Interactions between factor XIII and the αC region of fibrinogen

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Abstract

Fibrinogen αC residues 242-424 have been shown to have a major regulatory role in the activation of FXIII-A₂B₂, however the interactions underpinning this enhancing effect have not been determined. Here we have characterised the binding of rFXIII-A subunit and FXIII-A₂B₂ with fibrin(ogen) and fibrin αC residues 233-425. Using recombinant truncations of the fibrin αC region 233-425 and surface plasmon resonance, we found that activated rFXIII-A bound αC 233-425 (K_D of 2.35 ± 0.09μM) which was further localised to αC 389-403. Site directed mutagenesis of this region highlighted Glu396 as a key residue for binding of activated rFXIII-A. The interaction was specific for activated rFXIII-A and dependent on the calcium induced conformational change known to occur in rFXIII-A during activation. Furthermore non-activated FXIII-A₂B₂, thrombin cleaved FXIII-A₂B₂, and activated FXIII-A₂B₂ each bound fibrin(ogen) and specifically αC region 371-425 with high affinity (K_D < 35nM and K_D < 31nM respectively), demonstrating for the first time the potential involvement of the αC region in binding to FXIII-A₂B₂. These results suggest that in addition to fibrinogen γ chain binding; the fibrin αC region also provides a platform for the binding of FXIII-A₂B₂ and FXIII-A subunit.
Introduction

Fibrinogen is a 340,000 Dalton glycoprotein composed of two sets of disulphide linked non-identical polypeptide chains; Aα, Bβ and γ\(^1\,^2\). Thrombin catalyses the polymerisation of fibrinogen to fibrin by sequentially cleaving fibrinopeptide A (FpA) and fibrinopeptide B (FpB) initiating lateral aggregation of protofibrils and fibre formation\(^3\,^4\). Factor XIII is a 325.8 kDa heterotetramer comprised of two identical globular A subunits non-covalently bound to two FXIII-B subunits\(^6\,^8\). FXIII-A\(_2\)B\(_2\) is converted to its active form by the thrombin catalysed hydrolysis of the Arg37-Gly38 peptide bond at the N-terminal of the FXIII-A subunit\(^9\). In the presence of calcium, the thrombin cleaved FXIII-A\(_2\)B\(_2\) complex dissociates yielding FXIII-B\(_2\) and activated FXIII-A\(_2\)\(^10\,^12\). Recently calcium has been shown to cause small but significant conformational changes in FXIII-A during activation exposing potential exosites within FXIII-A\(^13\,^15\). Activated FXIII-A\(_2\) stabilises the forming protofibril by introducing ε-amino(γ-Glutamyl)Lysine cross-links between carboxyl terminal portions of adjacent fibrin γ chains, prior to lateral association of the protofibril\(^16\). Cleavage of FpB and subsequent release of the αC regions from the central E region initiates lateral aggregation of protofibrils, and enables activated FXIII-A\(_2\) to cross-link adjacent αC stabilising the developing fibre and making it more resistant to fibrinolysis\(^17\,^18\). Interactions between fibrin(ogen) and FXIII-A\(_2\)B\(_2\) are well documented; Greenberg and Shuman demonstrated that non-activated plasma FXIII-A\(_2\)B\(_2\) bound specifically to fibrinogen via the FXIII-A\(_2\) subunits with an equilibrium constant (K\(_D\)) of 10nM\(^19\). Greenberg also noted that binding of FXIII-A\(_2\)B\(_2\) was unaffected by fibrinogen polymerisation suggesting that the interaction must occur prior to thrombin cleavage of FpA and FpB\(^20\). Hornyak and Shafer compared activated, non-activated platelet FXIII-A and non-activated plasma FXIII-A\(_2\)B\(_2\) for binding to fibrin clots (K\(_D\) of 2.1μM, 14μM and 200nM respectively)\(^21\). In addition, Hornyak examined the effect of fibrin on the activation of platelet FXIII-A, a phenomenon previously described by Credo et al\(^22\,^23\). Hornyak found that fibrin did not promote activation of platelet FXIII-A alone, but did enhance FXIII-A\(_2\)B\(_2\) activation, suggesting that this effect might be mediated by the dissociation of the B chains\(^21\). Immunoblotting of fibrinogen plasmin degradation products identified binding regions in the Aα- and Bβ chains for platelet FXIII-A. Binding was not observed on the fibrinogen γ chains\(^24\).
Interestingly, Siebenlist et al. suggested that plasma FXIII preferentially bound to fibrinogen molecules containing the variant γ′ because fibrinogen containing γAγ′ co-purified with the non-activated FXIII-A2B2, and that this interaction takes place via the B subunit of FXIII(25).

The binding region on fibrin for placental FXIII-A was first localised by Procyk et al(26). Procyk utilised several antibodies specific to various regions of fibrinogen (T2G1 (Anti-Bβ15-21), 1D4/x1-f (Anti-Aα389-402), 4-2/x1-f (Anti-γ392-406), Fd4-7B3 (Anti-γ[Fragment D]), 1C2-2 (Anti-Aα529-539) to identify the key regions thought to be involved in FXIII-A binding. The results demonstrated that the antibodies against the Bβ chain and the C-terminal region of the γ chains (Anti-Bβ15-21, Anti-γ392-406 and Anti-γ[Fragment D] did not appreciably affect the binding of activated FXIII-A. The binding was however substantially lowered by an anti-Aα389-402 antibody and furthermore by cyanogen bromide fragment Hi2-DSK (Aα241-476) suggesting that the location of the FXIII-A binding site was within αC region Aα389-402. In agreement with Hornyak and Shafer(21), Procyk showed that the activation of FXIII-A by thrombin, in the presence of calcium was necessary to enable a binding interaction. This αC binding region is consistent with previous work by Credo et al(23), in which αC residues 242-424 were found to enhance FXIII-A2B2 activation. Therefore it would seem reasonable to speculate that binding of activated FXIII-A and/or FXIII-A2B2 would occur within this αC region.

The aim of the current study was to characterise the interactions between FXIII and the fibrin(ogen) αC region to better understand the role of the αC region in regulating FXIII activation. This was performed using recombinantly expressed truncations of the αC region α233-425, and investigating binding interactions with i) non-activated recombinant FXIII-A (rFXIII-A), ii) activated rFXIII-A, iii) non-activated plasma FXIII-A2B2, and iv) thrombin cleaved FXIII-A2B2 in the presence and absence of calcium by surface plasmon resonance. In addition, site directed mutagenesis of the αC binding region 389-402 has enabled us to identify for the first time a key amino acid residue involved in the binding of activated rFXIII-A. Furthermore we have confirmed a) its specificity for activated rFXIII-A, ii) that the binding is specific for the calcium induced conformational change observed during rFXIII-A activation and iii) the binding is independent of rFXIII-A cross-linking.
activity. Finally, we have identified a novel high affinity FXIII-A2B2 binding site on the αC region of fibrinogen.

Materials and Methods

Development of GSTα fragments and FXIII-A expression constructs

Nine human fibrinogen αC fragments: α233-425, α233-403, α233-388, α233-375, α233-341, α233-290, α233-265, α289-425 and α371-425 (termed α fragment 1-9 respectively) were recombinantly expressed in E. coli using pGEX-6P-1 glutathione S-transferase (GST) gene fusion system (GE Healthcare, Chalfont St. Giles, UK). Variants of the αC region 371-425 (Pro389Ala, Asp390Ala, Trp391Ala, Gly392Ala, Phe394Ala, Glu396Ala, Gly399Ala, Ser402Ala) were produced by introducing site directed point mutations of highly conserved residues using the QuickChangeII Site Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA) carried out according to the manufacturers’ instructions. DNA sequencing confirmed the αC fragments and the mutations. See supplemental material for further details.

Full length rFXIII-A was PCR amplified from rFXIII-A construct pGF13A2, a kind gift from Dr. C.S. Greenberg, Duke University Medical Centre, North Carolina, USA(27). DNA sequencing confirmed the region of interest to be consistent with FXIII-A subunit. The PCR product was digested with Bgl II and Not I, cloned into pGEX-6P-1 and transformed into DH5α E. coli for screening. A recombinant FXIII-A double thrombin cleavage variant R37A/K513A was produced by introducing site directed point mutations of wild-type rFXIII-A residues using the QuickChangeII Site Directed Mutagenesis Kit carried out according to the manufacturers’ instructions. DNA sequencing confirmed the mutations coding for R37A and K513A.

Expression and purification of recombinant proteins

All GST tagged recombinant proteins (GSTαC fragments 1-9, GSTα fragment 9 variants, GST-FXIII-A and GST-FXIII-A R37A/K513A) were expressed using BL21-Gold E.coli following the method detailed in the supplemental material. GST fusion proteins were purified by GST-affinity chromatography using an AKTAprome™ system according to the recommended method by GE healthcare. Details of the cell lysis, protein purification and GST PreScission cleavage protocols used in this investigation can be found in the supplemental material.
Purification of Fibrogammin-P FXIII-A2B2 using gel filtration chromatography

Fibrogammin P (CSL Behring, Marburg, Germany) was used as a source of plasma FXIII-A2B2 after isolation from other additives by gel filtration chromatography using a Biocad sprint automated chromatography system (Perspective Biosystems, Framingham, MAS, USA) as detailed previously by Standeven et al.\textsuperscript{(28)} For additional details see supplemental material.

Purification of fibrinogen

Plasminogen-free fibrinogen from human plasma (Calbiochem, San Diego, CA), was prepared by ammonium sulphate precipitation to remove bound FXIII as described by Siebenlist et al.\textsuperscript{(25)} For additional details see supplemental material. The FXIII-free fibrinogen was screened by western blotting and biotin labelled pentylamine incorporation FXIII-A activity assay to confirm the removal of any contaminating FXIII. The fibrinogen concentration was determined at A\textsubscript{280}nm using the extinction coefficient 1.51 for a 1mg/ml solution.

Biotin labelled pentylamine incorporation FXIII-A activity assay.

The cross-linking activity of purified FXIII-A2B2, rFXIII-A and rFXIII-A R37A/K513A variant was determined using a modified version of a 5-(biotinamido)pentylamine incorporation assay\textsuperscript{(29)}). Modifications to the method can be found in the supplemental material.

Activation of rFXIII-A and FXIII-A2B2

Recombinant FXIII-A and FXIII-A2B2 were activated using 5U/ml human α-thrombin with 1.5mM calcium for 2h at 37°C, unless stated otherwise. The sample was centrifuged at 13,500 rpm for 5 min to remove precipitate and the concentration determined as described above. When removal of thrombin was necessary; biotinylated thrombin (Merck, Darmstadt, Germany,) was used at 5U/ml for 2h at 37°C. Biotinylated thrombin was removed using streptavidin agarose according to the manufacturer’s protocol.

Surface plasmon resonance
In all cases Surface plasmon resonance was performed using a Biacore 3000 platform and data evaluated using BIAcore 3000 BIAevaluation 4.1 software (GE Healthcare, UK).

**Binding of rFXIII-A to fibrinogen αC region.** Using SPR-calcium running buffer (20mM Hepes, 140mM NaCl, 1.5mM CaCl$_2$, 0.05% (v/v) surfactant P20, pH 7.4) approximately 2500 response units (RU) of Goat anti-GST antibody (GE healthcare) was immobilised directly onto a CM5 sensor chip (flow cell 2) by amine coupling according to the manufacturers instructions. A second flow cell (flow cell 1) was immobilised with the same antibody but was used as a blank reference cell. 3.6μM of GSTα fragment 1-9 was injected for 300s at a flow rate of 20μl/min over flow cell (Fc) 2 to reach a capture level of approximately 500 RU. 100μg/ml rGST (Sigma-Aldrich, UK) was injected over Fc1 and 2 for 300s at a flow rate of 20μl/min to block any remaining anti-GST binding sites. PreScission cleaved rFXIII-A was dialysed for 16h at 4°C into SPR-calcium running buffer. Dialysed rFXIII-A was thrombin activated, centrifuged and concentration determined as described above. A 2-fold serial dilution of activated rFXIII-A (7.8-1000nM) was injected in ascending order for 60s at a flow rate of 30μl/min over Fc1 and Fc2. Regeneration was achieved using buffer flow after stabilisation for 300s. Due to the rapid binding of activated rFXIII-A to the αC, it was not possible to perform kinetic analysis. The $K_D$ was therefore obtained with a pre-defined steady state affinity model using blank reference (Fc2-1, Fc4-3) and buffer subtracted data. This interaction was also performed in the reverse orientation to confirm the $K_D$; Using SPR-calcium running buffer 5000 RU of activated rFXIII-A was directly immobilised onto a CM5 sensor chip as detailed above. αC fragments 1, 3 and 9 (cleaved from the GST tag) were dialysed into SPR-calcium running buffer. A 2-fold serial dilution of 0.01- 10μM αC fragment was injected in ascending order for 60s at a flow rate of 30μl/min over the immobilised activated rFXIII-A. Regeneration was achieved using buffer flow after stabilisation for 300s (n=3).

**Binding of α fragment 9 variants to activated rFXIII-A.** αC fragment 9 variants were cleaved from the GST tag and dialysed into SPR-calcium running buffer. A fixed concentration of 1μM was injected over immobilised activated rFXIII-A for 60s at a flow rate of 30μl/min. The binding response of each variant at 55s, after
buffer subtraction, was taken and plotted against the wild type α fragment 9 binding response for comparison (n=3).

**Binding of FXIII-A₂B₂ to fibrinogen αC region.** Approximately 500 RU of GSTα fragment 1 or GSTα fragment 9 were captured on a CM5 sensor chip as described above. Non-activated FXIII-A₂B₂ and thrombin cleaved FXIII-A₂B₂ activated in the presence and absence of calcium, were tested for binding to the captured GSTα fragment 1 and 9. Two aliquots of purified FXIII-A₂B₂ were dialysed for 16h at 4°C into SPR-EDTA buffer (20mM Hapes, 140mM NaCl, 5mM EDTA, 0.05% (v/v) surfactant P20, pH 7.4). A third aliquot was dialysed into SPR-calcium buffer pH 7.4. The FXIII-A₂B₂ dialysed into SPR-calcium buffer, and one of two FXIII-A₂B₂ aliquots dialysed into SPR-EDTA buffer were thrombin cleaved as described above. A 2-fold serial dilution (0.7-200nM) of non-activated FXIII-A₂B₂ or thrombin cleaved FXIII-A₂B₂ were injected for 60s over the captured GSTα fragment 1 or GSTα fragment 9 at a flow rate of 30µl/min. Removal of bound FXIII-A₂B₂ was achieved using two 50s pulses of 2M NaCl at a flow rate of 30µl/min. The surface was re-equilibrated in running buffer for 300s.

**Binding of FXIII-A₂B₂ to fibrinogen and fibrin.** 100 RU of purified fibrinogen was immobilised directly onto a CM5 sensor chip (Fc2) by amine coupling according to the manufacturers instructions. A blank reference surface (Fc1) was activated and deactivated as described above. The immobilised fibrinogen was converted to fibrin as previously described.³⁰ Purified FXIII-A₂B₂ was dialysed at 4°C for 16h into SPR-calcium running buffer and thrombin activated. A 2-fold serial dilution of thrombin and calcium activated FXIII-A₂B₂ (0.7-200nM) was injected over the immobilised fibrin at a flow rate of 30µl/min for 120s. Removal of bound FXIII-A₂B₂ was achieved using two 40s pulses of 5mM NaOH containing 100mM NaCl at a flow rate of 30µl/min. The surface was re-equilibrated in running buffer for 300s. Similar methodology was applied for analyses of the interactions between non-activated FXIII-A₂B₂ (in the absence of thrombin or calcium) and FXIII-A₂B₂ cleaved by thrombin in the presence of EDTA (without calcium) with fibrinogen and fibrin. The SPR-buffers contained EDTA or calcium to be consistent with the conditions employed for the cleavage of FXIII by thrombin. Non-activated, or thrombin only activated FXIII-A₂B₂ was never exposed to calcium to prevent non-proteolytic activation thought to occur in the presence of calcium alone. Data was fitted to a
bivalent analyte model as the 1:1 Langmuir binding model resulted in poor curve fitting (Chi² <5 and Chi² >20 respectively). The bivalent analyte model was chosen to take into account the FXIII-A₂ and FXIII-B₂ dimers in the analyte FXIII-A₂B₂. However, the KD obtained for this interaction is an estimate only as fibrinogen is also a dimer which, when bound to FXIII-A₂B₂, would result in a complex multivalent interaction which would be difficult to fit to a kinetics model. All SPR investigations were tested for mass transfer using a pre-defined Mass Transfer control wizard (BIAcore 3000 software version 4.1). The results confirmed that in all cases the interaction rates observed were not limited by mass transfer (data not shown).

**Competitive inhibition assay using surface plasmon resonance**

A CM5 sensor chip surface was used to capture GSTα fragment 1 using the same methodology as described above. GST-cleaved αC fragments 1-9 and rFXIII-A were dialysed for 16h at 4°C into SPR-calcium running buffer. To determine the binding response from thrombin activated rFXIII-A alone; 125nM was injected for 60s over captured GSTα fragment 1 at a flow rate of 30μl/min. The binding response was classified as 100% binding of activated rFXIII-A to the captured GSTα fragment 1. 125nM of thrombin activated rFXIII-A was pre-incubated for 1 minute at 25°C with the cleaved αC fragments 1-9 at increasing molar concentrations of 12.5nM, 125nM, 1250nM and 6250nM. The mix of activated rFXIII-A with αC fragment 1-9 was injected for 60s at a flow rate of 30μl/min over the captured GSTα fragment 1. The percentage binding response of the activated rFXIII-A binding to the captured GSTα fragment 1 was plotted against the molar concentration of the αC fragment employed in the pre-incubation with activated rFXIII-A. GST-cleaved α fragment 9 was also used as a competitive inhibitor to ascertain if the binding of activated FXIII-A₂B₂ to the captured GSTα fragment 1, and directly immobilised fibrin could be inhibited. PreScission cleaved α fragment 9 was pre-incubated, at increasing molar concentrations (0μM, 0.012μM, 0.125μM, 1.25μM and 12.5μM where 0μM refers to activated FXIII-A₂B₂ in the absence of α fragment 9 competitor) for 60s with 125nM thrombin activated FXIII-A₂B₂ in the presence of calcium. The sample was injected for 60s at a flow rate of 30μl/min over captured GSTα fragment 1 and immobilised fibrin. The binding response in RU was converted to percentage binding of FXIII-
A2B2 to the surface; captured GSTα fragment 1 or immobilised fibrin for comparison (n=3).

The role of calcium in rFXIII-A activation and its effects on binding to the αC region 233-425 by SPR

Non-activated rFXIII-A was dialysed for 16h at 4°C into SPR-calcium running buffer, and a separate rFXIII-A aliquot was dialysed into SPR-EDTA buffer. Both rFXIII-A aliquots were thrombin activated and binding analysis performed using captured GSTα fragment 1 as previously described, using SPR-calcium running buffer for the rFXIII-A sample activated in the presence of thrombin and calcium, and a second SPR-EDTA running buffer for rFXIII-A activated with thrombin only in the presence of EDTA. The rFXIII-A samples were injected for 60s over the captured GSTα fragment 1 at a flow rate of 30μl/min.

Effect of Iodoacetamide inhibition of rFXIII-A on binding to αC 233-425 by SPR

rFXIII-A was dialysed for 16h at 4°C into SPR-calcium running buffer. The rFXIII-A was thrombin activated and incubated with 50mM iodoacetamide (Sigma-Aldrich) for 15 min at 37°C. An aliquot of activated rFXIII-A in the absence of iodoacetamide was used for comparison. Prior to performing the binding study, 50mM iodoacetamide was injected for 60s at a flow rate of 30μl/min over the captured GSTα fragment 1. The iodoacetamide alone did not result in a binding response. Activated rFXIII-A (0.5μM) in the presence and absence of 50mM iodoacetamide was injected at a flow rate of 30μl/min for 60s over captured GSTα fragment 1.

Results

Expression of recombinant αC truncations, rFXIII-A and rFXIII-A-R37A/K513A

Recombinant GSTα fragments 1-9 and αC fragments 1-9 cleaved from the GST tag were visualised using SDS-PAGE (Figure 1A-C) and exhibited major bands of the expected molecular mass as shown in Table 1. SDS-PAGE analysis of thrombin activated rFXIII-A clearly demonstrated a decrease in molecular mass by approximately 4 kDa resulting from the N-terminal cleavage of the activation peptide by thrombin (Figure 1D; Lane 1-2). This effect was not observed in the rFXIII-A R37A/K513A variant (Figure 1D; Lane 3-4) as predicted since R37 and K513 have
been identified as thrombin cleavage sites\textsuperscript{(9,31)}. The FXIII-A pentylamine incorporation assay indicated that the wild-type rFXIII-A and FXIII-A\textsubscript{2B2} were active in contrast to variant rFXIII-A R37A/K513A which showed minimal cross linking activity (Figure 1E).

**Activated rFXIII-A subunit binds fibrinogen αC region 371-425**

Binding of activated rFXIII-A was observed for GSTα fragments 1, 2, 8 and 9 (α233-425, α233-403, α289-425 and α371-425 respectively). Negligible binding was observed for αC fragments 3 to 7 (α233-388, α233-375, α233-341, α233-290 and α233-265 respectively) which was too low to undertake formal analysis (Data not shown). Figure 2 shows the binding of activated rFXIII-A to GSTα fragment 1 and GSTα fragment 9. Applying a steady state affinity model showed that GSTα fragment 1 (α233-425) bound to activated rFXIII-A with a $K_D$ of $2.35 \pm 0.09 \mu M$ while GSTα fragment 2 (α233-403) bound with a $K_D$ of $2.64 \pm 1.21 \mu M$. Competitive inhibition studies using αC fragments 1 and 2 confirmed that the binding region for activated rFXIII-A was within αC 233-425. Complete inhibition of binding to captured GSTα fragment 1 could not be achieved using α fragments 3 to 7, supporting the SPR data that the αC region 233-388 is not sufficient to mediate the interaction with activated rFXIII-A (Figure 2C). Rather, these results suggest the presence of essential determinants within the C-terminal portion of residues α389-403.

To further localise the αC binding region for activated rFXIII-A, amino-terminal truncations α fragment 8 (α289-425) and 9 (α371-425) were employed. Activated rFXIII-A bound GSTα fragment 8 with a $K_D$ of $1.81 \pm 0.45 \mu M$ and GSTα fragment 9 with a $K_D$ of $3.19 \pm 0.57 \mu M$ (Figure 2B; Table 1). Competitive inhibition studies using α fragments 8 and 9 showed complete inhibition of rFXIII-A binding to captured GSTα fragment 1, comparable to the inhibition observed using cleaved α fragment 1 (Figure 2D).

To confirm the binding interaction, the experiment was performed in the reverse orientation by immobilising activated rFXIII-A and injecting αC fragment 1, 9 and 3. The results demonstrated that αC fragment 1 and 9 bound with a $K_D$ of $3.91 \pm 0.54 \mu M$ and $3.56 \pm 0.53 \mu M$ respectively. Negligible binding was observed for α.
fragment 3 (Figure 3). These data indicate that essential determinants of the fibrinogen $\alpha C$ binding region for activated rFXIII-A lie within $\alpha C$ residues 389-425 and are consistent with the localization 389-403 deduced above. The localisation is also in agreement with Procyk et al.$^{26}$.

Identification of a key amino acid residue involved in binding activated rFXIII-A

If the interaction between activated FXIII-A and the $\alpha C$ region is physiologically significant it would be anticipated that key residues would be conserved between species. Alignment showed strong conservation of residues within 389-403 (Figure 4A). Site directed mutagenesis of highly conserved amino acid residues within the $\alpha C$ region demonstrated that a glutamic acid residue at position $\alpha 396$ is involved in the binding of activated rFXIII-A (Figure 4B) whereas substitution of 7 adjacent conserved residues was without effect. This suggests that FXIII-A is not binding to a linear binding site that is wholly contained within residues 389-403, but is binding to an assembled determinant that also involves residues located between 371 and 389.

Non-activated rFXIII-A does not bind fibrinogen $\alpha C$ region 233-425

SPR binding analysis demonstrated that wild type non-activated rFXIII-A and the inactivate variant R37A/K513A did not bind to the captured GST$\alpha$ fragment 1 (Figure 5A). Furthermore, it was observed that the $\alpha C$ region 233-425 does not bind to rFXIII-A activated with thrombin only, but also required the presence of calcium which induces a conformational change that exposes portions of FXIII-A that are not structurally exposed in the non-activated form$^{13-15}$ (Figure 5B). Binding did not depend on catalytic activity as blocking the rFXIII-A active site Cys314 with iodoacetamide, did not inhibit binding to the $\alpha C$ region 233-425 which occurred to the same degree as activated rFXIII-A in the absence of iodoacetamide (Figure 5C).

FXIII-A$_2$B$_2$ binds with high affinity to fibrin(ogen) $\alpha C$ region 371-425

Surface plasmon resonance was used to determine if FXIII-A$_2$B$_2$ bound to GST$\alpha$ fragment 1 ($\alpha 233-425$) and GST$\alpha$ fragment 9 ($\alpha 371-425$). Using a bivalent analyte model the SPR results demonstrated that GST$\alpha$ fragment 1 ($\alpha 233-425$) bound to non-activated FXIII-A$_2$B$_2$ ($K_D 1 = 7.3 \pm 6.3nM$), thrombin cleaved FXIII-A$_2$B$_2$ (no calcium) ($K_D 1 = 3.7 \pm 0.3nM$), and thrombin cleaved FXIII-A$_2$B$_2$ activated in the
presence of calcium ($K_D = 21.9 \pm 2.2nM$) (Table 2). In addition, the binding of the different activation states of FXIII-A$_2$B$_2$ were further localised to fibrinogen $\alpha$C 371-425 (non-activated FXIII-A$_2$B$_2$: $K_D = 30.9 \pm 23nM$, thrombin cleaved FXIII-A$_2$B$_2$ (no calcium): $K_D = 5.4 \pm 1.8nM$, thrombin cleaved FXIII-A$_2$B$_2$ activated in the presence of calcium: $K_D = 7.3 \pm 1.8nM$) (Figure 6A; Table 2). The SPR sensograms for the binding of FXIII-A$_2$B$_2$ to GST$\alpha$ fragment 1 were identical to the those displayed for the binding of FXIII-A$_2$B$_2$ to GST$\alpha$ fragment 9 and therefore are not shown.

The various activation states of FXIII-A$_2$B$_2$ were also tested for binding to full length fibrinogen and fibrin. The results demonstrated that both fibrinogen and fibrin bound to non-activated FXIII-A$_2$B$_2$ with a $K_D$ of 11 ± 4.7nM and 35.2 ± 4nM respectively, thrombin cleaved FXIII-A$_2$B$_2$ (no calcium) with a $K_D$ of 2.9 ± 2.1nM and 6.1 ± 1nM and thrombin cleaved FXIII-A$_2$B$_2$ activated in the presence of calcium with a $K_D$ of 10.7 ± 0.9nM and 14.5 ± 3.1nM (Figure 6B-C; Table 2).

$\alpha$ Fragment 9 ($\alpha$371-425), in the absence of the GST tag, was employed as a competitive inhibitor to confirm the specificity of the interaction. The results demonstrated that $\alpha$ fragment 9 inhibited the binding of thrombin cleaved FXIII-A$_2$B$_2$ (activated in the presence of calcium), to the SPR captured GST$\alpha$ fragment 1 and full length fibrin (Figure 7). Furthermore these results confirm that the interaction between the $\alpha$ fragment 9 and activated FXIII-A$_2$B$_2$ occurs in solution in addition to binding to an immobilised surface.

**Discussion**

Previous studies have demonstrated the importance of the $\alpha$C region for FXIII activation. The aim of this study was to characterise the interactions between FXIII-A and FXIII-A$_2$B$_2$ with the fibrinogen $\alpha$C region 233-425 that could contribute to this process. Using recombinant truncations of the fibrinogen $\alpha$C region 233-425; we have identified a novel high affinity interaction between FXIII-A$_2$B$_2$ and fibrinogen $\alpha$C region 371-425 which was evident both in the zymogen and after activation with thrombin and calcium. Additionally, we have confirmed a previously described low affinity interaction between activated FXIII-A and the $\alpha$C region of fibrinogen, but have extended the analysis to confirm the domain involved and to implicate a key residue, Glu396. The low affinity interaction was dependent upon calcium, which
induces a conformational change in the β-barrel 1 and the β-sandwich domains of thrombin-cleaved FXIII-A\(^{(13-15)}\). Additionally, calcium could mediate the interaction with Glu396. However, the low affinity interaction was independent of catalytic activity showing that it did not arise from a transient covalent intermediate.

Low affinity and reversible binding of activated FXIII-A\(_2\) to fibrinogen was reported by Procyk \textit{et al.} using antibodies specific for the αC region 389-402 and by cyanogen bromide fragment Hi2-DSK (Aα241-476) suggesting that the location of the FXIII-A binding site was within αC region Aα389-402\(^{(26)}\). Our results obtained by mapping recombinant fragments implicate the same αC region, and in addition present a key residue, Glu396, as being critical for binding activated FXIII-A. Furthermore, our data also implies that additional residues upstream (α371-389) may contain secondary stabilising sites which cannot function in the absence of Glu396, since it is unlikely that only one amino acid residue could make up the contact site for activated FXIII-A. The role of this interaction is currently undefined, but it is notable that the αC region 389-402 is close to the key αC glutamine residues (Gln366, Gln328 and Gln237) required for FXIII-A cross-linking aiding clot formation and stabilisation. Mutagenesis of the key interacting residue Glu396 in full-length fibrinogen will enable the putative role of the interaction in FXIII-A-mediated cross-linking to be assessed, and will be of value in designing structural studies to identify the other residues comprising this binding site.

Our results also confirm high affinity interactions between non-activated and activated FXIII-A\(_2\)B\(_2\) and i) a recombinant αC fragment of fibrinogen and ii) the αC region on fibrin. The tetrameric nature of the SPR analyte FXIII-A\(_2\)B\(_2\), together with the dimeric structure of the ligand fibrin(ogen), which is thought to adopt a conformation similar to that of soluble fibrinogen when immobilised\(^{(32)}\), has the potential to result in a complex multivalent interaction. However SPR data are consistent with K\(_D\)\(_1\) dissociation constants in the range of 35nM. These values are in line with the K\(_D\) (10nM) deduced by Greenberg and Shuman for the binding of non-activated plasma FXIII-A\(_2\)B\(_2\) to full length fibrinogen, in this case monitoring the interaction on the surface of latex beads\(^{(19)}\). Interactions between FXIII-A\(_2\)B\(_3\) and FXIII-A\(_3\) with fibrin(ogen) have also been shown by immunoblotting\(^{(24)}\), clot binding assays\(^{(21)}\), and ELISA\(^{(20,33)}\).
Greenberg and co-workers have suggested that FXIII may circulate in plasma bound to the fibrinogen molecule, allowing the non-activated FXIII to be present for cross-linking activity when required. Interestingly, in our investigation all forms of FXIII bound similarly to the αC region suggesting that this region contributes to binding of FXIII to circulating fibrinogen, supporting the hypothesis proposed by Greenberg et al. However, Siebenlist et al. have proposed that circulating FXIII-A₂B₂ binds preferentially to a subset of fibrinogen molecules that contain an extended γ chain as a result of alternative splicing of the γ chain mRNA and which comprise about 10% of fibrinogen γ chains in the plasma. Fractionation of fibrinogen by anion exchange chromatography into pools enriched or depleted in γ’ chains have shown preferential association of FXIII-A₂B₂ with the enriched pool(25). However, it is notable that Gersh and Lord have reported that fibrinogen of γ’γ’, γ’γ’ and γ’/γ’ composition all bind FXIII-A₂B₂ with a K_D of 41nM, within the same order of magnitude as determined for the interaction between fibrinogen γ’γ’ and fibrin γ’γ’ with FXIII-A₂B₂ (K_D 11nM and 35nM respectively) observed in this investigation (34).

The existence of an interaction between FXIII-A₂B₂ and the αC region of fibrinogen is predicted by work from Credo et al. which reported that the presence of fibrinogen accelerates activation of FXIII(22), and more specifically the αC residues 242-244 enhanced the activation of FXIII to almost the same degree as full length fibrinogen(23). For this enhancement to occur an interaction between FXIII-A₂B₂ and the αC regions of fibrinogen must take place at an early stage of clot formation. Our results demonstrate i) that the αC region 371-425 binds with similar affinity to FXIII-A₂B₂ to that displayed by full length fibrinogen and ii) that the αC fragment 371-425 inhibited FXIII-A₂B₂ binding to fibrin. We were not able to unequivocally demonstrate FXIII-A₂B₂ binding to the αC region on full length fibrinogen, as opposed to fibrin or the recombinant αC fragment. In this context, probably the best interpretation of currently available data is that γ’ chain binding is important to promote a carrier protein function for FXIII-A₂B₂ and that the αC region:FXIII-A₂B₂ interactions may have an important separate role during fibrin formation which may be to enhance FXIII activation. However, further functional studies of αC region:FXIII-A₂B₂ interactions will need to be carried out to confirm this hypothesis.

In FXIII-A₂B₂, sushi domains 1-9 of FXIII-B mostly cover FXIII-A (35), making it likely that binding of the non-activated FXIII-A₂B₂ to the fibrinogen αC
region takes place via the B subunit. In ongoing studies, we have confirmed that the αC region binds to the B-subunit (unpublished data). Thrombin, in the absence of calcium, cleaves the activation peptide from the amino-terminus of FXIII-A but does not dissociate the complex\(^9\), so that the interaction between fibrin and FXIII-A\(_2\)B\(_2\) might be expected to be maintained. Accordingly, in our hands thrombin cleavage of FXIII-A\(_2\)B\(_2\) did not affect FXIII-A\(_2\)B\(_2\) binding to the αC region 371-425. Finally, thrombin, in the presence of calcium, both cleaves the activation peptide from the amino-terminus of FXIII-A and causes dissociation of the FXIII-A\(_2\)B\(_2\) complex. We observed that the binding interaction was preserved showing that it didn’t depend on the presence of both chains. We assume that FXIII-B may remain in complex with fibrinogen through a relatively tight interaction with a slow dissociation rate constant while activated FXIII-A is released. The relatively weak interaction of liberated FXIII-A with the αC is consistent with the need for the enzyme to be mobile to perform its cross-linking functions.

The results of this study localise the binding of FXIII-A\(_2\)B\(_2\) to a high affinity interaction site on the αC region 371-425, in addition to a low affinity αC binding site involving key amino acid residue Glu396 for activated FXIII-A. Activated rFXIII-A binding is dependent on the conformational change induced by calcium, and is independent of catalytic activity. These results support the view that interactions fundamental to FXIII-A\(_2\)B\(_2\) activation for αC cross-linking, fibrin formation and stabilisation are taking place in this region. Further studies are required to evaluate the effect of these interactions on thrombus formation in health and disease.

Acknowledgements
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Authorship
Contribution: K.A.S carried out the laboratory work, assisted in study design, analysed data and wrote the manuscript. P.J.A. made the FXIII-A constructs, R.J.P.
assisted in study design, data analysis and critically reviewed the manuscript. A.J.B. helped with data analysis and critically reviewed the manuscript. J.M.B. helped with laboratory work. R.A.S.A. supervised the work and assisted in study design. P.A.C. provided general advice during the study and critically reviewed the manuscript. H.P. supervised the work, assisted in study design, data analysis and critically reviewed the paper. P.J.G. provided overall supervision, assisted in study design and helped write the manuscript.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

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Reference List


Table 1

<table>
<thead>
<tr>
<th>Ligand GSTα Fragment</th>
<th>Molecular mass of GSTα fragments (kDa)</th>
<th>Molecular mass of GST removed α fragments (kDa)</th>
<th>Equilibrium dissociation constant (K_D) μM</th>
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<tr>
<td>GSTα fragment 1 (233-425)</td>
<td>50.0</td>
<td>20.02</td>
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<td>15.83</td>
<td>*Negligible binding</td>
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<td>GSTα fragment 8 (289-425)</td>
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<td>GSTα fragment 9 (371-425)</td>
<td>35.7</td>
<td>5.7</td>
<td>3.19 ± 0.57</td>
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Steady state affinity data for the binding of activated rFXIII-A to captured GSTα fragment 1-9 by SPR. Molecular mass of recombinant fibrinogen αC fragments 1 to 9 in the presence and absence of the GST tag are also shown. Using a CM5 sensor chip anti-GST antibody capture approach on a BIAcore 3000 platform; a 2-fold serial dilution of activated rFXIII-A was injected for 60s over captured GSTα fragments 1-9. The data was evaluated using BIAcore 3000 BIAevaluation 4.1 software fitted to a pre-defined steady state affinity model using blank reference and buffer subtracted data. *Negligible binding was observed for GSTα fragment 3, 4, 5, 6, and 7 which was too low to undertake formal analysis. (n=3).
Table 2

<table>
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<th>Analyte</th>
<th>Immobilised Ligand</th>
<th>Equilibrium dissociation constant (K_D1)*</th>
<th>Association constant k_a1 (1/Ms)</th>
<th>Dissociation constant k_d1 (1/s)</th>
<th>Association constant k_a2 (1/Ms)</th>
<th>Dissociation constant k_d2 (1/s)</th>
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<td>Unactivated GSTαF1</td>
<td>7.3 ± 6.3nM</td>
<td>1.97 ± 1.52E+05</td>
<td>1.43 ± 0.68E-03</td>
<td>4.78 ± 8.00E+02</td>
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<tr>
<td>FXIII-A2B2 (no calcium)</td>
<td>30.9 ± 23nM</td>
<td>1.32 ± 1.03E+05</td>
<td>4.09 ± 3.39E-03</td>
<td>2.22 ± 3.54E+03</td>
<td>1.85 ± 1.24E-01</td>
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<tr>
<td>(no calcium)</td>
<td>11.0 ± 4.7nM</td>
<td>5.97 ± 1.54E+05</td>
<td>6.57 ± 1.02E-03</td>
<td>2.68 ± 3.57E+03</td>
<td>1.50 ± 1.75E-01</td>
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<td>Fibrin</td>
<td>35.2 ± 4.0nM</td>
<td>3.66 ± 0.91E+05</td>
<td>1.29 ± 0.46E-02</td>
<td>9.01 ± 8.35E+03</td>
<td>3.68 ± 2.76E-01</td>
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<tr>
<td>Thrombin GSTαF1</td>
<td>3.7 ± 0.3nM</td>
<td>1.64 ± 0.06E+05</td>
<td>6.00 ± 0.33E-04</td>
<td>7.35 ± 2.78E+01</td>
<td>1.81 ± 0.72E-01</td>
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<tr>
<td>cleaved A2B2 (no calcium)</td>
<td>5.4 ± 1.8nM</td>
<td>1.27 ± 0.66E+05</td>
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<td>Fibrin</td>
<td>2.9 ± 2.1nM</td>
<td>8.67 ± 1.62E+05</td>
<td>2.48 ± 1.34E-03</td>
<td>6.09 ± 5.17E+03</td>
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<td>(no calcium)</td>
<td>6.1 ± 1.0nM</td>
<td>5.80 ± 3.15E+05</td>
<td>3.53 ± 3.22E-03</td>
<td>6.07 ± 4.61E+03</td>
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<tr>
<td>Thrombin GSTαF1</td>
<td>21.9 ± 2.2nM</td>
<td>1.10 ± 0.19E+06</td>
<td>2.41 ± 0.16E-02</td>
<td>5.20 ± 1.77E+02</td>
<td>1.61 ± 0.48E-01</td>
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</tr>
<tr>
<td>cleaved A2B2 (with Calcium)</td>
<td>7.3 ± 1.8nM</td>
<td>1.95 ± 0.07E+06</td>
<td>1.42 ± 0.32E-02</td>
<td>2.59 ± 2.17E+03</td>
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<tr>
<td>Fibrin</td>
<td>10.7 ± 0.9nM</td>
<td>1.19 ± 0.37E+06</td>
<td>1.28 ± 0.41E-02</td>
<td>1.41 ± 1.19E+04</td>
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<tr>
<td>(with Calcium)</td>
<td>14.5 ± 3.1nM</td>
<td>1.26 ± 0.32E+06</td>
<td>1.83 ± 0.69E-02</td>
<td>1.71 ± 1.07E+04</td>
<td>2.24 ± 1.24E-01</td>
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**SPR Kinetic analysis for the interaction between FXIII-A2B2 activation forms binding to immobilised fibrinogen, fibrin and the GST captured fibrinogen αC fragments 1 (233-425) and 9 (371-425).** The data was evaluated using BIAcore 3000 BIAevaluation 4.1 software fitted to a bivalent analyte model using blank reference and buffer subtracted data. The bivalent analyte model; A + B ↔ AB1: AB1 + B ↔ AB2, where A is the analyte and B is the ligand, results in two sets of rate constants; association (k_a1/k_a2), dissociation (k_d1/ k_d2) and equilibrium dissociation constant (K_D1/K_D2). K_D1 (AB1) is clearly stronger than K_D2 (AB2) and is therefore the primary interaction considered in this study. The low affinity second interaction (AB2 [K_D2]) is less relevant than AB1, as it can only occur following AB1 formation and is generally thought of as a stabilising interaction supporting the formation of AB1. For each rate constant the average and standard deviation of triplicate experiments are shown. *Chi^2 < 5 for all analysis shown.*
Figure legend:

**Figure 1: Development of recombinant proteins.** (A) Schematic representation of recombinant GSTα fragments 1-9. FXIII-A cross-linking glutamines Q237, Q328, Q366 and the α2 anti-plasmin lysine K303 are shown. The tandem repeat region (αC region 270-373) is represented by the shaded area. (B) Expression of recombinant GSTα fragments 1-9. 4-20% Bis-tris reducing SDS-PAGE. GSTα fragments 1-9 are shown left to right respectively. (C) 16% Tris-tricine SDS-PAGE of α fragment 1-9 (in the absence of the GST tag) shown left to right respectively. (D) 4-12% Bis-tris SDS-PAGE of representative samples used in this investigation. Lane 1: reduced non-activated rFXIII-A, Lane 2: reduced thrombin activated rFXIII-A in the presence of calcium, Lane 3: reduced non-activated rFXIII-A R37A/K513A double thrombin cleavage variant, Lane 4: reduced thrombin treated R37A/K513A variant in the presence of calcium, Lane 5: non-reduced purified FXIII-A2B2 displaying FXIII-A and –B subunit, Lane 6: reduced purified fibrinogen displaying αβγ chains. Dashed grey vertical lines have been inserted to indicate a repositioned gel lane. (E) Biotin labelled pentylamine incorporation FXIII-A activity assay comparing the activity of FXIII-A2B2 (▲) with wild-type rFXIII-A (■) and double thrombin cleavage mutant rFXIII-A R37A/K513A (●) (n=3).

**Figure 2: Localisation of the rFXIII-A binding region on the αC of fibrinogen using SPR.** Utilising a CM5 sensor chip anti-GST antibody capture approach; a serial dilution of activated rFXIII-A (7.8-1000nM) was injected for 60s over captured GSTα fragments 1-9 at a flow rate of 30μl/min. Sensorgram shown are representative of triplicate data and display the double subtracted binding data for GSTα fragment 1 (A) and 9 (B). Response units (RU) are plotted against time (n=3). (C) Competitive inhibition of activated rFXIII-A binding to captured GSTα fragment 1. Thrombin activated rFXIII-A (125nM) was pre-incubated for 5 min at 25°C with increasing molar concentrations (12.5nM, 125nM, 1250nM, 6250nM) of GST-cleaved α fragments 1-7. The mix of rFXIII-A/αFragment was injected for 60s at a flow rate of 30μl/min over captured GSTα fragment 1. The same experiment was performed using α fragments 1, 8 and 9 (D). The binding response (RU) for each sample was converted to percentage binding of activated rFXIII-A to the captured GSTα fragment 1 (Y-axis).
and plotted against the molar concentration of each αC fragment 1-7 (nM) added to the activated rFXIII-A (C) and α fragment 1, 8 and 9 (D), (n=3).

**Figure 3:** The binding of αC fragment 1, 3, and 9 to activated rFXIII-A by SPR in the reverse orientation. Utilising a CM5 sensor chip a serial dilution (0.01-10μM) of GST free α fragment 1(▲), 3 (♦) and 9 (■) was injected for 60s over directly immobilised activated rFXIII-A at a flow rate of 30μl/min. SPR response units (RU) are plotted against the concentration of fragment injected (μM) (n=3).

**Figure 4:** Identification of a key fibrinogen αC amino acid residue involved in binding activated rFXIII-A. (A) Multiple sequence alignment(36) of fibrinogen αC region 371-425 in 7 species to identify conserved residues for site directed mutagenesis within the activated rFXIII-A binding region of α389-403. Amino acid residues highlighted in bold are highly conserved. (B) Displays the binding of each α fragment 9 variant to activated rFXIII-A by SPR. Each α fragment 9 variant (1μM) was injected for 60s over immobilised activated rFXIII-A at a flow rate of 30μl/min. The SPR response at 55s was plotted for each of the variants and compared to wild type (WT) α fragment 9 (n=3).

**Figure 5:** Characterisation of the interaction between rFXIII-A and αC region 233-425 by SPR. (A) Comparison of activated wild-type rFXIII-A (red), with non-activated wild type (Green) and rFXIII-A double thrombin cleavage mutant R37A/K513A (Blue) binding to captured GST α fragment 1. Both wild-type rFXIII-A and R37A/K513A variant were treated with thrombin (5U/ml) and calcium (1.5mM) for 2h at 37°C. 1μM activated wild-type rFXIII-A, R37A/K513A variant and wild-type non-activated rFXIII-A were injected for 60s at a flow rate of 30μl/min over captured GSTα fragment 1. (B) The effect of calcium during rFXIII-A activation on binding to captured GSTα fragment 1. rFXIII-A (1μM), activated with 1.5mM calcium and 5U/ml thrombin (Red), and rFXIII-A (1μM) activated with 5U/ml thrombin in the presence of 5mM EDTA (Blue), were injected for 60s at a flow rate of 30μl/min over captured GSTα fragment 1 for comparison. (C) The effect of 50mM iodoacetamide on rFXIII-A binding to captured GSTα fragment 1. Wild-
type activated rFXIII-A (0.5μM) was pre-incubated with (blue) or without (red) 50mM iodoacetamide for 15 min at 37°C and injected for 60s at a flow rate of 30μl/min over captured GSTα fragment 1 for comparison. Sensorgram’s shown in A, B and C are representative of one experiment from triplicate runs (n=3). The binding response was observed using reference subtracted data. Response units (RU) are plotted against time in seconds.

**Figure 6: The binding of FXIII-A₂B₂ to fibrinogen, fibrin and fibrinogen αC region 371-425 (fragment 9) by SPR.** Increasing concentrations of FXIII-A₂B₂ (0.7-200nM) activated in the presence of 5U/ml biotinylated thrombin and 1.5mM calcium [α₂b₂], or 5U/ml thrombin in the presence of 5mM EDTA [Thr-A₂B₂], or non-activated FXIII-A₂B₂ in the presence of 5mM EDTA [non-activated A₂B₂], were injected over (A) GST α fragment 9, (B) fibrinogen and (C) fibrin for 120s (dissociation time 120s) at a flow rate of 30μl/min. SPR running buffers contained 140mM NaCl, 20mM HEPES, 0.05% P20 and either 1.5mM calcium or 5mM EDTA pH 7.4. Regeneration of binding was achieved using 2 x 50s injections of 2M NaCl for captured GST α fragment 9 binding the FXIII-A₂B₂ activation variants, and 2 x 40s injections of 5mM NaOH containing 100mM NaCl at a flow rate of 30μl/min for interactions between fibrin(ogen) and the FXIII-A₂B₂ activation variants (n=3). The SPR sensorgram’s for the binding of FXIII-A₂B₂ to GSTα fragment 1 were indistinguishable from the binding to GSTα fragment 9 and therefore are not shown. All sensorgram’s shown are representative of one experiment from triplicate runs (n=3).

**Figure 7: Competitive inhibition of FXIII-A₂B₂ binding to GSTα fragment 1 and fibrin using α fragment 9 (α371-425).** PreScission cleaved α fragment 9 was pre-incubated, at increasing molar concentrations (0μM, 0.012μM, 0.125μM, 1.25μM and 12.5μM where 0μM refers to activated FXIII-A₂B₂ in the absence of any α fragment 9 competitor) for 60s with 125nM thrombin activated FXIII-A₂B₂ in the presence of calcium. The sample was injected for 60s at a flow rate of 30μl/min over captured GSTα fragment 1 and immobilised fibrin. The binding response (RU) was converted to percentage binding of FXIII-A₂B₂ to captured GSTα fragment 1 (■) or
immobilised fibrin (♦). Results are normalised to binding in the absence of the competitor (n=3).
Figure 2

A) GSTαF1 (233-425)

B) GSTαF9 (371-425)

C) Percentage binding of rFXIII-a to GSTαF1

D) Percentage binding of rFXIII-a to GSTαF9
Figure 3

Concentration of αC fragment (μM)

SPR Response unit (RU)

- αFragment 1
- αFragment 9
- αFragment 3
Figure 4

A)  

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<tr>
<th>Variant</th>
<th>α371</th>
<th>α389</th>
<th>α403</th>
<th>α425</th>
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B)  

![Graph showing SPR response units (RU) for different αC Fragment 9 variants]
Figure 5

A)  

B)  

C)  

Response units (RU)

Time (s)

RU
Figure 6

A) $a_\beta_2$ binding $\alpha F 9$

Thr-A$_2$B$_2$ binding $\alpha F 9$

Unactivated A$_2$B$_2$ binding $\alpha F 9$

B) $a_\beta_2$ binding fibrinogen

Thr-A$_2$B$_2$ binding fibrinogen

Unactivated A$_2$B$_2$ binding fibrinogen

C) $a_\beta_2$ binding fibrin

Thr-A$_2$B$_2$ binding fibrin

Unactivated A$_2$B$_2$ binding fibrin
Figure 7

Concentration of α fragment 9 (μM)

% of activated FXIII-A,B binding to GSTα fragment 1 and Fibrin

Fibrin
GSTα fragment 1
Interactions between factor XIII and the αC region of fibrinogen

Kerrie A. Smith, Penelope J. Adamson, Richard J. Pease, Jane M. Brown, Anthony J. Balmforth, Paul A. Cordell, Robert A.S. Ariëns, Helen Philippou and Peter J. Grant