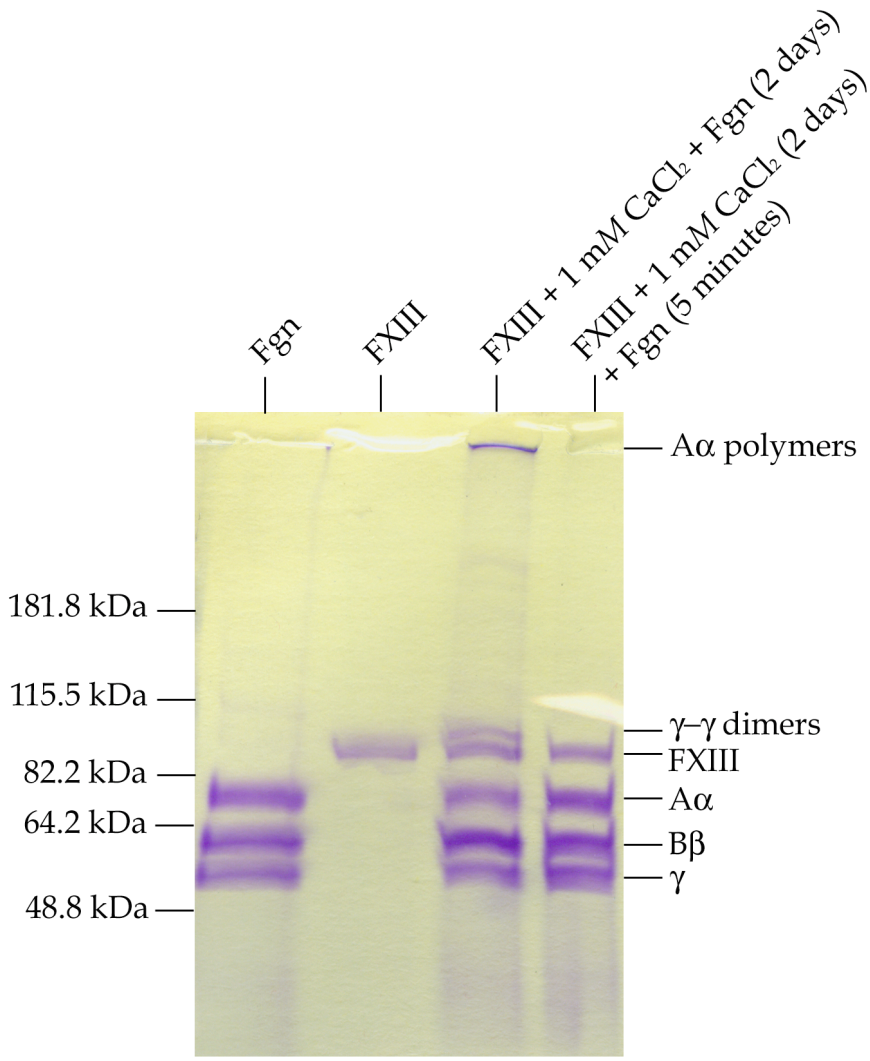

Figure A.4. Fibrinogen is a required cofactor for thrombin-independent activation of factor XIII. 8.8 μ*M* fibrinogen standard and 8.8 μ*M* factor XIII standard are in the first two wells. In the next sample, 8.8 μ*M* each of fibrinogen and factor XIII are incubated for 2 days with 1.0 m*M* $CaCl_2$. The last lane shows 8.8 μ*M* factor XIII + 1.0 m*M* $CaCl_2$ incubated for 2 days with 8.8 μ*M* fibrinogen added back in the last 5 minutes. The locations of γ-γ dimer and Aα-polymer bands are shown.

Because fibrinogen is both cofactor and substrate when factor XIII activation is measured by cross-linking of fibrinogen, it simplifies analysis to remove its role as substrate when attempting to study its cofactor activity. To do this, I have employed a dansylcadaverine incorporation assay, originally described by Lorand [11]. In this assay, factor XIII(a) (activated either with or without thrombin, under various conditions) catalyzes incorporation of the fluorescent substrate dansylcadaverine into the protein N,N′-dimethylcasein. Incorporation into the hydrophobic protein alters the fluorescence intensity of the dansylcadaverine and the rate of incorporation can be measured by the slope of fluorescence increase, monitored kinetically. Fibrinogen was included with factor XIII zymogen for 2 days prior to the dansylcadaverine incorporation experiment for thrombin-independent activation. The concentration of fibrinogen during the activation period was 0.88 μ*M*, but was diluted to 0.088 μ*M* before the fluorescence monitoring began. This dilution prevented fibrinogen from effectively competing with the 2.5 m*M* dansylcadaverine as a substrate for factor XIII. As an additional precaution, fibrinogen concentrations were equalized in all samples before the start of the fluorescence incorporation reaction, even if fibrinogen was not included in the initial activation mixture. DTT was not included during the activation period, but was added to the incorporation reaction to increase factor XIII's response to a non-physiological substrate. Figure A.5 shows how this experiment provides a quantitative measure of factor XIII activation under various conditions. Factor XIII zymogen activation in the presence of EDTA gave a slope of dansylcadaverine incorporation that was close to zero, similar to the no FXIII control sample. As the concentration of calcium increased, the slope of dansylcadaverine incorporation (measured as fluorescence versus time) increased until we reach 50 m*M* $CaCl_2$ which displayed the same rate of activity as

thrombin-activated factor XIIIa. A straight line at 65,535 fluorescence units indicates saturation of the instrument's sensitivity. Table A.1 demonstrates the relationship between calcium concentration and extent of activation of factor XIII zymogen.

To determine the need for fibrinogen as cofactor in thrombin-independent activation of factor XIII, factor XIII was activated at different concentrations of calcium with and without fibrinogen and then used in the dansylcadaverine incorporation assay. Figure A.6 shows the results of this experiment. To account for day-to-day variability of the fluorescent substrate, slopes were measured as a percent of the thrombin-activated factor XIIIa activity. At 1.0 m*M* $CaCl_2$, addition of fibrinogen produced a significant increase in the activity of factor XIII compared to 1.0 m*M* $CaCl_2$ in the absence of fibrinogen. At 5.0 m*M* $CaCl_2$ this effect was abolished. Fibrinogen also had no effect on activity at 50 m*M* $CaCl_2$, consistent with the observations of Credo and coworkers [1]. Thus, fibrinogen is only necessary at a low, physiological concentration of $CaCl_2$ (1 m*M*) to act as a cofactor in thrombin-independent activation of factor XIII.

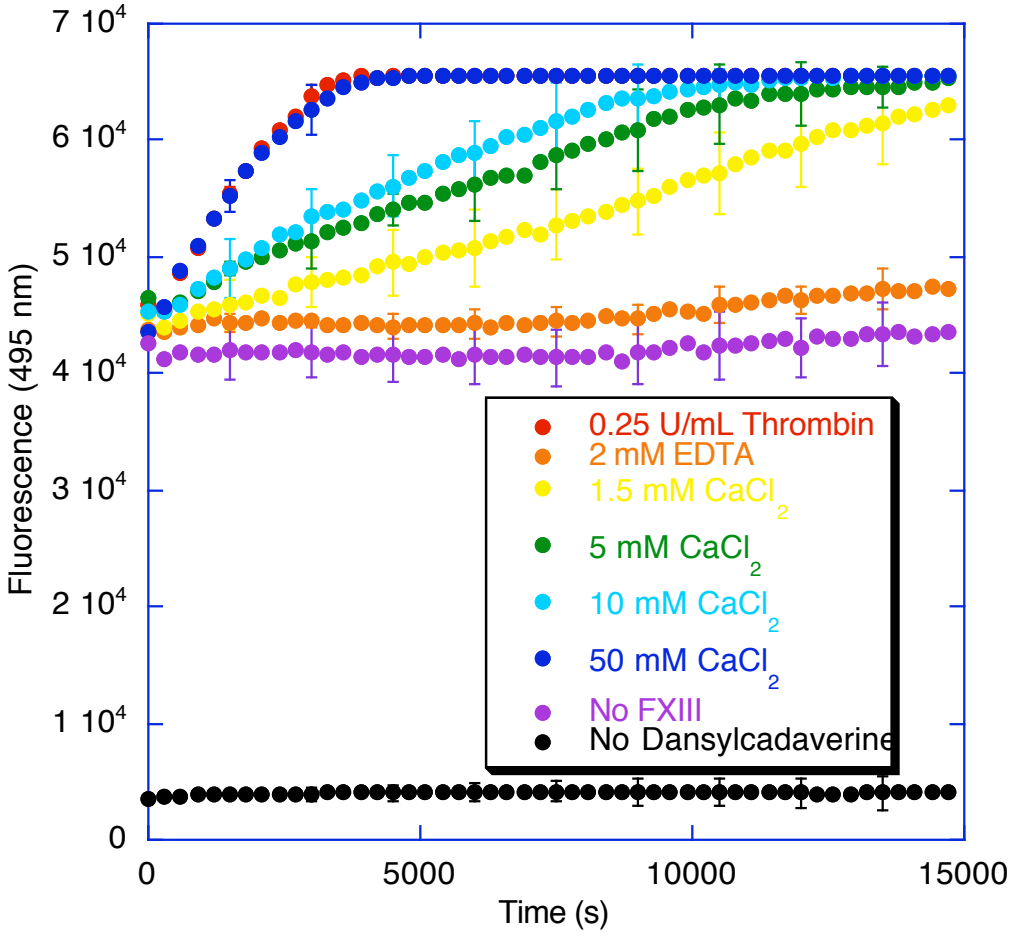

Figure A.5. Dansylcadaverine incorporation assay for thrombin-activated factor XIIIa and thrombin-independently activated factor XIII zymogen. Incorporation of dansylcadaverine into N,N′-dimethylcasein by factor XIII is measured as an increase in fluorescence intensity over time. Factor XIII was activated by thrombin (red) or incubation with 0.30 mg/mL fibrinogen + the concentration of $CaCl_2$ or EDTA listed in the legend. Samples lacking FXIII (purple) or fluorescent substrate (black) are included as controls.

Dansylcadaverine Incorporation by Factor XIII(a)

Method of FXIII Activation	Activity (slope of dansylcadaverine incorporation, s^{-1})
0.25 U/mL thrombin	6.0 +/- 1.3
2.0 m*M* EDTA	0.23 +/- 0.05
1.5 m*M* $CaCl_2$	1.3 +/- 0.2
5.0 m*M* $CaCl_2$	1.6 +/- 0.3
10 m*M* $CaCl_2$	2.0 +/- 0.3
50 m*M* $CaCl_2$	5.4 +/- 1.8
No FXIII control	0.12 +/- 0.06

Table A.1. Activation of factor XIII zymogen is directly related to the concentration of calcium with which it is incubated. Slopes determined from Figure A.5.

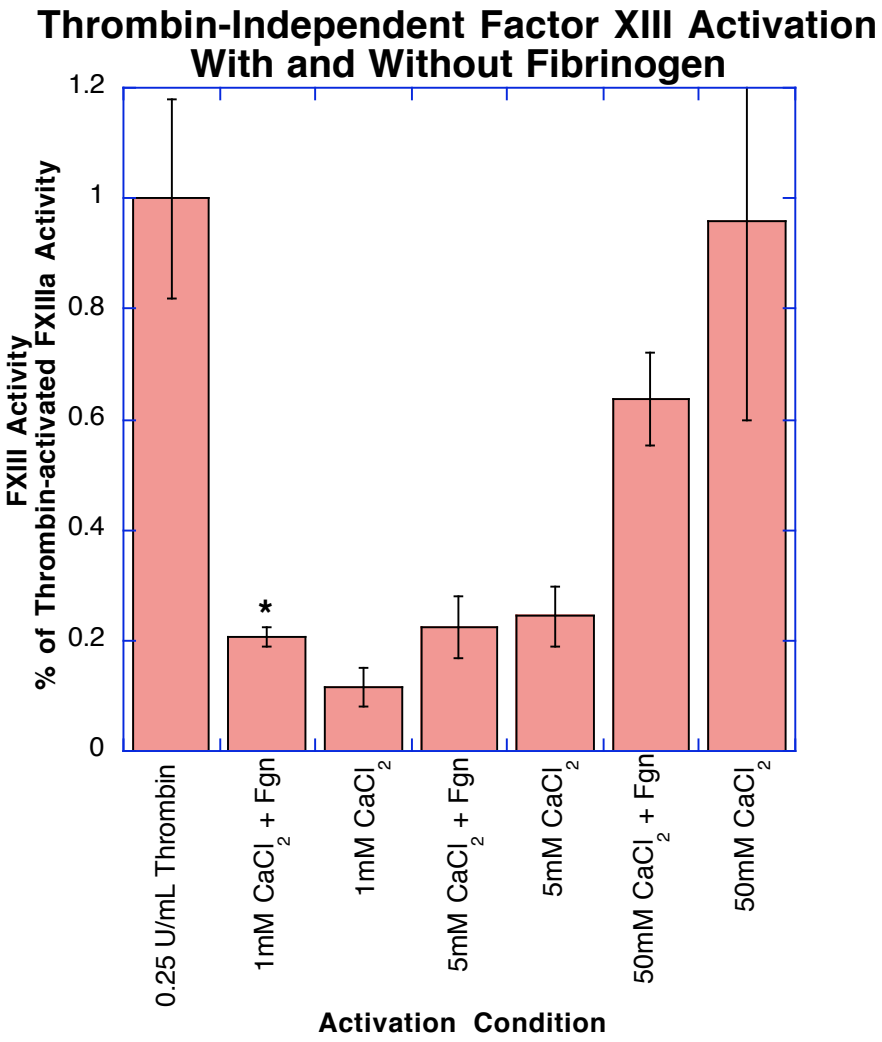

Figure A.6. Factor XIII activity generated by thrombin or thrombin-independently with and without fibrinogen at 1.0 m*M*, 5.0 m*M*, and 50 m*M* $CaCl_2$ determined by dansylcadaverine incorporation. Experiments were performed as described in Methods. The asterisk denotes a significant increase in activation in the presence of fibrinogen compared to its absence.

Catalysis of Thrombin-Independent Factor XIII Activation by Variant Fibrinogens

To locate the domain on fibrinogen responsible for catalyzing thrombin-independent activation of factor XIII at low concentrations of calcium, we employed recombinant fibrinogen variants in the dansylcadaverine incorporation assay. To see if the presence of the γ′ extension played a role in this process, recombinant γ′/γ′ fibrinogen was used. We included Aα251 fibrinogen, in which the Aα-chain is truncated at residue 251, to explore the role of the αC domain in activation. These recombinant fibrinogens were mixed with factor XIII zymogen at 0.30 mg/mL, along with 1.0 m*M* $CaCl_2$, with or without thrombin to activate the factor XIII. As can be seen in Figure A.7, the presence of thrombin increased the extent of factor XIII activation, but in its absence factor XIII still displayed significant dansylcadaverine incorporation. Table A.2 reports the slope of incorporation from Figure A.7, a measure of factor XIII activity. While the activity of thrombin-activated factor XIIIa was the same no matter which fibrinogen was present during activation, inclusion of both of the mutant fibrinogens led to statistically significant lowered activity of thrombin-independently activated factor XIII compared to normal recombinant fibrinogen. This finding suggested that both the γ′ region and the αC domain play a role in thrombin-independent factor XIII activation.

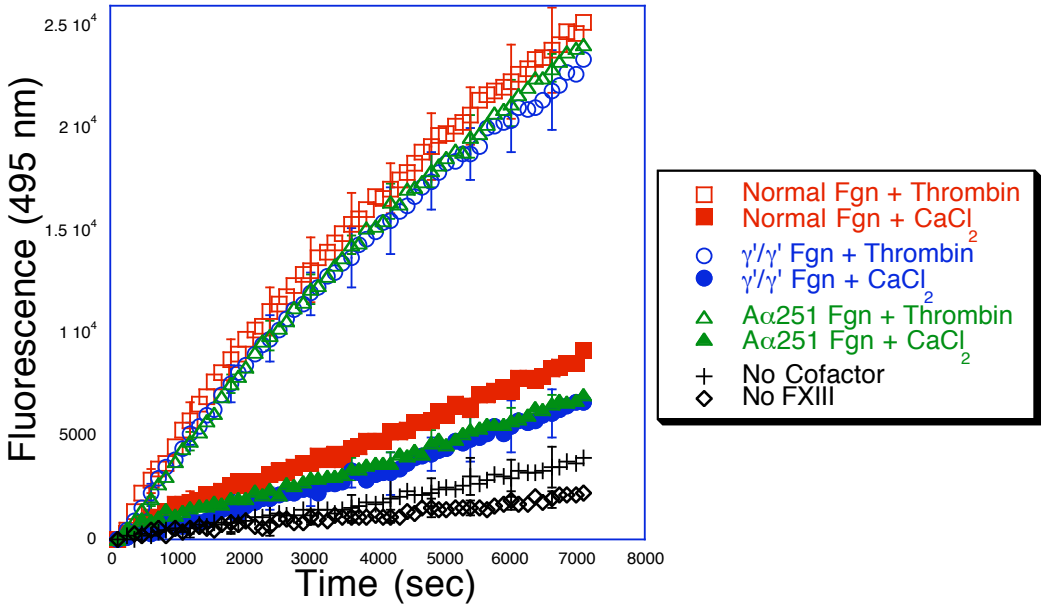

Figure A.7. Thrombin-cleaved (open symbols) and thrombin-independently activated (closed symbols) factor XIII(a) in dansylcadaverine incorporation assay. Normal recombinant fibrinogen (red), γ′/γ′ fibrinogen (blue), and Aα251 fibrinogen (green) were included in the assay. Experiments were performed as described in Methods with 0.30 mg/mL factor XIII, 0.30 mg/mL recombinant fibrinogen, and either 1.0 m*M* $CaCl_2$ or 2.5 U/mL thrombin.

Dansylcadaverine Incorporation for Factor XIII(a) Activated with Recombinant Fibrinogens

Fibrinogen	Rate of Incorporation for Thrombin-Catalyzed Factor XIIIa (slope x 10^{-5} +/- std dev)	Rate of Incorporation for Thrombin-Independently Activated Factor XIII (slope x 10^{-5} +/- std dev)
Normal	10 +/- 0.7	2.6 +/- 0.2
γ′/γ′	9.0 +/- 0.7	1.7* +/- 0.6
Aα251	9.6 +/- 0.5	2.1* +/- 0.3

Table A.2. The rate of dansylcadaverine incorporation into casein is proportional to the extent of factor XIII activation. The slope of dansylcadaverine incorporation from Figure A.7 is reported +/- standard deviation for each activation condition. An asterisk (*) denotes $p < 0.05$, compared to normal recombinant fibrinogen.

Visualization of Thrombin-Independent Activation of Factor XIII by Native Gel Electrophoresis

A native gel was run of factor XIII activated by 10 U/mL thrombin, 50 m*M* $CaCl_2$ + plasma fibrinogen, or 1 m*M* $CaCl_2$ + plasma fibrinogen. The subunits of factor XIII remain associated on a native gel, allowing us to probe the structure that results from each of these activation methods, compared to unactivated factor XIII. See Figure A.8.

All three methods of activation used to prepare samples for this gel have generated factor XIII capable of cross-linking as shown on reducing gels (see Figures A.1,2). Cross-linking of fibrinogen can be seen on this native gel by a loss of intensity in the fibrinogen bands. Factor XIII activated with 1.0 m*M* $CaCl_2$ cross-linked less of the fibrinogen than factor XIII activated by either 50 m*M* $CaCl_2$ or thrombin, but still showed some dissipation of the fibrinogen band compared to the sample treated with EDTA. Thus, each of these lanes contain activated factor XIII. The A and B subunits remained associated in the samples activated with $CaCl_2$, but dissociated in the sample activated by thrombin. This observation suggests a structural difference between the different methods of activation.

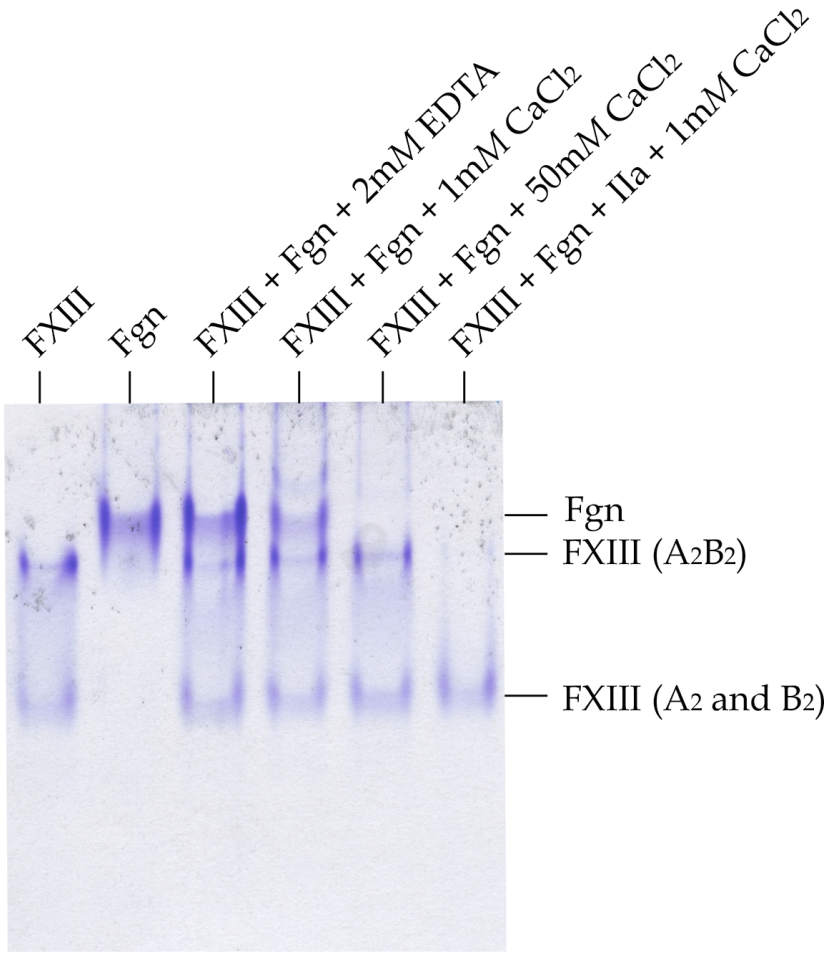

Figure A.8. Native 4-15% Tris gel of factor XIII incubated with 2 m*M* EDTA, 1 m*M* $CaCl_2$ + fibrinogen, 50 m*M* $CaCl_2$ + fibrinogen, or 10 U/mL IIa + 1 m*M* $CaCl_2$ + fibrinogen.

Discussion

The experiments described in this appendix confirm the assertions made by Siebenlist, Meh, and Mosesson in 2001 that factor XIII zymogen can become active and cross-link fibrinogen, without activation peptide cleavage by thrombin [6]. We have shown that γ-γ and Aα cross-links formed within one hour at physiological concentrations of fibrinogen, factor XIII, and calcium in the absence of thrombin (Figures A.1, 2). We have also shown that this thrombin-independent activation was dependent on the presence of calcium (Figure A.3) and the extent of activation was directly proportional to the concentration of calcium present during the activation period (Figure A.5).

Dansylcadaverine incorporation experiments have been used to demonstrate that at low, physiological concentrations of calcium, fibrinogen was a required cofactor for thrombin-independent factor XIII activation (Figure A.6). Various regions of the fibrinogen molecule have been implicated in this cofactor activity, as shown by the use of γ′/γ′ and Aα251 recombinant fibrinogens as cofactor in thrombin-independent factor XIII activation (Figure A.7). While the presence of γ′ chains inhibited this activation process, the αC-domain was necessary for it to take place to its full extent. Notably, however, inclusion of neither mutant in the preactivation period completely abrogated activation of factor XIII zymogen, suggesting that factor XIII may interact with multiple domains of fibrinogen during thrombin-independent activation.

Finally, a native gel showed that while thrombin-catalyzed activation of factor XIII led to subunit dissociation, thrombin-independent activation took place with the subunits remaining associated as A_2B_2 (Figure A.8). This conformational difference suggests that factor XIII activated in these two different ways is a completely different molecule; it is

therefore all the more remarkable that factor XIII activated by 50 m*M* $CaCl_2$ generates the same level of activity as factor XIII activated by 0.25 U/mL thrombin (Table A.1).

Thrombin-independent activation of factor XIII in the blood could potentially lead to catastrophic thrombotic effects. From the experiments described here, I cannot comment as to whether the activity I have characterized leads to a small amount of cross-linked fibrinogen molecules that circulate in the blood as suggested by Siebenlist *et al* [6], or if it is simply an *in vitro* effect as suggested by Shainoff and DiBello [7]. What is clear, however, is that this activity affects any experiments in which fibrinogen and factor XIII are mixed in the presence of calcium for more than just a few moments. Use of the lowest possible concentrations of calcium will minimize this effect. Some recombinant fibrinogen variants will catalyze less activation than others. But some conversion of factor XIII into an active form will necessarily take place in each experiment. I used this background to design experiments and interpret data from them in the most informed manner.

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