Interactions between factor XIII and the αC region of fibrinogen

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Fibrinogen αC residues 242-424 have been shown to have a major regulatory role in the activation of factor XIII-A2B2 (FXIII-A2B2); however, the interactions underpinning this enhancing effect have not been determined. Here, we have characterized the binding of recombinant (r)FXIII-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 (Kd of 2.35 ± 0.09 μM) which was further localized 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 depended on the calcium-induced conformational change known to occur in rFXIII-A during activation. Furthermore, nonactivated FXIII-A2B2, thrombin-cleaved FXIII-A2B2, and activated FXIII-A2B2 each bound fibrin(ogen) and specifically αC region 371-425 with high affinity (Kd < 35nM and Kd < 31nM, respectively), showing 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. (Blood. 2011;117(12):3460-3468)

Introduction

Fibrinogen is a 340 000-Da glycoprotein composed of 2 sets of disulphide linked nonidentical polypeptide chains; αA, βB, and γ.2,3 Thrombin catalyzes the polymerization of fibrinogen to fibrin by sequentially cleaving fibrinopeptide A and fibrinopeptide B (FpB), initiating lateral aggregation of protofibrils and fiber formation.4,5 Factor XIII (FXIII) is a 325.8-kDa heterotetramer (FpB), initiating lateral aggregation of protofibrils and fiber formation.4,5 Factor XIII (FXIII) is a 325.8-kDa heterotetramer composed of 2 identical globular A subunits noncovalently bound by the bond at the N-terminal of the FXIII-A subunit.9 In the presence of calcium, the thrombin-cleaved FXIII-A2B2 complex dissociates, yielding FXIII-B2 and activated FXIII-A.10-12 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-A2 stabilizes the forming protofibril by introducing ε-amino(γ-Glutamyl)Lysine cross-links between carboxyl terminal portions of adjacent fibrin chains, before lateral association of the protofibril.16 Cleavage of FpB and subsequent release of the αC regions from adjacent fibrin chains initiates lateral aggregation of protofibrils and enables activated FXIII-A2 to cross-link adjacent αC, stabilizing the developing fiber and making it more resistant to fibrinolysis.17,18

Interactions between fibrinogen and FXIII-A2B2 are well documented; Greenberg and Shuman19 demonstrated that nonactivated plasma FXIII-A2B2 bound specifically to fibrinogen by the FXIII-A2 subunits with an equilibrium constant (Kd) of 10nM. Greenberg et al20 also noted that binding of FXIII-A2B2 was unaffected by fibrinogen polymerzation, suggesting that the interaction must occur before thrombin cleavage of fibrinopeptide A and FpB. Hornyak and Shafer21 compared activated, nonactivated platelet FXIII-A and nonactivated plasma FXIII-A2B2 for binding to fibrin clots (Kd of 2.1μM, 14μM, and 200mM, respectively). In addition, Hornyak and Shafer examined the effect of fibrin on the activation of platelet FXIII-A, a phenomenon previously described by Credo et al.22,23 Hornyak and Shafer found that fibrin did not promote activation of platelet FXIII-A alone, but that it did enhance FXIII-A2B2 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 αA and βB 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’ copurified with the nonactivated FXIII-A2B2, and that this interaction takes place by the B subunit of FXIII.25

The binding region on fibrin for placentary FXIII-A was first localized by Procyk et al.26 Procyk used several antibodies specific to various regions of fibrinogen [T2G1 (anti-Bβ15-21), 1D4/x1-f (anti-α389-402), 4-2/x1-f (anti-γ392-406), Fd4-7B3 (anti-γ[Fragment D]), 1C2-2 (anti-α3529-539)] to identify the key regions thought to be involved in FXIII-A binding. The results have shown 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-α389-402 antibody and furthermore by cyanogen bromide fragment H12-DSK (αα241-476), suggesting that the location of the FXIII-A binding site was within αC region αα389-402. In


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agreement with Hornyak and Shafer,21 Procyk et al26 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 or FXIII-A2B2 or both would occur within this αC region.

The aim of the current study was to characterize 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 (1) nonactivated recombinant FXIII-A (rFXIII-A), (2) activated rFXIII-A, (3) nonactivated plasma FXIII-A2B2, and (4) thrombin-cleaved FXIII-A2B2 in the presence and absence of calcium by surface plasmon resonance (SPR). 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 (1) its specificity for activated rFXIII-A, (2) that the binding is specific for the calcium-induced conformational change observed during rFXIII-A activation, and (3) 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.

Methods

Development of glutathione-S-transferase αC 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 Escherichia coli with the use of pGEX-6P-1 glutathione-S-transferase (GST) gene fusion system (GE Healthcare). Variants of the αC region 371-425 (Pro389Ala, Asp390Ala, Trp391Ala, Gly392Ala, Phe394Ala, Gly399Ala, Ser402Ala) were produced by introducing site-directed point mutations of highly conserved residues with the use of the QuickChangeII Site Directed Mutagenesis Kit (Stratagene) carried out according to the manufacturers instructions. DNA sequencing confirmed the αC fragments and the mutations. See supplemental Data for further details (available on the Blood Web site; see the Supplemental Materials link at the top of the online article).

Full-length rFXIII-A was amplified by polymerase chain reaction from rFXIII-A construct pGF13A2, a kind gift from Dr C.S. Greenberg, Duke University Medical Center.27 DNA sequencing confirmed the region of interest to be consistent with the FXIII-A subunit. The polymerase chain reaction product was digested with BglII and NotI, 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 with the use of 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 (GSTC fragments 1-9, GSTα fragment 9 variants, GST–FXIII-A and GST–FXIII-A R37A/K513A) were expressed with the use of BL21-Gold E. coli, following the method detailed in the supplemental material. GST fusion proteins were purified by GST-affinity chromatography with the use of an AKTAprime 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 supplemental Data.

Purification of Fibrogammin P FXIII-A2B2 with the use of gel filtration chromatography

Fibrogammin P (CSL Behring) was used as a source of plasma FXIII-A2B2 after isolation from other additives by gel filtration chromatography with the use of a Biocad sprint automated chromatography system (Perseptive Biosystems) as detailed previously by Standeven et al.25 For additional details see supplemental Data.

Purification of fibrinogen

Plasminogen-free fibrinogen from human plasma (Calbiochem) was prepared by ammonium sulfate precipitation to remove bound FXIII as described by Siebenlist et al.24 For additional details see supplemental Data. The FXIII-free fibrinogen was screened by Western blotting and biotin-labeled pentylamine incorporation FXIII-A activity assay to confirm the removal of any contaminating FXIII. The fibrinogen concentration was determined at 280nm with the use of the extinction coefficient 1.51 for a solution of 1 mg/mL.

Biotin-labeled pentylamine incorporation FXIII-A activity assay

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

Activation of rFXIII-A and FXIII-A2B2

Recombinant FXIII-A and FXIII-A2B2 were activated with the use of 5 U/mL human α-thrombin with 1.5 mM calcium for 2 hours at 37°C, unless stated otherwise. The sample was centrifuged at 13 500 rpm for 5 minutes to remove precipitate, and the concentration was determined at 280nm with the use of the extinction coefficient 1.58 for a solution of 1 mg/mL. When removal of thrombin was necessary, biotinylated thrombin (Merck) was used at 5 U/mL for 2 hours at 37°C. Biotinylated thrombin was removed with the use of streptavidin agarose according to the manufacturer’s protocol.

Surface plasmon resonance

In all cases SPR was performed with the use of a Biacore 3000 platform, and data were evaluated with Biacore 3000 BIAevaluation 4.1 software (GE Healthcare).

Binding of rFXIII-A to fibrinogen αC region

With the use of SPR–cal:ium running buffer [20mM HEPES (N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid), 140mM NaCl, 1.5mM CaCl2, 0.05% (vol/vol) surfactant P20, pH 7.4] ~ 2500 response units (RU) of goat anti-GST antibody (GE Healthcare) was immobilized directly onto a CMS sensor chip (flow cell 2) by amine coupling according to the manufacturer’s instructions. A second flow cell (flow cell 1) was immobilized with the same antibody but was used as a blank reference cell. GSTα fragment 1-9 (3.6μM) was injected for 300 seconds at a flow rate of 20 μL/min over flow cell (Fc) 2 to reach a capture level of ~500 RU. rGST (100 μg/mL; Sigma-Aldrich) was injected over Fc1 and Fc2 for 300 seconds at a flow rate of 20 μL/min to block any remaining anti-GST binding sites. PreScission cleaved rFXIII-A was dia lyzed for 16 hours at 4°C into SPR–cal:ium running buffer. Dialyzed rFXIII-A was thrombin activated and centrifuged, and the concentration was determined as described above. A 2-fold serial dilution of activated rFXIII-A (7.8–1000nM) was injected in ascending order for 60 seconds at a flow rate of 30 μL/min over Fc1 and Fc2. Regeneration was achieved with buffer flow after stabilization for 300 seconds. Because of the rapid binding of activated FXIII-A to the αC, it was not possible to perform kinetic analysis. The Ka was therefore obtained with a predefined steady state affinity model with the use of a blank reference (Fc2-1, Fc4-3) and buffer subtracted data. This interaction was also performed in the reverse orientation to confirm the Ka. With the use of SPR–cal:ium running buffer 5000 RU of activated rFXIII-A was directly immobilized onto a CMS sensor chip as detailed above. αC fragments 1, 3, and 9 (cleaved from the GST tag) were dialyzed into SPR–cal:ium running buffer. A 2-fold serial dilution of 0.01-10μM αC fragment was injected in ascending order for 60 seconds at a flow rate of

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30 µL/min over the immobilized activated rFXIII-A. Regeneration was achieved with buffer flow after stabilization for 300 seconds (n = 3).

**Binding of α fragment 9 variants to activated rFXIII-A.** αC fragment 9 variants were cleaved from the GST tag and dialed into SPR-calcium running buffer. A fixed concentration of 1µM was injected over immobilized activated rFXIII-A for 60 seconds at a flow rate of 30 µL/min. The binding response of each variant at 55 seconds, 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. Nonactivated 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 dialyzed for 16 hours at 4°C into SPR-EDTA (ethylendiaminetetraacetic acid) buffer (20mM HEPES, 140mM NaCl, 5mM EDTA, 0.05% (vol/vol) surfactant P20, pH 7.4). A third aliquot was dialyzed into SPR-calcium buffer pH 7.4. The FXIII-A2B2 dialyzed into SPR-calcium buffer, and 1 of 2 FXIII-A2B2 aliquots dialyzed into SPR-EDTA buffer were thrombin cleaved as described above. A 2-fold serial dilution (0.7-200nM) of nonactivated FXIII-A2B2 or thrombin-cleaved FXIII-A2B2 were injected for 60 seconds over the captured GSTα fragment 1 or GSTα fragment 9 at a flow rate of 30 µL/min. Removal of bound FXIII-A2B2 was achieved with the use of two 50-second pulses of 2M NaCl at a flow rate of 30 µL/min. The surface was reequilibrated in running buffer for 300 seconds.

**Binding of FXIII-A2B2 to fibrinogen and fibrin.** Purified fibrinogen (100 RU) was immobilized directly onto a CM5 sensor chip (Fc2) by amine coupling according to the manufacturer’s instructions. A blank reference surface (Fc1) was activated and deactivated as described above. The immobilized fibrinogen was converted to fibrin as previously described.30 Purified FXIII-A2B2 was dialyzed at 4°C for 16 hours 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 immobilized fibrin at a flow rate of 30 µL/min for 120 seconds. Removal of bound FXIII-A2B2 was achieved with the use of two 40-second pulses of 5mM NaOH containing 100mM NaCl at a flow rate of 30 µL/min. The surface was reequilibrated in running buffer for 300 seconds. Similar method was applied for analyses of the interactions between nonactivated 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 used for the cleavage of FXIII by thrombin. Nonactivated or thrombin-only activated FXIII-A2B2 was never exposed to calcium alone. The Kd obtained for this interaction is an estimate (0.7-200nM) where 0µM refers to activated FXIII-A2B2 in the absence of α fragment 9 competitor) for 60 seconds with 125nM thrombin-activated FXIII-A2B2 in the presence of calcium. The sample was injected for 60 seconds at a flow rate of 30 µL/min over captured GSTα fragment 1 and immobilized fibrin. The binding response in response units was converted to percentage binding of FXIII-A2B2 to the surface; captured GSTα fragment 1 or immobilized fibrin was used for comparison (n = 3).

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

Nonactivated rFXIII-A was dialyzed for 16 hours at 4°C into SPR-calcium running buffer, and a separate rFXIII-A aliquot was dialyzed into SPR-EDTA buffer. Both rFXIII-A aliquots were thrombin activated, and binding analysis was performed with the use of 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 FXIII-A activated with thrombin only in the presence of EDTA. The rFXIII-A samples were injected for 60 seconds over the captured GSTα fragment 1 at a flow rate of 30 µL/min.

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

rFXIII-A was dialyzed for 16 hours at 4°C into SPR-calcium running buffer. The rFXIII-A was thrombin activated and incubated with 50nM iodoacetamide (Sigma-Aldrich) for 15 minutes at 37°C. An aliquot of activated rFXIII-A in the absence of iodoacetamide was used for comparison. Before performing the binding study, 50nM iodoacetamide was injected for 60 seconds at a flow rate of 30 µL/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 50nM iodoacetamide was injected at a flow rate of 30 µL/min for 60 seconds 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 visualized with the use of sodium dodecyl sulfate–polyacrylamide gel electrophoresis (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 showed a decrease in molecular mass by ~ 4 kDa, resulting from the N-terminal cleavage of the activation peptide by thrombin (Figure 1D lanes 1-2). This effect was not observed in the rFXIII-A R37A/K513A variant (Figure 1D lanes 3-4) as predicted because R37 and K513 have been identified as thrombin cleavage sites.33 The FXIII-A pentamely incorporation assay indicated that the wild-type rFXIII-A and FXIII-A2B2 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 3-7 (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 $2.35 \pm 0.09 \mu M$, whereas GSTα fragment 2 (α233-403) bound with a $K_d$ of $2.64 \pm 1.21 \mu M$. Competitive inhibition studies that used αC fragments 1 and 2 confirmed 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 with the use of α fragments 3-7, 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.

Table 1. Steady state affinity data for the binding of activated rFXIII-A to captured GSTα fragment 1-9 by SPR

<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>
</tr>
</thead>
<tbody>
<tr>
<td>GSTα fragment 1 (233-425)</td>
<td>50.0</td>
<td>20.02</td>
<td>2.35 ± 0.09</td>
</tr>
<tr>
<td>GSTα fragment 2 (233-403)</td>
<td>47.4</td>
<td>17.43</td>
<td>2.64 ± 1.21</td>
</tr>
<tr>
<td>GSTα fragment 3 (233-388)</td>
<td>45.8</td>
<td>15.83</td>
<td>*</td>
</tr>
<tr>
<td>GSTα fragment 4 (233-375)</td>
<td>44.6</td>
<td>14.57</td>
<td>*</td>
</tr>
<tr>
<td>GSTα fragment 5 (233-341)</td>
<td>41.2</td>
<td>11.18</td>
<td>*</td>
</tr>
<tr>
<td>GSTα fragment 6 (233-290)</td>
<td>36.4</td>
<td>6.41</td>
<td>*</td>
</tr>
<tr>
<td>GSTα fragment 7 (233-266)</td>
<td>34.1</td>
<td>4.1</td>
<td>*</td>
</tr>
<tr>
<td>GSTα fragment 8 (289-425)</td>
<td>43.7</td>
<td>13.7</td>
<td>1.81 ± 0.45</td>
</tr>
<tr>
<td>GSTα fragment 9 (371-425)</td>
<td>35.7</td>
<td>5.7</td>
<td>3.19 ± 0.57</td>
</tr>
</tbody>
</table>

*Negligible binding was observed for GSTα fragments 3, 4, 5, 6, and 7, which was too low to undertake formal analysis. (n = 3).
Identification of a key amino acid residue involved in binding activated rFXIII-A

If the interaction between activated FXIII-A and the αC 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 αC region showed that a glutamic acid residue at position 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 it is binding to an assembled determinant that also involves residues located between 371 and 389.

Nonactivated rFXIII-A does not bind fibrinogen αC region 233-425

SPR binding analysis showed that wild-type nonactivated rFXIII-A and the inactive variant R37A/K513A did not bind to the captured GSTα fragment 1 (Figure 5A). Furthermore, it was observed that the αC region 233-425 does not bind to rFXIII-A activated with thrombin only but also required the presence of calcium that induces a conformational change that exposes portions of FXIII-A that are not structurally exposed in the nonactivated form13-15 (Figure 5B). Binding did not depend on catalytic activity because blocking the rFXIII-A active site Cys314 with iodoacetamide did not inhibit binding to the αC region 233-425, which occurred to the same degree as activated rFXIII-A in the absence of iodoacetamide (Figure 5C).

FXIII-A2B2 binds with high affinity to fibrin(ogen) αC region 371-425

SPR was used to determine whether FXIII-A2B2 bound to GSTα fragment 1 (α233-425) and GSTα fragment 9 (α371-425). With the use of a bivalent analyte model the SPR results showed that GSTα fragment 1 (α233-425) bound to nonactivated FXIII-A2B2 (Kd1 = 7.3 ± 6.3nM), thrombin-cleaved FXIII-A2B2 (no calcium; Kd1 = 3.7 ± 0.3nM), and thrombin-cleaved FXIII-A2B2 activated in the presence of calcium (Kd1 = 21.9 ± 2.2nM; Table 2). In addition, the binding of the different activation states of FXIII-A2B2 were further localized to fibrinogen αC 371-425 [nonactivated FXIII-A2B2: Kd1 = 30.9 ± 23nM; thrombin-cleaved FXIII-A2B2 (no calcium): Kd1 = 5.4 ± 1.8nM; thrombin-cleaved FXIII-A2B2 activated in the presence of calcium: Kd1 = 7.3 ± 1.8nM] (Figure 6A; Table 2). The SPR sensorgrams for the binding of FXIII-A2B2 to GSTα fragment 1 were identical to the those displayed for the binding of FXIII-A2B2 to GSTα fragment 9 and therefore are not shown.

The various activation states of FXIII-A2B2 were also tested for binding to full-length fibrinogen and fibrin. The results showed that both fibrinogen and fibrin bound to nonactivated FXIII-A2B2 with a Kd1 of 11 ± 4.7nM and 35.2 ± 4nM, respectively, thrombin-cleaved FXIII-A2B2 (no calcium) with a Kd1 of 2.9 ± 2.1nM and 6.1 ± 1nM, and thrombin-cleaved FXIII-A2B2 activated in the presence of calcium with a Kd1 of 10.7 ± 0.9nM and 14.5 ± 3.1nM (Figure 6B-C; Table 2).

α Fragment 9 (α371-425), in the absence of the GST tag, was used as a competitive inhibitor to confirm the specificity of the interaction. The results showed that α fragment 9 inhibited the binding of thrombin-cleaved FXIII-A2B2 (activated in the presence of calcium) to the SPR-captured GSTα fragment 1 and full-length fibrin (Figure 7). Furthermore, these results confirm that the interaction between the α fragment 9 and activated FXIII-A2B2 occurs in solution in addition to binding to an immobilized surface.
Discussion

Previous studies have shown the importance of the αC region for FXIII activation. The aim of this study was to characterize the interactions between FXIII-A and FXIII-A2B2 with the fibrinogen αC region 233-425 that could contribute to this process. Using recombinant truncations of the fibrinogen αC region 233-425, we have identified a novel high-affinity interaction between FXIII-A2B2 and fibrinogen αC region 371-425 which was evident both in the zymogen and after activation with thrombin and calcium. In addition, we have confirmed a previously described low-affinity interaction between activated FXIII-A and the αC region of fibrinogen, but we have extended the analysis to confirm the domain involved and to implicate a key residue, Glu396. The low-affinity interaction was dependent on calcium, which induces a conformational change in the β-barrel 1 and the β-sandwich domains of thrombin-cleaved FXIII-A. In addition, 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-A2 to fibrinogen was reported by Procyk et al. with the use of antibodies specific for the αC region 389-402 and with the cyanogen bromide fragment Hi2-DSK (Ac241-476), suggesting that the location of the FXIII-A binding site was within αC region Ac389-402. 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 imply that additional residues upstream (α371-389) may contain secondary stabilizing sites that cannot function in the absence of Glu396, because 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 the tetrameric nature of the SPR analyte FXIII-A2B2, together with the dimeric structure of the ligand fibrin(ogen), which is thought to adopt a conformation similar to that of soluble fibrinogen when immobilized, has the potential to result in a complex multivalent interaction between FXIII-A and fibrinogen.

Figure 4. Identification of a key fibrinogen αC amino acid residue involved in binding activated rFXIII-A. (A) Multiple sequence alignment 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 αC389-403. Amino acid residues highlighted in bold are strongly 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 60 seconds over immobilized activated rFXIII-A at a flow rate of 30 μL/min. The SPR response at 55 seconds was plotted for each of the variants and compared with wild-type (WT) α fragment 9 (n = 3). Error bars show ± 1 SD.

Figure 5. Characterization of the interaction between rFXIII-A and αC region 233-425 by SPR. (A) Comparison of activated wild-type rFXIII-A (red), with nonactivated wild-type (green), and rFXIII-A double thrombin cleavage mutant R57AK513A (blue) binding to captured GSTα fragment 1. Both wild-type rFXIII-A and R377A/K513A variant were treated with thrombin (5 U/mL) and calcium (1.5mM) for 2 hours at 37°C. Activated wild-type rFXIII-A, R377A/K513A variant and wild-type nonactivated rFXIII-A (1μM) were injected for 60 seconds at a flow rate of 30 μL/min over captured GSTα fragment 1. (B) The effect of calcium during rFXIII-A activation on binding to captured GSTα fragment 1. Wild-type activated rFXIII-A (0.5μM) was preincubated with (blue) or without (red) 5mM EDTA at 37°C and injected for 60 seconds at a flow rate of 30 μL/min over captured GSTα fragment 1 for comparison. Sensograms shown in panels A, B, and C are representative of one experiment from triplicate runs (n = 3). The binding response was observed with reference subtracted data. Response units are plotted against time in seconds.
interaction. However, SPR data are consistent with $K_d1$ 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 nonactivated plasma FXIII-A2B2 to full-length fibrinogen, allowing the nonactivated FXIII to be present for cross-linking activity when required. Interestingly, in our investigation all forms of FXIII bound similarly to the αC region, fibrin(ogen) have also been shown by immunoblotting,24 clot binding assays,23 and enzyme-linked immunosorbent assay.20,33

Greenberg et al have suggested that FXIII may circulate in plasma bound to the fibrinogen molecule, allowing the nonactivated FXIII to be present for cross-linking activity when required.\(^\text{19}\) Interestingly, in our investigation all forms of FXIII bound similarly to the αC region,


References


Figure 7. Competitive inhibition of FXIII-A2B2 binding to GSTα fragment 1 and fibrin with the use of α fragment 9 (α371-425). PreScission-cleaved α fragment 9 was preincubated, at increasing molar concentrations (0 μM, 0.0125 μM, 0.125 μM, 1.25 μM, and 12.5 μM) with α fragment 9 competitor for 60 seconds with 125 mM thrombin-activated FXIII-A2B2 in the presence of calcium. The sample was injected for 60 seconds at a flow rate of 30 μL/min over captured GSTα fragment 1 and immobilized fibrin. The binding response (RU) was converted to the percentage of binding of FXIII-A2B2 to captured GSTα fragment 1 (*) or immobilized fibrin (●). Results are normalized to binding in the absence of the competitor (n = 3).

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