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The interaction between fibrinogen and zymogen FXIII-A_2B_2 is mediated by fibrinogen residues γ390-396 and the FXIII-B subunits

Running Title: FXIII-A_2B_2 binds fibrinogen γ390-396 via FXIII-B_2

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KEY POINTS

-Fibrinogen γ-chain residues 390-396 bind FXIII-A_2B_2 and mediate its activation in a FXIII-B subunit-dependent mechanism.

-Excess FXIII-B_2 in plasma circulates bound to fibrinogen.

ABSTRACT

The coagulation transglutaminase factor XIII (FXIII) exists in circulation as the heterotetrameric proenzyme FXIII-A_2B_2. Effectively all FXIII-A_2B_2 circulates bound to fibrinogen, and excess FXIII-B_2 circulates in plasma. The motifs that mediate interaction of FXIII-A_2B_2 with fibrinogen have been elusive. We recently detected reduced binding of FXIII-A_2B_2 to murine fibrinogen that has γ-chain residues 390-396 mutated to alanines (Fibγ$^{390\text{-}396A}$). Here, we evaluated binding features using human components, including recombinant fibrinogen variants, FXIII-A_2B_2, and isolated FXIII-A_2 and -B_2 homodimers. FXIII-A_2B_2 co-precipitated with wild-type (γA/γA), alternatively-spliced (γ′/γ′), and αC-truncated (Aα251) fibrinogens, whereas co-precipitation with human Fibγ$^{390\text{-}396A}$ was reduced by 75% ($P < 0.0001$). Surface plasmon resonance showed γA/γA, γ′/γ′, and Aα251 fibrinogens bound FXIII-A_2B_2 with high affinity (nM); however, Fibγ$^{390\text{-}396A}$ did not bind FXIII-A_2B_2. These data indicate fibrinogen residues γ390-396 comprise the major binding motif for FXIII-A_2B_2. Compared to γA/γA clots, FXIII-A_2B_2 activation peptide release was 2.7-fold slower in Fibγ$^{390\text{-}396A}$ clots ($P<0.02$). Conversely, activation of recombinant FXIII-A_2 (lacking FXIII-B_2) was similar in γA/γA and Fibγ$^{390\text{-}396A}$ clots, suggesting fibrinogen residues γ390-396 accelerate FXIII-A_2B_2 activation in a FXIII-B_2-dependent mechanism. Recombinant FXIII-B_2 bound γA/γA, γ′/γ′, and Aα251 with similar affinities as FXIII-A_2B_2, but did not bind or co-precipitate with Fibγ$^{390\text{-}396A}$. FXIII-B_2 also co-precipitated with fibrinogen in plasma from F13a$^{-/-}$ mice. Collectively, these data indicate that FXIII-A_2B_2 binds fibrinogen residues γ390-396 via the B subunits, and that excess plasma FXIII-B_2 is not free, but rather, circulates bound to fibrinogen. These findings provide insight into assembly of the fibrinogen/FXIII-A_2B_2 complex in both physiologic and therapeutic situations.

INTRODUCTION

Factor XIII (FXIII) is a plasma protransglutaminase that circulates at 14-28 µg/mL (43-86 nM, reviewed in [1]). Zymogen FXIII is composed of two A subunits (FXIII-A_2) and two carrier B subunits (FXIII-B_2) assembled as a non-covalent heterotetramer (FXIII-A_2B_2). In plasma, FXIII-A_2 is tightly-associated (K_D ~100 pM)[2] with FXIII-B_2. Excess FXIII-B_2 (43-62 nM) is present in circulation.[2,3] During coagulation, FXIII-A_2B_2 is activated by thrombin-mediated cleavage of an N-terminal, 37-amino acid activation peptide from the FXIII-A subunits (FXIII-A_2'). After activation peptide release, calcium promotes the dissociation of the inhibitory FXIII-B subunits, yielding fully activated FXIII-A_2* (FXIIIa). Once activated, FXIIIa catalyzes the formation of ε-N-(γ-glutamyl)-lysyl crosslinks between γ- and α-chains of fibrin and between fibrin and other plasma proteins. Crosslinking is essential for clot mechanical and biochemical stability (reviewed in [1]). Fibrin α-chain crosslinking also promotes red blood cell retention in venous thrombi and consequently, mediates thrombus composition and size.[4,5]

FXIII-A_2B_2 circulates in complex with fibrinogen (K_D ~10 nM)[6], and these proteins are readily co-precipitated from plasma.[7] However, the fibrinogen residues that mediate binding to FXIII-A_2B_2 in humans have not been defined. Early studies suggested the alternatively-spliced fibrinogen γ′-chain contained the FXIII-A_2B_2 binding site.[8,9] However, studies using recombinant fibrinogen showed that FXIII-A_2B_2 binds to γ- and γ′-containing fibrinogen with similar affinity[10], suggesting the γ′-extension is not necessary for FXIII-A_2B_2 binding. More recently, Smith et al. observed high affinity binding of non-activated FXIII-A_2B_2 to a glutathione-S-transferase-fused peptide containing amino acid residues 371-425 of the fibrinogen αC domain.[11] However, whether the fibrinogen αC domain fulfills the carrier function of FXIII-A_2B_2 remains unclear.

We recently observed decreased co-precipitation of FXIII-A_2B_2 with murine fibrinogen that has alanine substitutions within residues γ390-396 (NRLSIGE to AAAAAAA, Fib$\gamma^{390\text{-}396A}$), suggesting these γ-chain residues mediate the FXIII-A_2B_2 carrier function in mice.[4] Accordingly, Souri et al. subsequently detected binding of FXIII-A_2B_2 to the human fibrinogen γ-chain at residues C-terminal of γLys356.[12] Notably, fibrinogen residues γ390-396 are highly conserved in mammals (NRLTIGE [human, gorilla, dog], NRLSIGD [rat], and NRLAIGE [giant panda]), suggesting these residues fulfill this essential, conserved function across species.

Herein, we used entirely human components, including recombinant human fibrinogen variants and human FXIII heterotetramers and homodimers, as well as FXIII-deficient mice, to define the interaction between these proteins. Our data reveal a direct interaction between human fibrinogen residues γ390-396 and the FXIII-B subunits, and uncover a fundamental mechanism mediating FXIII-fibrinogen complex assembly in blood. These data have important implications for both physiologic assembly of this complex in healthy individuals, and assembly in FXIII-A_2-deficient patients receiving therapeutic recombinant FXIII-A_2.

METHODS

Proteins and Materials. Anti-human fibrinogen antibody was from Dako (Carpinteria, CA) and AlexaFluor 488 anti-rabbit and anti-sheep secondary antibodies were from Jackson Immunoresearch (West Grove, PA). Two rabbit polyclonal anti-FXIII-B antibodies were used: HPA003827 (Sigma Aldrich, St. Louis, MO) and A074 (Zedira, Darmstadt, Germany), as indicated. Plasma FXIII-A_2B_2, anti-human FXIII-A antibody, and peak 1 human fibrinogen (FXIII-depleted) were from Enzyme Research Laboratories (ERL, South Bend, IN). Recombinant FXIII-A_2 (rFXIII-A_2) was a generous gift of Novo Nordisk (Bagsværd, Denmark). FXIII-A_2B_2 (plasma-derived) used for surface plasmon resonance and recombinant FXIII-B_2 (rFXIII-B_2, produced in insect cells) were from Zedira. Insect cell-derived FXIII-B_2 undergoes different post-translational modification than human plasma-derived FXIII-B_2 and migrates slightly faster on SDS-PAGE. Murine studies were approved by the Institutional Animal Care and Use Committees of the Cincinnati Children's Hospital Medical Center and the University of North Carolina at Chapel Hill. Additional materials and sources are detailed in the Supplemental Materials.

Expression of recombinant fibrinogen variants. A vector expressing recombinant Fib$\gamma^{390\text{-}396A}$ was constructed as detailed in Supplemental Materials. Recombinant human wild-type (γA/γA), alternatively-spliced (γ'/γ'), αC-truncated (Aα251), and Fib$\gamma^{390\text{-}396A}$ (Fib$\gamma^{390\text{-}396A}$) fibrinogen variants were expressed in Chinese Hamster Ovary cells and affinity-purified as previously described.[13-15]

Fibrinogen precipitation experiments. Fibrinogen (1 mg/mL [2.9 μM], final) was incubated with FXIII-A_2B_2 (20 μg/mL [60 nM], final), rFXIII-A_2 (10 μg/mL [60 nM], final), or rFXIII-B_2 (10 μg/mL [63 nM], final) at room temperature for 15 minutes. Glycine (165 mg/mL) was then added and samples were rotated for 1 hour, after which the precipitate was pelleted by centrifugation (7000xg, 15 minutes) and resuspended in HEPES-buffered saline (HBS; 20 mM HEPES [pH 7.4], 150 mM NaCl). Fibrinogen, FXIII-A, and FXIII-B content of the initial sample, pellet, and supernatant were assessed by Western blotting under reducing conditions, unless otherwise specified. Briefly, samples were separated by SDS-PAGE on 10% Tris-Glycine gels, transferred to polyvinylidene fluoride membranes, and probed with primary antibodies

(rabbit anti-human fibrinogen [1:7000], sheep anti-human FXIII-A [1:1000], or rabbit anti-human FXIII-B [HPA003827, 1:500]) overnight at 4°C before incubation with fluorescently-labeled secondary antibodies for 1 hour at room temperature. Blots were visualized using a Typhoon FLA9000 Imager (GE Healthcare, Little Chalfont, UK). Densitometry was performed with ImageJ 1.48v. Percentage of FXIII-A or FXIII-B in the pellet was determined by dividing the intensity of the subunit band in the pellet by the sum of the band intensities in both the pellet and the supernatant.

For experiments with mouse plasma, blood was drawn via the inferior vena cava and processed to platelet-poor plasma by centrifugation (5000xg, 10 minutes). Fibrinogen was isolated from wild-type ($F13a^{+/+}$), heterozygous ($F13a^{+/-}$), FXIII-deficient ($F13a^{-/-}$)[4,16], or afibrinogenemic[17] mouse plasma by glycine precipitation[4]. For experiments with immunodepleted human plasma (Affinity Biologicals, Ancaster, ON, lacks both FXIII-A and FXIII-B), plasma was first reconstituted with 20 µg/mL rFXIII-B_2 prior to glycine precipitation. Precipitated fibrinogen and FXIII-A were detected as above. Both murine and human FXIII-B were detected using anti-human FXIII-B antibody (A074, 1:1000 overnight at room temperature).

Surface plasmon resonance (SPR). SPR ligands and analytes were prepared as detailed in Supplemental Materials. SPR was performed on a SensiQ Pioneer platform described in the Supplemental Materials. Fibrinogen analytes were diluted to 50 nM or 1 µM using the same batch of running buffer for blanks and the OneStep® titration function. Fibrinogen analytes were injected into the sensor chamber at a flow-rate of 30 µL/min using the OneStep® titration function[18,19] with a loop-inject of 75% following 5 leadoff blanks and 3 bulk standard injections of 3% sucrose in running buffer. The 50 nM and 1 µM samples had dissociation times of 500 and 1000 seconds, respectively. The chip surface was regenerated with 2 M NaCl (30 µL/min, 60 seconds), followed by 3 M NaCl (30 µL/min, 60 seconds).

Data were analyzed with Qdat data analysis software (SensiQ Technologies Inc., Oklahoma City, OK). Binding data were fit using a simple k_a/k_d model and aggregation/retention parameters adjusted per binding curve according to goodness of fit and curve type. Sensorgrams from experiments with 50 nM fibrinogen analytes (maximum) recognized one binding site and were fit using a one-site model. Sensorgrams with 1 µM fibrinogen analyte (maximum) identified two binding sites for some FXIII ligands, so these data were fit using a two-site binding model; however, given the plasma concentrations of FXIII-A_2B_2 and fibrinogen, affinities for the second binding sites (0.7-21 µM) were considered too weak to be physiologically-relevant. Thus, only data from the first, high-affinity site are reported.

FXIII activation and fibrin crosslinking. FXIII-A_2B_2 (20 µg/mL [60 nM], final) or rFXIII-A_2 (10 µg/mL [60 nM], final) were incubated with fibrinogen (0.15 mg/mL [440 nM], final) at room temperature for 15 minutes. Reactions were triggered with thrombin (2 nM, final) and calcium (10 mM, final). This thrombin concentration enabled us to visualize early FXIII activation and fibrin crosslinking. Reactions were quenched and clots dissolved with 50 mM dithiothreitol/12.5 mM EDTA in 8 M urea (60°C, 1 hour). Samples were boiled in SDS-containing sample buffer and separated using SDS-PAGE on 10% Tris-glycine gels before transfer to polyvinylidene fluoride membranes. Membranes were probed with primary antibodies (sheep anti-human FXIII-A [1:1000] or rabbit anti-human fibrinogen [1:7000]; overnight, 4°C) before incubation with fluorescently-labeled secondary antibodies (1 hour, room temperature). Blots were visualized using a Typhoon FLA9000 Imager. Densitometry was performed with ImageJ 1.48v. FXIII activation was determined by dividing the intensity of the FXIII-A′ band by the sum of the FXIII-A and FXIII-A′ bands to obtain % of FXIII-A′. Crosslinking of fibrin γ-chains was determined as previously described.[12,20]

Statistics. Descriptive statistics (mean, standard deviation [SD], standard error of the mean [SEM]) were calculated and Lilliefors test was used to assess normality. FXIII-A_2 and FXIII-B_2 co-precipitation with each fibrinogen variant was compared to γA/γA using ANOVA with Dunnett's post-hoc testing (Kaleidagraph, Synergy Software, v4.5). FXIII activation and fibrin crosslinking rates were compared using 2-tailed Student's t-tests for equal or unequal variances, as appropriate. $P<0.05$ was considered statistically significant.

RESULTS

Fibrinogen residues γ390-396 mediate FXIII-A_2B_2 binding to soluble human fibrinogen. Previous studies have suggested FXIII-A_2B_2 binds to fibrinogen at the alternatively-spliced γ′-chain[8,9], residues in the αC domain[11], or residues in the γ-chain[4,12]. To compare FXIII-A_2B_2 binding to these regions of fibrinogen, we expressed and purified recombinant human fibrinogen proteins: wild-type (γA/γA), alternatively-spliced (γ′/γ′), αC-truncated (Aα251), and Fibγ$^{390\text{-}396A}$. As expected[13-15,21], each of these fibrinogen variants contained all three chains, polymerized normally, and were >95% clottable (Figure 1 and data not shown). We pre-incubated these fibrinogen variants with FXIII-A_2B_2, precipitated fibrinogen with glycine, and performed SDS-PAGE and Western blotting to identify FXIII-A_2B_2 present in the pellets and supernatant. FXIII-A_2B_2 co-precipitated with γA/γA, γ′/γ′, and Aα251 fibrinogen constructs (Figure 1). However, co-precipitation of FXIII-A_2B_2 with Fibγ$^{390\text{-}396A}$ was reduced by 75% ($P < 0.0001$, Figure 1).

We also quantified binding of the fibrinogen variants to FXIII-A_2B_2 using SPR. Consistent with the co-precipitation data, γA/γA and γ′/γ′ fibrinogens bound FXIII-A_2B_2 with similar, high affinity (Table 1, Supplemental Figure 1). Compared to γA/γA and γ′/γ′ fibrinogens, Aα251 fibrinogen binding to adherent FXIII-A_2B_2 was slightly reduced, consistent with the finding that the αC domain contributes to FXIII-A_2B_2-binding function[11]. Regardless, given these affinity constants (Table 1), greater than 99% of circulating FXIII-A_2B_2 (plasma concentration ~43-86 nM[2,3]) would bind each of these variants. Notably, Fibγ$^{390\text{-}396A}$ fibrinogen did not bind FXIII-A_2B_2 (Table 1, Supplemental Figure 1). Collectively, these data indicate the primary binding site for zymogen FXIII-A_2B_2 is present in γA/γA, γ′/γ′, and Aα251 fibrinogens, but absent in Fibγ$^{390\text{-}396A}$ fibrinogen. Together with previous findings[4], these results suggest the highly-conserved fibrinogen residues γ390-396 mediate binding of zymogen FXIII-A_2B_2 to fibrinogen in both mice and humans.

Human fibrin(ogen) residues γ390-396 mediate the ability of fibrin to accelerate FXIII-A_2B_2 activation. During fibrin formation, FXIII-A_2B_2 binding to the fibrin D:E:D/thrombin complex accelerates FXIII activation peptide cleavage and FXIII activation.[12,22-28] Using mice expressing murine Fibγ$^{390\text{-}396A}$ fibrinogen, we previously detected delayed FXIII activation and fibrin crosslinking in plasma.[4] To directly determine the contribution of fibrin(ogen) residues γ390-396 to these functions, we now assessed the ability of human Fibγ$^{390\text{-}396A}$ fibrin(ogen) to

support FXIII-A_2B_2 activation in a purified system. Compared to reactions with human γA/γA fibrin(ogen), reactions with Fibγ$^{390\text{-}396A}$ fibrin(ogen) showed 2.7-fold slower release of the FXIII activation peptide (2.35 ± 0.46% vs. 0.87 ± 0.20% FXIII-A′/minute, respectively, P<0.02, Figure 2A-C), and was similar to that observed in the absence of fibrinogen (0.96 ± 0.16% FXIII-A′/minute, P=0.8, Figure 2A-C).

The rate of fibrin formation was similar for γA/γA and Fibγ$^{390\text{-}396A}$ (Figure 2D and data not shown). However, the rate of fibrin crosslinking was slower for Fibγ$^{390\text{-}396A}$. Specifically, γ-γ dimer formation was 2.5-fold slower (6.7 ± 1.5 versus 17.0 ± 1.3% γ-γ/minute, for Fibγ$^{390\text{-}396A}$ versus γA/γA, respectively, Figure 2D-F). The delays in both FXIII-A_2B_2 activation and crosslinking activity are consistent with a lack of binding of zymogen FXIII-A_2B_2 to Fibγ$^{390\text{-}396A}$ fibrinogen.

The ability of fibrin(ogen) residues γ390-396 to accelerate FXIII activation is FXIII-B subunit-dependent. Souri et al. previously suggested the acceleratory effect of fibrin(ogen) on FXIII-A_2B_2 activation is FXIII-B subunit-dependent.[12] We therefore also measured activation of FXIII-A_2 (rFXIII-A_2) in the absence of FXIII-B_2 and compared these rates in the presence of γA/γA and Fibγ$^{390\text{-}396A}$ fibrin(ogen). Compared to activation of FXIII-A_2B_2, activation of rFXIII-A_2 was slower (Figure 2A-C and Figure 3A-C, P<0.02), consistent with a critical role for the FXIII-B subunits in this reaction. Interestingly, however, in contrast to that seen with FXIII-A_2B_2, activation of rFXIII-A_2 was similar in the presence of γA/γA and Fibγ$^{390\text{-}396A}$ fibrin(ogen) (0.60 ± 0.06 vs. 0.46 ± 0.06% FXIII-A′/minute, respectively, Figure 3A-C). Moreover, in the presence of rFXIII-A_2, the formation rate of γ-γ dimers was more similar for γA/γA and Fibγ$^{390\text{-}396A}$ (15.6 ± 0.5 versus 12.6 ± 0.5% γ-γ/minute, respectively, Figure 3D-F), relative to reactions in the presence of FXIII-A_2B_2. These findings show Fibγ$^{390\text{-}396A}$ can be crosslinked, indicating the delayed crosslinking seen with FXIII-A_2B_2 (Figure 2D-F) was not due to a substantial disruption of structure in this region. Rather, these data attribute the delay seen with FXIII-A_2B_2 (Figure 2D-F) to decreased interaction between fibrin(ogen) residues γ390-396 and the FXIII-B subunit. Together, these data suggest the presence of fibrin(ogen) residues γ390-396 accelerate FXIII activation, and do so in a FXIII-B subunit-dependent mechanism.

The FXIII-B subunit binds fibrinogen residues γ390-396. We then directly tested the

hypothesis that FXIII binding to fibrinogen residues γ390-396 is mediated by the B subunits using both precipitation and SPR assays. First, we precipitated recombinant γA/γA or Fibγ$^{390-396A}$ fibrinogen in the presence of rFXIII-A_2, rFXIII-B_2, or rFXIII-A_2 plus rFXIII-B_2. rFXIII-A_2 did not co-precipitate with either γA/γA or Fibγ$^{390-396A}$ fibrinogen (Figure 4A, B). However, rFXIII-B_2 readily co-precipitated with γA/γA fibrinogen (Figure 4A), but not Fibγ$^{390-396A}$ fibrinogen (Figure 4B). Addition of rFXIII-B_2 to rFXIII-A_2 promoted co-precipitation of rFXIII-A_2 with γA/γA fibrinogen (Figure 4A), likely through the formation of rFXIII-A_2B_2 heterotetramers. Conversely, rFXIII-B_2 did not promote rFXIII-A_2 co-precipitation with Fibγ$^{390-396A}$ (Figure 4B). Experiments using purified, plasma-derived γA/γA (peak 1) fibrinogen fully recapitulated findings with recombinant γA/γA (data not shown).

Second, we examined binding of the fibrinogen variants to surface-bound rFXIII-B_2. These data revealed that γA/γA bound rFXIII-B_2 with similar affinity as seen with FXIII-A_2B_2 (Table 1, Supplemental Figure 2). γ′/γ′ and Aα251 fibrinogens also bound rFXIII-B_2 (Table 1, Supplemental Figure 2), indicating both that the binding motif for FXIII-B_2 is present on each of these fibrinogen molecules and that the presence of FXIII-A_2 does not enhance FXIII-B_2 binding to fibrinogen. These data also confirm that rFXIII-B_2 co-precipitation with fibrinogen in the previous experiments was not due to the presence of glycine. Notably, however, we were unable to detect binding of Fibγ$^{390-396A}$ fibrinogen to rFXIII-B_2 (Table 1, Supplemental Figure 2). Collectively, these co-precipitation and SPR data indicate that the FXIII-B subunit(s) of FXIII-A_2B_2 mediate binding to fibrinogen residues γ390-396.

Excess FXIII-B_2 in plasma circulates bound to fibrinogen. FXIII-B_2 is present in ~2-fold molar excess over FXIII-A_2 in plasma and is thought to circulate as free (unbound) FXIII-B_2 homodimer.[2,3] However, the co-precipitation and SPR data indicating FXIII-B_2 can bind fibrinogen in the absence of FXIII-A_2 (Figure 4A, Table 1) raise the interesting possibility that "free" FXIII-B_2 in plasma actually circulates bound to fibrinogen. Indeed, given the measured affinity of FXIII-B_2 to fibrinogen (0.4 nM, Table 1) and the estimated plasma concentration of "free" FXIII-B_2 (~43-62 nM)[2,3], greater than 99% of circulating FXIII-B_2 should be bound to fibrinogen. Therefore, to determine whether FXIII-B_2 circulates with fibrinogen in plasma, we precipitated fibrinogen from plasma from FXIII-A_2-sufficient (F13a$^{+/+}$) and FXIII-A_2-deficient (F13a$^{+/-}$ and F13a$^{-/-}$) mice[4,16] and used SDS-PAGE and Western blotting to detect FXIII-B in the

precipitate. Consistent with previous observations[29], there was a FXIII-A subunit dose effect on the total amount of FXIII-B present in the initial plasma sample (Figure 5A), suggesting FXIII-A_2 influences the circulating level of FXIII-B_2. Regardless, Figure 5A shows that FXIII-B_2 co-precipitated with fibrinogen from FXIII-A-deficient plasma. Experiments using human FXIII-A-deficient plasma fully recapitulated findings with FXIII-A-deficient mouse plasma (Figure 5B). To test the specificity of the precipitation protocol, we subjected afibrinogenemic mouse plasma to glycine precipitation. As expected, no fibrinogen was precipitated (Figure 5C). Importantly, glycine did not precipitate FXIII-B_2 from afibrinogenemic mouse plasma (Figure 5C), indicating that FXIII precipitation in these experiments is fibrinogen-dependent. Thus, these data suggest excess FXIII-B_2 in plasma does not circulate in a "free" state, but instead circulates bound to fibrinogen.

DISCUSSION

The observation that FXIII-A_2B_2 circulates in complex with fibrinogen is well-established; however, the motifs on fibrinogen and FXIII-A_2B_2 that mediate this interaction have been controversial. Our study that integrates both solid- and solution-phase binding experiments and functional assays reveals critical components of both fibrinogen and FXIII-A_2B_2 necessary for binding. First, our data indicate that the primary binding site for zymogen FXIII-A_2B_2 on human fibrinogen is not found in the alternatively-spliced γ′-extension or the αC region, but instead lies within γ-chain residues 390-396. Together with data from mice[4], this finding suggests these highly-conserved fibrinogen residues mediate this interaction in multiple species. Second, we showed that FXIII-A_2B_2 binds fibrinogen residues γ390-396 via the FXIII-B subunits. These data support previous studies suggesting an interaction between the FXIII-B subunit and fibrinogen[8,12], and extend these findings by defining the FXIII-B binding motif on fibrinogen. Third, we showed that FXIII-B_2 can bind fibrinogen in the absence of FXIII-A_2. This intriguing finding suggests "free" FXIII-B_2 in plasma actually circulates bound to fibrinogen, and has important implications for understanding assembly of the fibrinogen/FXIII-A_2B_2 complex in both physiologic and therapeutic situations.

Our finding that fibrinogen residues γ390-396 support FXIII-A_2B_2 binding are consistent with several prior studies implicating the D-domain[30], residues C-terminal to residue Lys356 of the γ-chain[12], and residues γ390-396 of murine fibrinogen[4] in this interaction. However, our findings are discordant with studies implicating the alternatively-spliced γ′-chain[8,9] as the primary mediator of this interaction. The reasons for this discord may relate to differences in the assay systems used. For example, although experiments using anion exchange chromatography suggested FXIII-A_2B_2 elutes in the same fraction as γA/γ′, this study did not directly compare binding of FXIII-A_2B_2 to these fibrinogen variants.[8] In contrast we tested this interaction by both co-precipitation and direct binding assays that enabled us to maintain fibrinogen in solution during the binding events. This assay design may be particularly important because the same residues we have implicated in FXIII-A_2B_2 binding have previously been shown to support fibrin(ogen) binding to the CD11b (α_M) subunit of CD11b/CD18 (Mac-1) integrin present on monocytes, macrophages, and neutrophils.[21,31] The observation from those studies that CD11b binds to residues γ390-396 in insoluble fibrin and adherent fibrin(ogen), but not soluble fibrinogen, has led to the hypothesis that fibrin formation or fibrinogen adherence to a surface

induces structural changes within these residues. Thus, previous experimental designs that used surface (or resin)-bound fibrin(ogen) or fibrin[6,8,26,32,33] may not have recapitulated the conformation of residues γ390-396 that would bind zymogen FXIII when fibrinogen is in solution. This possibility is also interesting when considering the role of the nearby fibrin(ogen) γ′ extension (residues 407-427) in FXIII-A_2B_2 binding. If residues in the γ′-extension influence structure within residues γ390-396, data from assays using surface- or resin-bound fibrin(ogen) or fibrin may have indicated the alternatively-spliced γ′-chain has different affinity for FXIII-A_2B_2. Similarly, ultracentrifugation experiments with γA/γ′ fibrinogen[9] may have been confounded by the presence of gel-like fibrin(ogen) dimers[34,35]. Thus, these conditions may have led to the conclusion that the zymogen binding motif is contained within the γ′-extension.

More recent findings that FXIII-A_2B_2 binds to a peptide derived from the fibrinogen αC domain (residues α371-425) suggested FXIII-A_2B_2 binds to the fibrinogen αC region.[11] In that study[11] it was not possible to distinguish the relative contributions of the αC region versus the D-region to this interaction in full-length fibrinogen. Our SPR data suggesting Aα251 fibrinogen has weaker binding to FXIII-A_2B_2 than does γA/γA fibrinogen suggests some FXIII-binding character is also derived from the αC domain. Future studies using variant fibrinogens with combined mutations in the γ- and α-chains may resolve the relative contribution of the αC region in this interaction.

The high degree of inter-species homology within fibrinogen residues γ390-396 has traditionally been attributed to their other essential function in supporting fibrin(ogen) binding to CD11b.[21,31] Interestingly, the earliest FXIII-fibrinogen system[36] and the α_M I-domain that binds fibrin[37,38] appeared together with the rise of vertebrates over 400 million years ago. No homozygous mutations have been identified in this region, emphasizing the physiologic importance of this fibrin(ogen) sequence. Therefore, the high homology in this region may result from strong evolutionary pressure to maintain both of these functions. Although the same fibrinogen residues mediate binding to both FXIII-A_2B_2 and CD11b, it is unlikely that these binding events compete. First, there is a vast excess of fibrinogen relative to FXIII-A_2B_2. Second, FXIII-A_2B_2 circulates with soluble fibrinogen[6], whereas CD11b binds insoluble fibrin[39]. Thus, these interactions likely occur in distinct physiologic settings.

Based on our and published findings, we propose the following model (Figure 6). FXIII-A_2B_2 circulates bound to fibrinogen at a site comprised of residues γ390-396 and supported by

the αC region.[11] During coagulation, fibrinogen transports FXIII-A_2B_2 into the nascent clot via its interaction with the FXIII-B subunits. As fibrin polymerizes, FXIII-A_2B_2 bound to the D-domain of one fibrin monomer contacts thrombin bound to the E-domain of another fibrin monomer at the D:E:D interface.[40] Formation of this complex promotes FXIII activation peptide cleavage and release from the FXIII-A subunits[22-28,40], followed by dissociation of the FXIII-B subunits from the FXIII-A subunits[12,22]. These sequential steps yield fully-activated FXIII-A_2*. Generation of FXIII-A_2* at residues γ390-396 conveniently localizes FXIIIa near the γ-chain crosslinking sites (residues γQ398/399 and γK406), which are the first fibrin residues to undergo crosslinking.[20,41-43] This γ-chain crosslinking also promotes FXIII-B_2 dissociation from the fibrin clot.[12] The FXIII-A_2* interaction with αC residue E396[11,44,45] then facilitates the translocation of its active site to the fibrin α-chain, where it catalyzes the formation of crosslinks between fibrin α-chains and between α-chains and other plasma proteins. Formation of these crosslinks is critical for the ability of FXIII(a) to promote resistance of clots to biomechanical and biochemical disruption. Moreover, we recently showed that the spatio-temporal regulation of FXIII activation kinetics during coagulation is also critical for determining red blood cell retention in contracted clots.[4] Thus, this model reconciles data from studies on the binding, activation, and activity of FXIII and reveals the importance of the FXIII-B subunits in fibrinogen/FXIII-A_2B_2 interactions, in FXIII-A_2B_2 activation, and consequently, in fibrin crosslinking and clot composition and stability. Although we did not identify the specific FXIII-B residues that mediate this interaction, previous studies have implicated sushi domains 1 and/or 10 in this interaction.[12] Further studies are ongoing to localize the FXIII-B residues that support binding to fibrinogen residues γ390-396.

A major finding from this work is the observation that FXIII-B_2 can bind fibrinogen in the absence of FXIII-A_2. Notably, the tight affinity of FXIII-B_2 binding to fibrinogen, together with the plasma concentrations of FXIII-B_2 and fibrinogen, suggests essentially all FXIII-B_2 in plasma is bound to fibrinogen. This observation appears to contradict the tenet that "free B" circulates in plasma.[3] However, FXIII-B_2 used in the previous report[3] was prepared by ammonium sulfate precipitation and heat denaturation to specifically remove fibrinogen. Thus, that study was not designed to characterize FXIII binding to other plasma proteins, and "free B" was likely only meant to imply "not bound to FXIII-A_2." Description of this material has been (incorrectly) interpreted more broadly since that time. Since both FXIII-B_2 and fibrinogen are

synthesized by hepatocytes, these proteins may associate during or immediately following their secretion. Subsequent association of the FXIII-A_2 subunits, which are synthesized by cells of bone marrow origin, with the fibrinogen/FXIII-B_2 complex would then result in formation of the complete fibrinogen/FXIII-A_2B_2 complex. Consequently, our data that suggest FXIII-B_2 circulates bound to fibrinogen may reveal part of a step-wise mechanism that leads to production of fibrinogen/FXIII-A_2B_2 complexes. In addition, these data may have important implications for understanding the mechanism of action of therapeutic rFXIII-A_2 infusion for FXIII-A-deficiency. Binding of infused rFXIII-A_2 to fibrinogen-bound FXIII-B_2 (versus "free" FXIII-B_2) ensures that rFXIII-A_2 will become incorporated into a functional, fibrinogen-bound complex, which is crucial for normal FXIII activation and function.

Our study has potential limitations. First, the loss of FXIII binding to the Fib$\gamma^{390\text{-}396A}$ mutant may reflect disrupted structure within the γ-domain. However, this possibility seems unlikely because crystallographic studies suggest this region is inherently disordered even in the native molecule.[46-48] Moreover, although the pattern of fibrin crosslinking is subtly altered in both mouse[4] and human Fib$\gamma^{390\text{-}396A}$ clots, both murine[4,21] and human Fib$\gamma^{390\text{-}396A}$ can be fully crosslinked at the canonical residues located immediately C-terminal to γ390-396 (γQ398/399 and γK406). Thus, mutations within these residues do not appear to catastrophically alter structure within this domain. Second, the protocol used to precipitate fibrinogen may also promote FXIII precipitation and/or FXIII interaction with fibrinogen, and we and others[8] have observed spontaneous precipitation of isolated FXIII-B subunits in certain experiments. However, glycine did not precipitate FXIII from afibrinogenemic plasma or in the presence of non-binding Fib$\gamma^{390\text{-}396A}$ fibrinogen. Moreover, data from the precipitation experiments were supported by both SPR analyses and functional FXIII activation assays performed in the absence of glycine.

In summary, our data expose critical molecular interactions mediating FXIII binding to fibrinogen. Identification of these motifs advances our understanding of this interaction in both physiologic and pathophysiologic situations.

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AUTHORSHIP

Contribution: J.R.B. designed and performed experiments, analyzed and interpreted the data, and wrote the manuscript; C.W., A.M.B., and C.B.B. performed experiments and analyzed data; M.J.F. provided vital reagents; H.P. designed experiments and interpreted data; A.S.W. designed the research, analyzed and interpreted the data, and wrote the manuscript. All authors reviewed and approved the manuscript.

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TABLE

Immobilized Ligand	Analyte	N	Equilibrium dissociation constant (K_D), nM
FXIII-A_2B_2	γA/γA	6	3.8 ± 2.4
	γ′/γ′	6	10.4 ± 11.1
	Aα251	3	71.0 ± 16.2
	Fibγ$^{390\text{-}396A}$	6	No Binding
rFXIII-B_2	γA/γA	4	0.4 ± 0.3
	γ′/γ′	6	53.0 ± 75.1
	Aα251	4	58.6 ± 26.5
	Fibγ$^{390\text{-}396A}$	6	No Binding

Table 1. SPR analysis of fibrinogen variant binding to immobilized FXIII-A_2B_2 and rFXIII-B_2. Equilibrium dissociation constant values are mean ± SD for the number of experiments indicated.

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FIGURES

Figure 1. Fibrinogen residues γ390-396 are necessary for FXIII-A_2B_2 binding. Recombinant human fibrinogen variants (γA/γA, γ′/γ′, Aα251, and Fibγ$^{390\text{-}396A}$) were mixed with FXIII-A_2B_2 (1 mg/mL and 20 µg/mL, final, respectively) and precipitated with glycine. (**A**) Representative Western blots for fibrinogen (Fgn), FXIII-A, and FXIII-B in the initial sample (I), pellet (P), or supernatant (S). Note that P and S samples were taken after the addition of glycine and are therefore subject to a dilution effect relative to the I sample. (**B**) Quantitation of all blots, indicating percent of FXIII-A or FXIII-B in the pellet, relative to total FXIII in the pellet and supernatant. Bars are means ± SEM, n=3.

Figure 2. Fibrin(ogen) residues γ390-396 mediate the acceleratory effect of fibrin(ogen) on FXIII-A_2B_2 activation. FXIII-A_2B_2 (20 µg/mL [60 nM], final) was mixed with recombinant fibrinogens (γA/γA or Fibγ$^{390\text{-}396A}$, 150 µg/mL, final) or buffer (No Fgn). Reactions were triggered by addition of thrombin (2 nM, final) and $CaCl_2$ (10 mM, final), quenched at the indicated time points, and analyzed by SDS-PAGE with Western blotting and densitometry. Activation peptide cleavage was detected using anti-FXIII-A antibody. Fibrin crosslinking was detected using anti-fibrinogen antibody. (**A**) Representative Western blots and (**B**) quantitation of FXIII-A_2B_2 activation over time from all blots. (**C**) Maximal rates of FXIII-A_2B_2 activation calculated from panel B. (**D**) Representative Western blots of fibrin crosslinking, and quantitation of (**E**) γ-γ dimer formation and (**F**) γ-γ dimer formation rate. Data are means ± SEM, n=3-6 replicates per time point.

Figure 3. Fibrin(ogen) residues γ390-396 do not accelerate FXIII-A_2 activation. Recombinant FXIII-A_2 (10 µg/mL [60 nM], final) was mixed with γA/γA or Fibγ$^{390\text{-}396A}$ fibrinogen (150 µg/mL, final). Reactions were triggered by addition of thrombin (2 nM, final) and $CaCl_2$ (10 mM, final), quenched at the indicated time points, and analyzed by SDS-PAGE with Western blotting and densitometry. Activation peptide cleavage was detected using anti-FXIII-A antibody. Fibrin crosslinking was detected using anti-fibrinogen antibody. (**A**) Representative Western blots and (**B**) quantitation of rFXIII-A_2 activation over time from all blots. (**C**) Maximal rates of rFXIII-A_2 activation were calculated from quantified Western blots. (**D**) Representative Western blots of fibrin crosslinking and quantification of (**E**) γ-γ dimer

formation and (**F**) formation rate. Data are means ± SEM, n=4 experiments.

Figure 4. FXIII-A_2B_2 binds fibrinogen residues γ390-396 via the FXIII-B subunits. Recombinant FXIII-A_2 (10 µg/mL [60 nM], final), rFXIII-B_2 (10 µg/mL [62 nM], final), or both, were mixed with (**A**) γA/γA or (**B**) Fibγ$^{390\text{-}396A}$ fibrinogen (1 mg/mL, final) and precipitated with glycine. (**A**-**B**) Representative Western blots for fibrinogen (Fgn), FXIII-A, and FXIII-B in the initial sample (I), pellet (P), or supernatant (S). Note that P and S samples were taken after the addition of glycine and are therefore subject to a dilution effect relative to the I sample. Blots are representative of n=3 experiments.

Figure 5. In the absence of FXIII-A_2, FXIII-B_2 co-precipitates with plasma fibrinogen. Fibrinogen was precipitated from (**A**) F13a$^{+/+}$, F13a$^{+/-}$, or F13a$^{-/-}$ mouse plasma, (**B**) FXIII-depleted human plasma, or (**C**) afibrinogenemic mouse plasma using glycine. Panels show representative Western blots for fibrinogen (Fgn), FXIII-A, and FXIII-B in the initial plasma (I), pellet (P), or supernatant (S) under reducing (Fgn) or non-reducing (FXIII-A, FXIII-B) conditions. Note in panel A, plasma albumin in I and S samples causes the Aα-chain to migrate faster than in the P samples. Blots are representative of n=3 experiments with mouse plasmas, and n=2 experiments with human plasma.

Figure 6. FXIII-A_2B_2 binding to fibrinogen residues γ390-396 promotes FXIII-A_2B_2 activation and activity. (**A**) Fibrinogen is comprised of two Aα- (medium gray), two Bβ- (dark gray), and two γ-chains (light gray) arranged in a trinodular structure with two distal D-domains and a central E-domain. The Aα-chains have a C-terminal domain (αC) that extends beyond the D-domain. FXIII-A_2B_2 circulates bound to fibrinogen γ-chain residues 390-396 via the FXIII-B subunits. (**B**) Once coagulation is initiated, thrombin interacts with the fibrinogen E-domain and cleaves fibrinopeptides A from the Aα-chains. As fibrin monomers polymerize, FXIII-A_2B_2 associated with γ390-396 is brought into contact with thrombin at the D:E:D interface to form a ternary complex.[40] This complex facilitates thrombin-mediated activation peptide cleavage from the FXIII-A subunits.[22-28,40], Following activation peptide cleavage, fibrin promotes the calcium-mediated FXIII-B subunit dissociation from the FXIII-A subunits to yield FXIIIa.[12,22] (**C**) FXIIIa then crosslinks (black line) the nearby γ-chains yielding γ-γ dimers.[20] This γ-chain crosslinking

also promotes dissociation of FXIII-B_2 from the fibrin clot.[12] (**D**) FXIIIa translocates from the γ-chain to the αC region, binding at or near α-chain residue E396[11,44,45], and catalyzes the formation of crosslinks between fibrin α-chains and between fibrin and other plasma proteins.