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The role of barrels 1 and 2 in the enzymatic activity of factor XIII-A

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Running title: Barrels 1 and 2 in factor XIII-A activity

Essentials

- The role/s of β-barrels 1 and 2 in factor XIII (FXIII) are currently unknown
- FXIII truncations lacking β-barrel 2, both β-barrels, or full length FXIII, were made
- Removing β-barrel 2 caused total loss of activity, removing both β-barrels returned 30% activity
- β-barrel 2 is necessary for exposure of the active site cysteine during activation

Summary

Background: Factor XIII (FXIII) is composed of an activation peptide segment, a β-sandwich domain, a catalytic core, and finally β-barrels 1 and 2. FXIII is activated following cleavage of its A-subunits by thrombin. The resultant transglutaminase activity leads to increased resistance of fibrin clots to fibrinolysis. *Objectives:* To assess the functional roles of β-barrels 1 and 2 in FXIII, we expressed and characterised the full-length FXIII-A subunit (FXIII-A) and variants truncated to residue 628 [truncated to β-barrel 1 (TB1)], 515 [truncated to catalytic core (TCC)] and 184 [truncated to β-sandwich (TBS)]. *Methods:* Proteins were analysed by gel electrophoresis, circular dichroism, fluorometric assays, colorimetric activity assays, clot structure by turbidity measurements, confocal microscopy, and clot formation by Chandler Loop. *Results and Conclusions:* Circular dichroism spectroscopy and tryptophan fluorometry indicate that full length FXIII-A and the truncations TCC and TB1 retain their secondary and tertiary structure. Removal of β-barrel 2 (TB1) resulted in total loss of transglutaminase activity, whilst the additional removal of β-barrel 1 (TCC) restored enzymatic activity to approximately 30% of full length FXIII-A. These activity trends were observed with physiological substrates and smaller model substrates. Our data suggest that the β-barrel 1 domain protects the active site cysteine in the FXIII protransglutaminase while the β-barrel 2 domain is necessary for exposure of the active site cysteine during activation. This study

demonstrates the importance of individual β-barrel domains in modulating access to the FXIII active site region.

Keywords

Catalytic domain, enzyme activation, factor XIII, protein conformation, transglutaminases

Introduction

In the final step of the blood coagulation cascade, fibrin monomers polymerise to generate a fibrin clot. Activated factor XIII (FXIIIa) catalyses the formation of ε-(γ-glutamyl)lysine covalent bonds between glutamine and lysine residues of adjacent fibrin molecules [1]. FXIIIa is also capable of cross-linking other substrates into the fibrin clot network whose functions include inhibition of fibrinolysis (e.g. α_2-antiplasmin [2, 3]), increased thrombin generation at the clot surface (e.g. factor V [4, 5]), and platelet adhesion to the clot (e.g. collagen [6, 7]). FXIII is a 320 kDa heterologous tetramer comprising two A-subunits, which contain the active site of the enzyme [8], and two B-subunits, which stabilise the hydrophobic A-subunits in the plasma [9, 10] (Figure 1A). The A-subunits are folded into four distinct domains, from N- to C-terminus: the activation peptide, β-sandwich, catalytic core (containing the active site), β-barrel 1 and β-barrel 2 domains [8] (Figure 1B). The dimer folds with the β-barrel domains arranged around the outside of the protein structure. Thrombin cleaves the activation peptide from the N-terminus of each A-subunit monomer and, in the presence of calcium, the B-subunits of FXIII dissociate from the A-subunits exposing the active sites of the A-subunits in the catalytic core to substrates [11-13].

Previous studies have shown that FXIII-A_2 undergoes conformational changes upon thrombin cleavage of the activation peptide [12, 14] and in the presence of calcium [15-17]. It

is also known that fibrin enhances thrombin cleavage of the activation peptide and contributes a binding surface for FXIII [18-22]. Fibrin thus both aids in FXIII activation and itself serves as a transglutaminase substrate. Although the precise changes that occur to FXIII are not fully understood, there is strong evidence that the β-sandwich, β-barrel 1, and β-barrel 2 all play a role in the conformation of the catalytic core [13, 23, 24]. Using a recombinant FXIII-A_2 truncation variant that lacks either barrel 2 or both barrel domains, we are able to show that β-barrels 1 and 2 are crucial for full enzymatic activity of the protein.

Materials and Methods

Production of recombinant FXIII A subunit and truncations

Recombinant FXIII A subunit (FXIII-A) was expressed in *E. coli* and purified as described previously [25]. Further experimental details for the expression of the truncation variants are provided in Data S1.

SDS-PAGE and densitometry analysis

Recombinant proteins were subjected to SDS-PAGE gel analysis under reducing conditions in pre-cast 4-12% bis-tris gels (Life Technologies, Paisley, UK). Gels were stained using Coomassie blue and subjected to densitometry analysis using ID 3.1 Image Software supplied with the Kodak IS2000R Imager (Eastman Kodak Company, New Haven, USA).

Fluorometry

Fluorescence emission spectra of recombinant full-length FXIII-A subunit (FXIII-A) and variants truncated to residue 628 [truncated to β-barrel 1 (TB1)], and 515 [truncated to catalytic core (TCC)] at 1.2 μM in 10 mM 3-(N-Morpholino)propanesulfonic acid (MOPS), pH 7.4, were collected using a Varioskan Flash fluorescence plate reader (Thermo Fisher Scientific, Waltham, MA, USA) at 25°C. Tryptophan residues of samples were excited at 280 nm and emission spectra were collected in triplicate from 300 to 400 nm in 1 nm steps. Blanks in the absence of proteins were measured in triplicate and subtracted from the protein spectra. Precise sample concentrations were determined by quantitative amino acid analysis and spectra compensated accordingly.

Circular dichroism

Purified full length recombinant FXIII-A and truncations TB1 and TCC were dialysed into 10 mM MOPS, pH 7.4 and the concentrations were adjusted to 0.14 mg/ml. Far UV-circular dichroism (CD) spectra were recorded in a Jasco J-715 Spectropolarimeter (Jasco, Great Dunmow, UK) at 21°C at 0.2 nm intervals over a wavelength range from 190 to 300 nm, in a 1 cm quartz Suprasil cuvette (Hellma Ltd, Southend on Sea, UK). Three scans of each spectra were averaged, baseline subtracted against buffer, and corrected to equal molar concentrations. Baseline subtracted spectra were zeroed between 263 and 270 nm. Spectra were smoothed using the Savitsky-Golay algorithm in the CDtool software [26]. Sample concentrations were determined by quantitative amino acid analysis and spectra were converted to mean residue ellipticity (degrees cm^2 $dmol^{-1}$ $residue^{-1}$).

Determination of transglutaminase activity by pentylamine incorporation

Activity of recombinant full-length FXIII-A subunit (FXIII-A) and variants truncated to residue 628 [truncated to β-barrel 1 (TB1)], 515 [truncated to catalytic core (TCC)] and 184 [truncated to β-sandwich (TBS)] was determined based on a pentylamine incorporation assay as described previously [27]. Briefly, microtiter plates were coated with either 100 μg/ml casein (Sigma Aldrich, Gillingham, Dorset, UK) at 4°C overnight or 40 μg/ml human fibrinogen (Enzyme Research Laboratories, Swansea, UK) at 37°C for 1 hour. After blocking with 1% BSA, plates were incubated with triplicates of 3.5 nM recombinant FXIII-A sample, 0.27 μM 5-(Biotinamido) pentylamine (Thermo Fisher Scientific Inc, Rockford, IL USA), 1 U/ml human thrombin (Calbiochem, Merck KGaA, Darmstadt, Germany), 100 μM DTT, and 1 mM $CaCl_2$. Incorporation of 5-(Biotinamido) pentylamine was stopped with 133 mM EDTA over a time course of 25 or 120 minutes for the fibrinogen or casein substrates, respectively. Cross-linking of the 5-(Biotinamido) pentylamine into the fibrin by recombinant FXIII was detected using streptavidin-alkaline phosphatase (Life Technologies) and p-nitrophenyl phosphate (Sigma Aldrich). Plates were measured at OD 405 nm in a ELx808 Bio-tek multiwell plate reader (Winooski, Vermont, USA).

Determination of protein activity by α2-antiplasmin incorporation

Activity of recombinant FXIII-A, TB1, TCC, and TBS was also assayed by α_2-antiplasmin incorporation, based on a method previously described [28]. Briefly, microtiter plates were coated with 40 μg/ml human fibrinogen (Enzyme Research Laboratories) at 37°C for 1 hour. After blocking with 1% BSA, plates were first incubated with 1 U/ml human thrombin (Calbiochem) and 5 mM $CaCl_2$ to convert fibrinogen to fibrin and then treated in triplicate with 3.5 nM recombinant FXIII-A sample, 10 μg/ml α_2-antiplasmin (Calbiochem), 1 U/ml human thrombin, 0.1 mM DTT, and 1 mM $CaCl_2$. Incorporation of α_2-antiplasmin was

stopped with 133 mM EDTA over a time course of 50 minutes. Cross-linking of the α_2-antiplasmin into the fibrin by recombinant FXIII was detected using goat anti-human α_2-antiplasmin antibody with a horse radish peroxidase conjugate (Enzyme Research Laboratories) and 1,2-Diaminobenzene *o*-Phenylenediamine (OPD; Dako, Ely, UK). Plates were measured at 490 nm in a multiwell plate reader (ELx808, Bio-tek).

Determination of transglutaminase activity using a Q-containing substrate peptide

A MALDI-TOF mass spectrometry assay was employed to monitor FXIIIa- catalyzed depletion of a model peptide into its cross-linked product [29]. K9 peptide (LGPGQSKVIG) served as the glutamine-containing substrate and glycine ethyl ester (GEE) as a lysine mimic. Each reaction mix contained 220 nM FXIII-A (full length, TB1, or TCC), 3 mM $CaCl_2$ and 5 mM GEE, all in Tris-acetate buffer. 7 U/ml bovine thrombin (Sigma Aldrich) was introduced and incubated at 37°C for 5 minutes. 400 μM of K9 peptide (Peptides International, Louisville, KY, USA) was then added. After 5, 10, and 30 minutes, aliquots were quenched with 5 mM EDTA. Samples were later run on a MALDI-TOF mass spectrometer and the percent reactant left at each time was calculated as follows:

$$\frac{\sum \text{Reactant Peak Height}}{\sum \text{Reactant Peak Height} + \sum \text{Product Peak Height}} * 100$$

Assays were done in triplicate and standard deviations calculated.

Depletion of FXIII from fibrinogen

FXIII depleted fibrinogen was purified from human fibrinogen (Enzyme Research Laboratories) by ammonium sulphate precipitation, as previously described [30].

Turbidity

Polymerisation of fibrinogen in the presence of full-length or truncated recombinant FXIII-A was measured in a microtiter plate turbidity assay as previously described [31]. Clots were formed in triplicate with 1 mg/ml FXIII-depleted fibrinogen, 65 nM recombinant FXIII-A wildtype or truncation, 0.125 U/ml human thrombin (Calbiochem), and 5 mM $CaCl_2$. Increase of turbidity was continually monitored at 340 nm every 12 seconds in a multiwell plate reader (ELx808, Bio-tek) for 60 minutes at 37°C.

Confocal microscopy

Fibrinolysis rates of fibrin clots formed in the presence of full length or truncated recombinant FXIII-A were measured using non-flourescent confocal microscopy as previously described [32]. Clots were formed in triplicate, using the same concentrations of reactants as for the turbidity experiments. Lysis was then initiated by 280 µg/ml plasminogen (Enzyme Research Laboratories) and 1 µg/ml tPA (Technoclone, Vienna, Austria). The clot was visualised under a low magnification every 20 seconds using a Leica TCS SP-2 laser scanning 1072 confocal microscope (Leica Microsystems, Heidelberg, Germany) and the time taken for the lysis front to migrate from a fixed point was measured. Lysis front velocity was determined and used to calculate the mean overall lysis rate in µm/sec.

Labelling fibrinogen with Alexa fluor-488

FXIII depleted fibrinogen was labelled with fluorophore Alexa Fluor® 488 (Life Technologies). 1 mg of the fluorophore was mixed with 24 mg of FXIII-depleted fibrinogen and incubated on a roller at room temperature for 60 minutes. The unreacted fluorophore was then removed by exhaustive dialysis into Tris-buffered saline, pH 7.4. The degree of labelling was determined to be 3-4 molecules of dye per 1 molecule of fibrinogen, according to the manufacturer's protocol.

Chandler loop

Fibrinolysis rates of fibrin clots formed in the presence of FXIII-A were also measured under flow using a Chandler loop system as previously described [32, 33]. Clots were formed using FXIII-depleted fibrinogen containing 5% Alexa Fluor® 488 conjugated FXIII depleted fibrinogen. The same concentrations of reactants as for the turbidity experiments were used. After 2 hours, clots were retained within the tubing and washed with tris-buffered saline. Lysis was initiated by 28 µg/ml plasminogen (Enzyme Research Laboratories) and 0.1 µg/ml tPA (Technoclone, Vienna, Austria).

Cross-linking of fibrin

Clots were formed at 37°C using 400 µg/ml FXIII-depleted fibrinogen, 26 nM recombinant FXIII-A wildtype or truncation, 0.125 U/ml human thrombin (Calbiochem), and 5 mM $CaCl_2$. The reaction was stopped after 0, 5, 30, 60, 90, and 180 minutes by the addition of reducing sample buffer (Life Technologies) and heating the samples for 10 minutes at 95°C. Samples were run and visualised as described above.

Statistical analysis

All statistical analyses were carried out using PASW 21.0, (SPSS Inc, Chicago, IL, USA). Data are expressed as mean and standard error of the mean (SEM). The one-way ANOVA with Bonferroni *post hoc* analysis was used and *P*-values < 0.05 were considered statistically significant.

Results

Recombinant Protein Expression and Structural Analysis

Recombinant full-length FXIII-A subunit (FXIII-A) and variants truncated to residue 628 [truncated to β-barrel 1 (TB1)], 515 [truncated to catalytic core (TCC)] and 184 [truncated to β-sandwich (TBS)] (Figure 1B-D) were successfully expressed and purified (Figure 2A). Full-length recombinant FXIII-A has a λ_{max} of 328 nm, corresponding to predominantly buried tryptophan side chains in agreement with the crystal structure (Figure 2B; 1GGU [34]). The recombinant FXIII-A truncations TB1 and TCC have reduced fluorescence yield (area under spectra) as a result of the loss of three tryptophan residues located within β-barrel 2. A λ_{max} of 329 nm was observed with full length FXIII-A, TB1, and TCC, indicating the TB1 and TCC tryptophans are maintained in a buried environment. Since both truncations have identical tryptophan locations, within the catalytic core and beta sandwich, similarity in λ_{max} and fluorescence yield suggests their folding is highly comparable and thus the enzymatically important catalytic core retains its tertiary structure in both proteins.

Circular dichroism spectroscopy (CD) was used to examine the secondary structure of the full-length recombinant FXIII-A and truncations TB1 and TCC. The CD spectra of recombinant FXIII-A has a negative CD signal at 215 nm, indicative of ordered secondary structure [35, 36] (Figure 2C). Both truncations have increased CD signal and double minima

at 208 and 220 nm, characteristic of an increased percentage of alpha helical content, consistent with the loss of the beta sheet barrels 1 and 2 [35-37]. In conjunction, fluorescence and CD spectroscopy indicate that removal of barrels 1 and 2 does not cause loss of secondary or tertiary structure to the remaining protein.

Recombinant Protein Activity

Activity of recombinant FXIII-A, TB1, TCC, and TBS was determined by their ability to incorporate either α_2-antiplasmin into plates coated with fibrinogen, or 5-(Biotinamido) pentylamine into plates coated with fibrinogen or casein (Figure 3A). The TCC truncation (shortened to residue 513) also displays activity although it is reduced to approximately 30% of that of the full-length FXIII-A. The truncation to residue 628 which eliminates β-barrel 2 (TB1) shows very little activity, whilst the truncation to residue 184 (TBS) shows no activity, due to the absence of the catalytic core domain.

Determination of transglutaminase activity using a Q-containing substrate peptide

The MALDI-TOF MS based assay was used to assess whether the thrombin-activated recombinant FXIII-A and the two truncations TB1 and TCC could covalently cross-link the lysine mimic GEE to the glutamine-containing K9 peptide (Fig 3B). Over the course of the assay, the MALDI-TOF MS peak for the K9 peptide (954 m/z) decreased in intensity over time whereas the K9 peptide – GEE product (1040 m/z) increased. The percent of reactant remaining was then calculated. As shown in Figure 3B, 53 ± 2 % of K9 substrate remained after 5 minutes of reaction with recombinant FXIII-A. By 30 minutes, only 5 ± 0.7 % of free K9 remained. The FXIII-A truncations TB1 and TCC, missing one or both β-barrels, could still recognize and catalyse cross-linking reactions at the active site, however, with reduced activity compared to full-length FXIII-A. The results further demonstrate that the catalytic

cores of FXIII-A TB1 and TCC are able to accommodate a Q-containing substrate peptide of 10 amino acids.

Clot Polymerisation

The effect of recombinant FXIII-A, TB1, TCC, TBS, and control (buffer only) on clot fibre thickness was investigated using the turbidity technique. Only clots with full length FXIII-A had a significant decrease in final maximum absorbance compared to control ($n=3$, $P < 0.05$). The final turbidity for the truncations was not significantly different from the control ($n=3$, $P > 0.05$; Figure 4A).

Clot Lysis

Lysis rates of clots formed in the presence of recombinant FXIII-A, TB1, TCC, TBS or control were investigated using a static confocal microscopy method and under flow in the Chandler loop. After the addition of fibrinolytic agents to the clot, we observed a significant decrease in the rate of fibrinolysis only for clots formed in the presence of FXIII-A compared with the control in both the confocal microscopy ($n=3$, $P < 0.05$; Figure 4B) and the Chandler loop methods ($n=3$, $P < 0.05$; Figure 4C-D). None of the truncations showed a significant decrease in fibrinolysis rate by either method ($n=3$, $P > 0.05$; Figure 4B-D).

Cross-linking of Fibrin

A time course of fibrin clots formed in the presence of recombinant FXIII-A, TB1, TCC, TBS or control, was run on 4-12% bis-Tris SDS-PAGE gels under reducing conditions to determine the degree of α- and γ-chain crosslinking. After 30 minutes, 90% of the γ-chain had been incorporated into the clot formed with full-length FXIII-A and approximately 25% of

the γ-chain had been incorporated into the clot formed with TCC truncation, all forming γ-γ dimers. The amount of unconverted γ-chain remained at 100% in the clots formed with TB1 and TBS, relative to the control clot formed without any FXIII (Figure 5A and B).

Discussion

Efficient activation of factor XIII (FXIII) is essential for the development of a mechanically stable fibrin clot which is resistant to lysis. The majority of this fibrinolysis resistance by FXIII is a result of the cross-linking of α_2-antiplasmin to fibrin [38]. However, the effect of FXIII on fibrin structure itself may also contribute in part to increased resistance to fibrinolysis [32]. Factors involved in the regulation of FXIII activation include thrombin, calcium and fibrin [12, 15, 18, 39-41]. However, the function of each individual domain of the A-subunit with respect to FXIII activity is hitherto undetermined. In this study, we have investigated the role of the two β-barrel domains in FXIII activity.

Recombinant full length factor XIII A subunit (FXIII-A) and the truncations TCC (both β-barrels removed) and TB1 (β-barrels 2 removed) all retained secondary and tertiary structure, thus the β-barrels are not required to maintain the overall, global conformations of the catalytic core and the β-sandwich domain. Furthermore, the four FXIII domains have been reported to be independent folding units [42]. Assays performed showed that removal of β-barrel 2 (TB1) leads to near total loss of transglutaminase activity, whereas the additional removal of β-barrel 1 (TCC) returns the enzymatic activity to about 30% of activated full length FXIII-A. This effect is observed not only with large, more physiological substrates (fibrin, casein, and α_2-antiplasmin) but also with smaller model substrates (K9 peptide and biotinamidopentylamine). Moreover, these transglutaminase assays made it possible to monitor the reactive glutamine and the reactive lysine residues cross-linked by activated FXIII-A. Influences on clot formation and clot lysis were also important to consider.

The full length FXIII-A remained the best at supporting clot polymerisation and reducing clot lysis. Similar to the 30% enzymatic activity mentioned above, the FXIII mutant TCC catalysed fibrin γ-γ formation with a reactivity that was 25% of wild-type FXIII. Little to no fibrin γ-γ formation occurred with TB1 and TBS.

FXIII contains a secondary thrombin cleavage site at the K513-S514 peptide bond located within the C-terminal portion of the catalytic domain [43, 44]. The 51 kDa protein that results from this cleavage has been reported to still bind fibrin and exhibit transglutaminase activity despite it lacking the two β-barrels [44, 45]. Interestingly, fibrin crosslinking was reduced to about 30% of activated full length FXIII-A, consistent with the results shown here for the recombinant TCC. Lai and coworkers proposed that the truncated FXIII could no longer promote effective binding and alignment of the reactive Q and K substrates [45]. The data with TB1 vs TCC presented in this manuscript suggests, for the first time, that the β-barrel 2 domain plays an important role in maintaining the proper conformational environment for the transglutaminase reaction. Without the structural support of β-barrel 2, β-barrel 1 hinders actions within the FXIII-A catalytic core domain. Prior studies indicated that the 19kDa proteolytic product (residues 514-731, β–barrels 1 &2) is unable to bind fibrin [44]. This result demonstrates that the two β–barrel domains exert their influences as part of the full FXIII-A molecule and not through the supporting fibrin scaffold [44].

Solvent accessibility studies involving amide proton hydrogen-deuterium exchange (HDX) have revealed that both FXIII β-barrel domains participate in conformational changes occurring during both proteolytic (thrombin with calcium) and nonproteolytic (calcium only) activation of FXIII [13, 16, 23]. The β-barrels of transglutaminase 2 have also been shown via HDX studies to undergo similar alterations upon calcium dependent enzyme activation [46]. Members of the transglutaminase family all have a tyrosine residue (FXIII: Y560, TG2: Y516, and TG3: Y525) whose hydroxyl group is H-bonded to the thiolate group on the active site cysteine [24]. As part of the activation process, this tyrosine must be displaced from the

active site region and the movement of both β-barrels are proposed to promote this conformational change [24, 47]. Studies involving HDX coupled with mass spectrometry have revealed that the FXIII-A β-barrel 1 segments 533-551, 556-559, and 560-573 [48, 49] become more exposed to solvent upon activation in the presence of increasing concentrations of calcium. The increased exposure of FXIIII-A residues 533-573 is consistent with this region of β-barrel 1 no longer having close interactions with the catalytic core surface. This FXIII-A segment may thus aid in displacing Tyr 560 from the FXIII-A active site. In response, the β-barrel 2 domain could serve as a lever to help direct β-barrel 1 away from the catalytic core domain of FXIII.

Without this β-barrel 2 lever action, there may be greater difficulties exposing the active site C314 leading to almost no enzymatic activity. Such a loss has been observed in this project with the TB1 truncation that contains β-barrel 1 but not β-barrel 2. With truncated TB1, cross-linking reactions involving fibrin, casein, α_2-antiplasmin, and model substrates are all greatly hindered. Furthermore, transglutaminase influences on rate of clot formation and clot lysis are lost. The additional removal of β-barrel 1 then causes enzymatic activity to return to 30% of full length FXIII-A. Once again the different glutamine and lysine containing substrates can better access the catalytic core regions involved in the transglutaminase reaction. An unresolved question is why TCC, lacking both β-barrels, exhibits reduced activity relative to wild type FXIII-A. The β-barrels may play a protective role in the zymogen form of FXIII-A. Once the β-barrels are lost, transglutaminase activity is possible but the catalytic core may become more vulnerable to biochemical attack at the active site or surrounding regions.

The first crystal structure of FXIII-A_2 trapped in an active conformation by a bound ligand was recently published by Stieler *et al* [50]. ZED1301 [Ac-Asp-MA(Michael Acceptor)-Nle-Nle-Leu-Pro-Trp-Pro-OH] was used as the inhibitory peptide to target FXIII. The MA group serves as a glutamine side chain analog that attaches covalently to the catalytic Cys

314. The FXIII-A_2 was nonproteolytically activated with calcium and subjected to the ZED1301 peptide [50]. The resultant crystal structure shows the FXIII-A_2 dissociated into two monomeric A-subunits. The FXIII β-barrel 1 and 2 domains rotated away from the catalytic core region and were directed upwards towards the β-sandwich domain. The exposed FXIIIa active site region containing the bound peptide could be viewed for the first time. This X-ray crystal structure helps support the proposed models for how TB1 and TCC work. Without the β-barrel 2 lever, there may be difficulties in moving β-barrel 1 into its correct position to help expose the FXIII catalytic site region.

The FXIII truncations highlight the roles of the individual β-barrel domains found within the FXIII A subunit. Current results suggest that β-barrels 1 and 2 are not required to maintain the overall, global conformation of the catalytic core domain. These two barrel domains are, however, hypothesized to exert an influence over to the active site region. In the zymogen state, the β-barrel 1 domain is proposed to protect the FXIII-A active site cysteine and surrounding residues. Later, the β-barrel 2 domain serves as a lever to help move β-barrel 1 away and expose the active site. The FXIII truncation mutant TB1 is therefore proposed to be such a poor transglutaminase since its β-barrel 1 domain can no longer take advantage of the lever action provided by the β-barrel 2 domain. Transglutaminase activity is regained with TCC, a mutant lacking both β-barrels 1 and 2. This study demonstrates that the individual β-barrels play a critical role in regulating substrate access to the FXIII active site region.

Addendum

E.L. Hethershaw participated in study design, performed the majority of the experiments, analysed the data, and co-wrote the manuscript. P.J. Adamson and W.N. Goldsberry performed some experiments and reviewed the manuscript. K.A. Smith, R.J. Pease, P.J. Grant, R.A.S. Ariens, S.E. Radford participated in study design and interpretation, and reviewed the manuscript. M.C. Maurer aided in data analysis and interpretation and co-wrote the manuscript. H. Philippou participated in study design, data interpretation, and co-wrote the manuscript.

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Disclosure of Conflict of Interests

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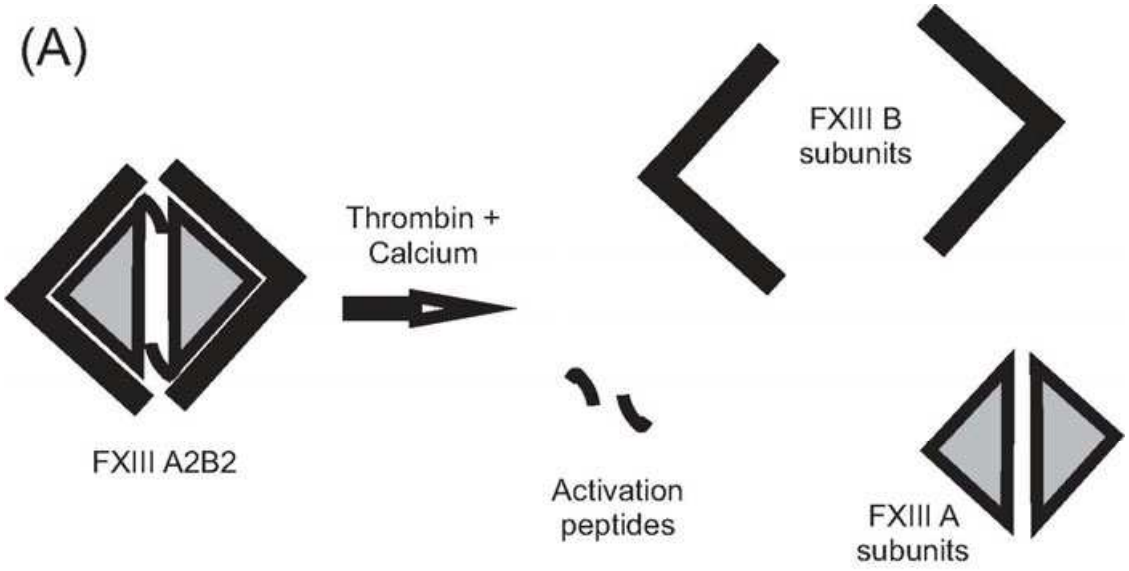
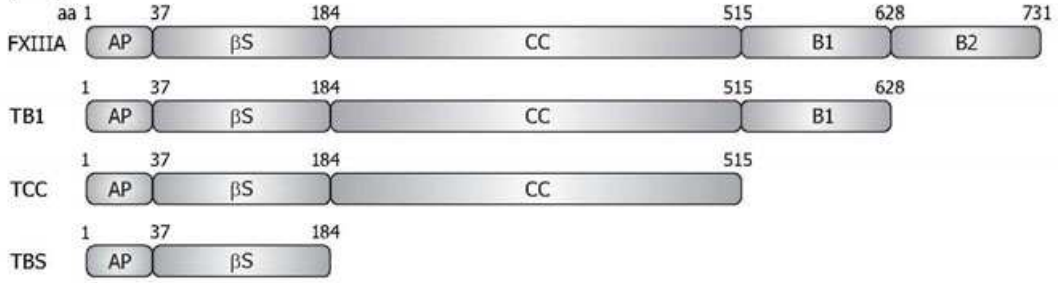
(A)



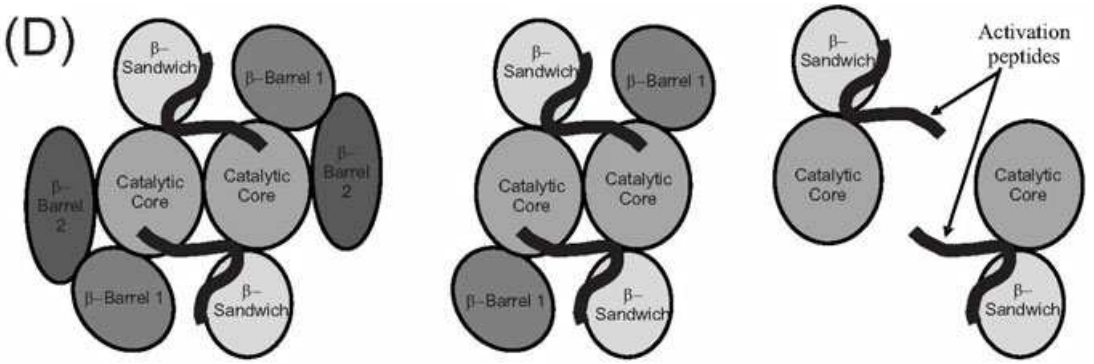
(B)



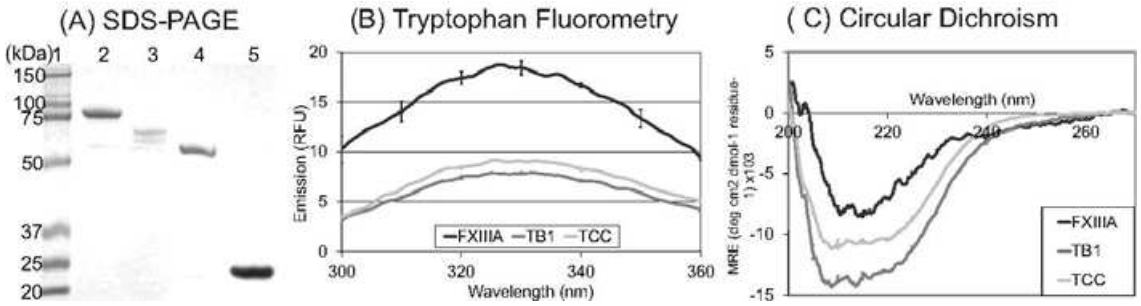
(C)

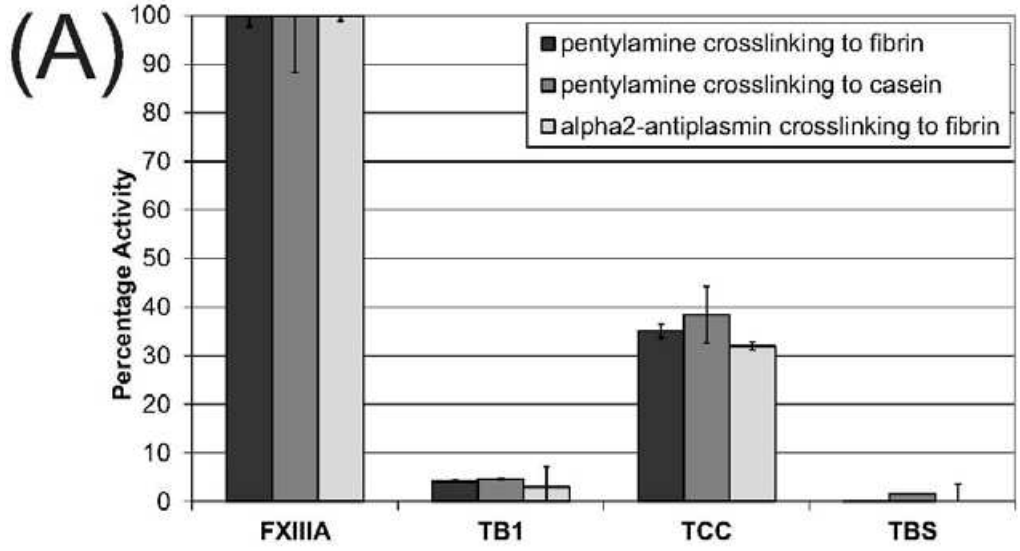
Region of Interest/Protein	Abbrev.	Amino Acids	Molecular Weight (Da)	Extinction Coefficient (mg/ml^{-1} cm^{-1})
Recombinant FXIIIA	rFXIIIA	1-731 (731)	83000	1.58
Trunc. Barrel 1	TB1	1-629 (629)	71400	1.52
Trunc. Catalytic Core	TCC	1-516 (516)	58500	1.75
Trunc. Beta Sandwich	TBS	1-184 (184)	21400	1.4

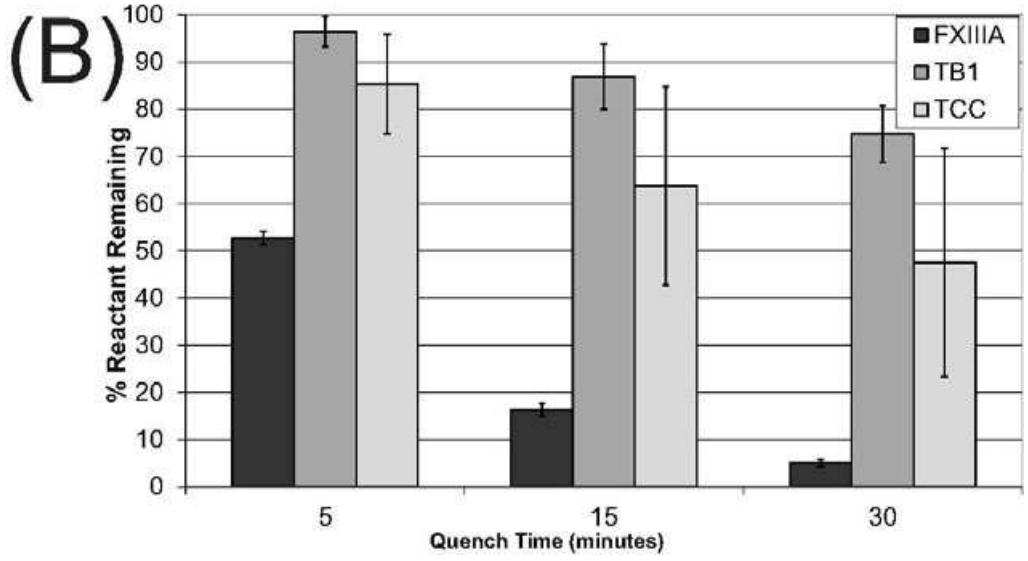
(D)

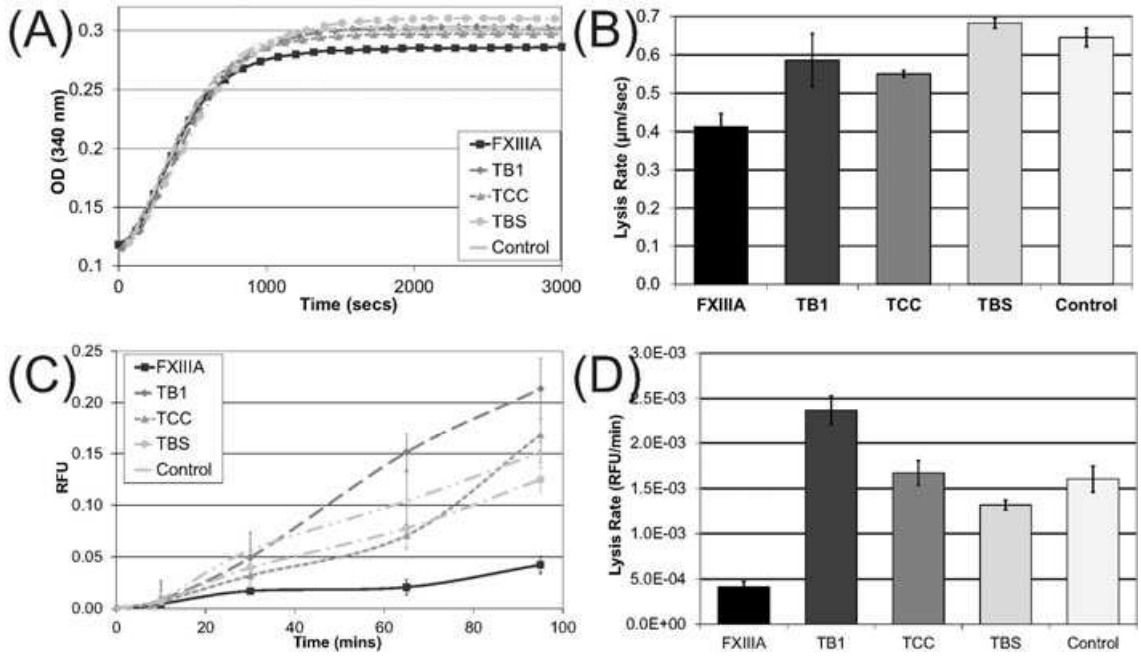


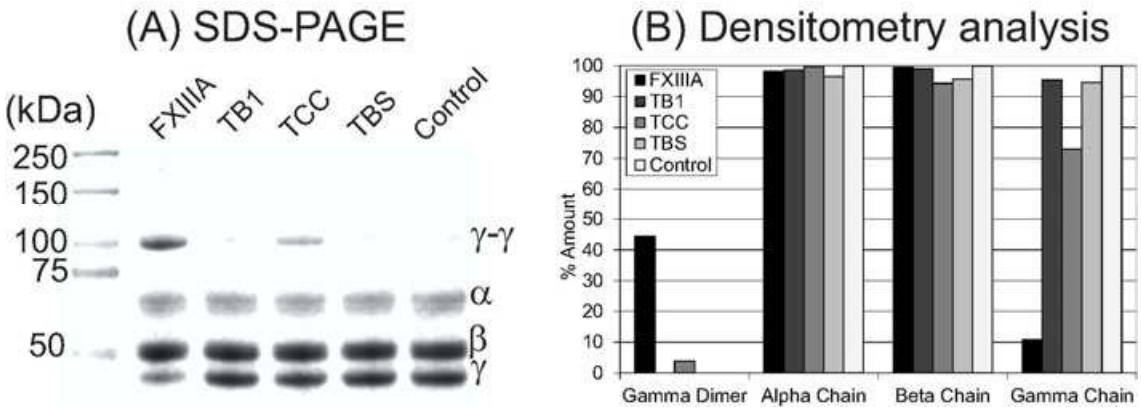
FXIIIA **TB1** **TCC**


(A) SDS-PAGE
(kDa)1 2 3 4 5
150
100
75
50
37
25
20
(B) Tryptophan Fluorometry
Emission (RFU)
Wavelength (nm)
FXIIIA TB1 TCC
(C) Circular Dichroism
Wavelength (nm)
MRE (deg cm2 dmol-1 residue-1) x103
FXIIIA
TB1
TCC


(A)
pentylamine crosslinking to fibrin
pentylamine crosslinking to casein
alpha2-antiplasmin crosslinking to fibrin
Percentage Activity
FXIIIA
TB1
TCC
TBS


(B)
FXIIIA
TB1
TCC
% Reactant Remaining
Quench Time (minutes)


(A)
OD (340 nm)
Time (secs)
FXIIIA
TB1
TCC
TBS
Control
(B)
Lysis Rate (µm/sec)
FXIIIA
TB1
TCC
TBS
Control
(C)
RFU
Time (mins)
FXIIIA
TB1
TCC
TBS
Control
(D)
Lysis Rate (RFU/min)
FXIIIA
TB1
TCC
TBS
Control


(A) SDS-PAGE
(kDa)
FXIIIA
TB1
TCC
TBS
Control
250
150
100
75
50
γ-γ
α
β
γ
(B) Densitometry analysis
% Amount
FXIIIA
TB1
TCC
TBS
Control
Gamma Dimer
Alpha Chain
Beta Chain
Gamma Chain