HHS Public Access

Author manuscript

Thromb Haemost. Author manuscript; available in PMC 2019 December 01.

Published in final edited form as:

Thromb Haemost. 2018 December ; 118(12): 2037–2045. doi:10.1055/s-0038-1675600.

Proline 36 of the Factor XIII Activation Peptide Plays a Crucial Role in Substrate Recognition and Zymogen Activation

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Abstract

The activation peptide of blood coagulation factor XIII (AP-FXIII) has important functions in stabilising the FXIII-A_2 dimer and regulating FXIII activation. Contributions of many of its 37 amino acids to these functions have been described. However, the role of proline 36, which is adjacent to the thrombin cleavage site at Arg37, has not yet been studied in detail. We approached this question when we came across a patient with congenital FXIII deficiency in whom we detected a novel Pro36Ser mutation. We expressed the mutant FXIII-A Pro36Ser protein in CHO cells and found that this mutation does not influence FXIII-A expression but significantly inhibits proteolytic activation by thrombin. The enzymatic transglutaminase activity is not affected as it can be induced in the presence of high Ca^{2+} concentrations. We performed Nuclear Magnetic Resonance (NMR) analysis to investigate AP-FXIII-thrombin interactions, which showed that the mutant Ser36 peptide binds less well to the thrombin surface than the native Pro36 peptide. The Arg37 at the P_1 position still makes strong interactions with the active site cleft but the P_4-P_2 residues (^{34}VVS^{36}) appear to be less well positioned to contact the neighbouring thrombin active site region. In conclusion, we have characterised a novel mutation in AP-FXIII representing only the fourth case of the rare FXIII-A type II deficiency. This case served as a perfect *in vivo* model to shed light on the crucial role of Pro36 in the proteolytic activation of FXIII-A. Our results contribute to the understanding of structure-function relationship in FXIII.

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Authors' contributions

BL, RB, and MCM performed the experiments; BL, RB, MCM, and VS analysed the results and prepared the figures; PRM and LA characterised the patient; HPK and VS designed the research; BL, RB, MCM, and VS wrote the paper; all authors revised the manuscript.

Conflict of Interest

None

Keywords

Coagulation factor XIII; factor XIII activation peptide; congenital factor XIII deficiency

Introduction

Blood coagulation factor XIII (FXIII) circulates in plasma as a heterotetramer (FXIII A_2B_2) that consists of two catalytic A subunits (FXIII-A) and two carrier/regulatory B subunits (FXIII-B). Its main function is to cross-link fibrin polymers and protect the fibrin clot from premature fibrinolytic degradation.[1]

Plasma FXIII is activated at the last stage of coagulation by thrombin and Ca^{2+}. During activation of plasma FXIII A_2B_2, thrombin first catalyses the cleavage of the FXIII activation peptide (AP-FXIII) from the N-terminus of the FXIII-A subunit by hydrolysing the Arg37-Gly38 peptide bond. AP-FXIII dissociates from the truncated molecule and appears in serum.[2] The proteolytic cleavage of FXIII-A by thrombin considerably weakens the interaction between A and B subunits. Then, in the presence of Ca^{2+}, the FXIII-B subunits dissociate of from the cleaved FXIII-A followed by conformational change of FXIII-A into an enzymatically active conformation.[3] In addition to the typical proteolytic activation pathway, plasma FXIII can also be activated by high Ca^{2+} concentrations (> 50 mM) without prior proteolytic cleavage.[4] It has been reported that the binding of Ca^{2+} ions to the high affinity Ca^{2+} binding sites on the A subunits was sufficient to dissociate the subunits and activate FXIII independently of thrombin.[5] Cellular FXIII (cFXIII) is present in platelets and monocytes/macrophages as A-subunit dimer (FXIII-A_2). Because cFXIII does not contain FXIII-B, low Ca^{2+} concentrations (2 mM) are sufficient to induce a slow progressive non-proteolytic activation of cFXIII *in vitro*[6], and this non-proteolytic activation pathway seems to be the physiological activation pathway in platelets.[7]

The AP-FXIII is a special feature among transglutaminase enzymes. It regulates FXIII activation by blocking its active site cavity.[8] In addition, we have shown earlier that AP-FXIII has also a crucial role in stabilising the FXIII-A dimer.[9] For proteolytic cleavage of AP-FXIII during FXIII activation, the FXIII-A cleavage site at Arg37-Gly38 (corresponding to the P_1-P_1' residues) must be recognised by thrombin. (The P nomenclature system is used to assign the individual amino acid positions on the substrate peptides. The scissile bond is designated by P_1-P_1'. The substrate amino acids N-terminal of the hydrolysis site are labeled P_2, P_3, P_4, etc. whereas those that are C-terminal are labeled P_2', P_3', P_4', etc.) A consensus recognition sequence of thrombin has been identified with a strong preference for arginine at position P_1 and for proline at position P_2.[10] Proline at position P_2 is also conserved across FXIII homologues including Pro36 in human FXIII-A.[9] Several amino acids of AP-FXIII near the thrombin cleavage site have been mutated to study their effects on substrate recognition by thrombin and FXIII activation. However, the role of Pro36 has never been specifically investigated, despite of the fact that proline with its unique side-chain has significant effects on polypeptide chain conformation and plays a special role in protein structure and sometimes even protein function[11].

Here we studied the role of Pro36 in FXIII activation by means of a novel Pro36Ser mutation we have identified in a patient with congenital heterozygous FXIII deficiency. We have expressed the mutant FXIII-A Ser36 protein and investigated its activation both via the proteolytic and non-proteolytic pathway. We have performed NMR analyses to study the interactions of Pro36 and Ser36 peptides with thrombin. Our results attribute a crucial role to Pro36 in substrate recognition and FXIII activation.

Materials and Methods

Visualisation of Pro36 in the molecular context

Structural data of the FXIII-A_2 dimer[12] and the AP-FXIII (28–37) in complex with thrombin[13] were obtained from the protein data bank (PDB-ID 1F13 and 1DE7; www.rcsb.org). Visualisation of Pro36 within the molecular context and preparation of the figures was done with DeepView/Swiss-Pdb Viewer v.4.1.0 (www.expasy.org/spdbv/).[14]

Determination of plasma FXIII-A antigen levels and FXIII activity

Informed consent for FXIII analysis including FXIII genetic analysis was obtained from a patient with suspected FXIII deficiency who was initially seen at the Division of Haematology, Cantonal Hospital of Lucerne, Switzerland. Plasma FXIII-A antigen levels were measured by ELISA as described earlier[15]. Plasma FXIII activity was measured with the Berichrom Factor XIII Chromogen assay (Siemens Healthcare, Erlangen, Germany) and with a fluorescent FXIII assay (Zedira, Darmstadt, Germany).

DNA isolation, amplification and sequencing

Genomic DNA of the patient was isolated from EDTA-anticoagulated whole blood using the QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany). According to our protocol we developed earlier[16], all exons of the F13A gene (including intron/exon boundaries) were individually amplified by polymerase chain reaction (PCR), followed by agarose gel electrophoresis, and purification of PCR products using the PureLink™ Quick Gel Extraction & PCR Purification Combo Kit (Invitrogen, Thermo Fisher Scientific, Waltham, MA). The purified PCR products were sequenced by Microsynth (Balgach, Switzerland) with the same primers as used for PCR. Sequence analysis was performed with NCBI Nucleotide BLAST. For the nomenclature of the mutation, we start numbering of the nucleotides with the ATG start codon, and the numbering of the amino acids with serine, the N-terminal amino acid in the mature FXIII-A protein.

Wild-type and mutant *F13A* expression plasmids

We used the pcDNA5/FRT expression plasmid containing the human wild-type *F13A* cDNA sequence (pcDNA5/FRT-WT-F13A) we had cloned earlier[9]. The mutant *F13A* expression plasmid with a mutation in exon 2 at codon 36 (c.109C>T, Pro36Ser) was constructed by site-directed mutagenesis of the wild-type expression plasmid (pcDNA5/FRT-WT-F13A) using the GeneArt® Site-Directed Mutagenesis PLUS Kit (Invitrogen). The mutagenic primers were designed using the web-based GeneArt® Primer and Construct Design Tool (http://www.thermofisher.com/order/oligoDesigner). The following primers were synthesised by Microsynth: forward primer 5’-

CTTCAGGGCGTGGTGTCCCGGGGCGTCAACC-3', and reverse primer 5'-GGTTGACGCCCCGGGACACCACGCCCTGAAG-3'. The mutagenesis reaction was performed according to the manufacturer's instruction using One Shot® MAX Efficiency® DH5α™-T1R competent cells (Invitrogen). Plasmids were purified using the PureLink™ HiPure Plasmid Midiprep Kit (Invitrogen) and then sequenced by Microsynth using their standard CMV-forward and BGH-reverse primers. The plasmids with correct wild-type (pcDNA5/FRT-WT-F13A) and mutant (pcDNA5/FRT-MU-F13A) sequences were used for transfection.

Stable expression of wild-type and mutant FXIII-A proteins

Stable expression of wild-type (Pro36) and mutant (Ser36) FXIII-A was achieved (as described earlier[9]) by co-transfection of Flp-In™ Chinese hamster ovary (CHO) cells (Invitrogen) with either expression plasmid (pcDNA5/FRT-WT-F13A or pcDNA5/FRT-MU-F13A) and the pOG44 plasmid encoding a specific recombinase, followed by selection with 600 μg mL^{-1} hygromycin (Invitrogen) over two weeks. After culture for three weeks, cells were lysed.

Analysis of wild-type and mutant FXIII-A proteins

Lysates were used for qualitative analysis of FXIII-A proteins by Western blot and quantitative analysis by ELISA, as described earlier[9].

Briefly, for Western blot, cells were lysed directly in 1X Bio-Rad SDS–Laemmli sample buffer (about 200 μl/10^6 cells). Proteins were separated by electrophoresis on Bolt™ 8% Bis-Tris Plus Gels (Invitrogen) with 1X Bolt™ MES SDS Running Buffer (Invitrogen), and transferred onto Immun-Blot® PVDF Membrane (Bio-Rad, Hercules, CA) with 1X Bolt™ Transfer Buffer (Invitrogen). The membrane was incubated with a primary monoclonal anti-FXIII-A antibody (ab1834; Abcam, Cambridge, UK), followed by a horseradish peroxidase-conjugated goat anti-mouse secondary antibody (Thermo Fisher Scientific), and developed with the WesternBright Quantum HRP chemiluminescent substrate (Advansta, Menlo Park, CA).

For ELISA, cells were lysed in a non-denaturing buffer (140 mM NaCl, 10 mM HEPES, pH 7.4, 1% Triton X-100, containing a protease inhibitor cocktail without EDTA (Pierce, Thermo Fisher Scientific)). Lysates were frozen on dry ice and kept at −80°C until analysis. The ELISA was based on the protocol by Ariëns et al.[15]. Lysates were diluted 1:50 in Tris-buffered saline (TBS) (140 mM Tris, 40 mM NaCl, pH 7.4) with 0.1% bovine serum albumin (BSA). The standard curve was obtained with serial dilutions of recombinant FXIII-A_2 (Zedira). For comparison between different samples, the total protein was measured using Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific) according to the manufacturer's protocol.

FXIII-A activity assay

FXIII transglutaminase activity of wild-type (Pro36) and mutant (Ser36) FXIII-A was measured with a biotin incorporation assay[17] with the following modifications: Cell lysates were diluted in TBS in order to load 12 ng wild-type or mutant FXIII-A protein per well. In

the activation/reaction mix, varying concentrations of thrombin (1, 10, 20 U mL^{-1}) and/or Ca^{2+} (10 or 50 mM) were used and the reactions were run for up to 60 min. FXIII activity was expressed relative to the first time point. Cell lysates from untransfected cells were used as control.

Synthetic peptides and thrombin for NMR

Synthetic peptides based on the human AP-FXIII were employed. Wild-type AP-FXIII (28–37) Pro36 [Ac-TVELQGVVPR-OH] and mutant AP-FXIII (28–37) Ser36 [Ac-TVELQGVVSR-OH] were custom synthesised by SynPep (Dublin, CA) and New England Peptide (Gardner, MA), respectively. Initial stock solutions of peptide were solubilised in deionised water, and the concentrations were determined by amino acid analysis (AAA Service Laboratory, Inc., Damascus, OR). Peptide substocks were later prepared into 25 mM H_3PO_4, 150 mM NaCl, 0.2 mM EDTA and at pH 5.6.

Bovine plasma thrombin was purchased from Haematologic Technologies, Inc (Essex Junction, VT). For the NMR experiments, thrombin was buffer exchanged into NMR buffer (25 mM H_3PO_4, 150 mM NaCl, 0.2 mM EDTA, pH 5.6) using a Vivaspin 2 ultrafiltration unit with a 5000 Da molecular weight cutoff (Sartorius, Göttingen, Germany). Protein concentrations were determined using an extinction coefficient $E^{1\%}_{280\ nm}$ of 18.3. In this research, bovine thrombin was used as the enzyme and the peptides sequences were derived from human sequences. Thrombin exhibits high sequence conservation between human and bovine forms.[18] No amino acid differences exist in the active site region, the thrombin β-insertion loop, or the allosteric sodium binding site. Any minor differences that exist between these two thrombin species are not anticipated to interfere with the binding of substrate peptides to the active site region.[19]

NMR sample preparation

AP-FXIII - thrombin complexes with ratios of at least 10:1 were prepared. Complexes included 1.5 mM AP-FXIII (28–37) Ser36 with 91 μM thrombin and then 1.2 mM AP-FXIII (28–37) Pro36 with 122 μM thrombin. At least 1 mM of free peptide was used as a control for each ligand-protein complex. For all samples, the NMR buffer (25 mM H_3PO_4, 150 mM NaCl, 0.2 mM EDTA, pH 5.6) also contained 10% D_2O. The proton chemical shift values for all the AP-FXIII amino acid residues were determined using a combination of standard 2D-TOCSY and 2D-transferred NOESY NMR methods.

NMR analysis of the peptide-thrombin complexes

The 1D ^1H line broadening experiments were carried out on a Varian Inova 700 MHz with a triple resonance cold probe equipped with pulsed field z-axis gradients. The NMR parameters included 128 transients, a sweep width of 8503.4 Hz, and 4096 complex points. Water was suppressed with the WET pulse sequence. The ^1H NMR results were processed using Mnova NMR (Mestrelab Research software). The spectra of free peptides were compared to their respective peptide-thrombin complexes.

For ligand-protein complexes with ratios of at least 10:1, any peptide resonances that come in direct contact with the enzyme surface and exchange rapidly on/off will exhibit line

broadening. Atoms of peptides that make this contact experience changes in transverse relaxation time and/or chemical shift position. The resultant changes in line width/shape reflect weighted contributions of bound structural information transferred to the free population. This NMR line broadening approach can help map the peptide residue protons that come in direct contact with the protein surface.[20,21]

Results

The thrombin substrate recognition and cleavage site on the FXIII-A molecule lies within an unstructured loop that protrudes from the molecule to enable thrombin to access it (Fig. 1, panels A and B). Being highly flexible, part of this loop is not resolved in the crystal structure. However, since proline exhibits conformational rigidity due to its cyclic pyrrolidine side-chain, we assumed that Pro36 has a crucial function in introducing some stability into the loop and holding it in place for thrombin to bind. Furthermore, Pro36 interacts with several thrombin residues via hydrogen bonds and van der Waals interactions[13] (Fig. 1, panels C and D). When we came across a patient with FXIII deficiency carrying a mutation at this very position, this case served as a perfect *in vivo* model for us to test our hypothesis on the role of Pro36 in the AP-FXIII.

Pro36Ser leads to a rare FXIII-A type II deficiency

The 35 year old female patient with a life-long history of numerous bleeding complications (including epistaxis, bruising, prolonged bleeding after minor injuries, delayed wound healing, bleeding from the gums, hypermenorrhea, severe bleeding after pregnancy loss, severe bleeding postpartum, severe bleeding after hysterectomy), some requiring blood transfusions and surgical interventions, showed repeatedly normal coagulation and platelet function parameters. Only the specific functional FXIII analysis revealed abnormal FXIII activity levels of 44% on average (45% and 49% measured on two occasions with the Berichrom FXIII assay, and 39% measured with the fluorescent FXIII assay). A mixing test (measuring FXIII activity after mixing the patient's plasma with normal control plasma)[22] gave no evidence of a FXIII inhibitor. At the same time, we found normal FXIII-A antigen levels of 120% measured by ELISA. These results indicated a heterozygous FXIII-A type II deficiency[22], an extremely rare case of a dysfunctional protein. Sequence analysis of the *F13A* gene revealed a heterozygous c.109C>T mutation in exon 2 (Fig. 2), changing the codon from CCC to TCC which encodes for a proline to serine exchange of amino acid 36 (Pro36Ser) in the activation peptide of the mature FXIII-A protein.

Mutant FXIII-A (Pro36Ser) is expressed normally *in vitro*

In order to confirm that the Pro36Ser mutation gives rise to a dysfunctional protein, we expressed wild-type (Pro36) and mutant (Ser36) FXIII-A *in vitro* after developing stable isogenic cell lines. As shown in Fig. 3, FXIII-A was expressed in both FXIII-A Pro36 and FXIII-A Ser36 transfected CHO cells, but not in non-transfected CHO cells.

For quantification of the FXIII-A expression, we performed a FXIII-A ELISA. Consistent with the qualitative Western blot results, FXIII-A was detected in both FXIII-A Pro36 and FXIII-A Ser36 transfected CHO cells, but not in non-transfected CHO cells. We normalised

the absolute amounts of FXIII-A expressed against the total protein concentration of each cell lysate. The amount of mutant FXIII-A Ser36 was 0.71±0.07 μg/100μg total protein, while the amount of wild-type FXIII-A Pro36 was 0.95±0.09 μg/100μg total protein (mean ±SD, n=3). The expression of mutant FXIII-A was slightly lower than wild-type FXIII-A, but the difference was not significant.

Proteolytic and non-proteolytic activation and transglutaminase activity of FXIII variants

Since both FXIII-A variants were expressed to a similar extent, the next step was to investigate their activation and transglutaminase activity. For this purpose we performed a FXIII activity assay under various conditions that allowed us to distinguish between possible defects in FXIII activation and/or FXIII transglutaminase activity. Normal proteolytic activation of FXIII occurs at 10 mM Ca^{2+} in the presence of thrombin, whereas non-proteolytic activation occurring without thrombin can be achieved at 50 mM Ca^{2+}. This non-proteolytic activation can be used to prove that transglutaminase activity is exhibited even when the protein cannot be activated proteolytically by thrombin. Since a proteolytic reaction can be a question of concentration, we also performed the assay with increasing thrombin concentrations to exclude the possibility that activation of the FXIII mutant is less sensitive than the wild-type and can be achieved at higher thrombin concentrations. As shown in Fig. 4, FXIII activity assays were performed at standard (10 mM) and high (50 mM) Ca^{2+} concentrations with and without thrombin, and with increasing thrombin concentrations. The quantitative data of relative FXIII activity and statistical comparison between FXIII-A Pro36 wild-type and Ser36 mutant are provided in the supplementary material. At standard Ca^{2+} concentration (Fig. 4A), wild-type FXIII-A Pro36 exhibited rapid and high transglutaminase activity developing upon thrombin activation, while the mutant FXIII-A Ser36 only showed a moderate increase in transglutaminase activity after 40 min. Similarly, in the absence of thrombin, both FXIII-A variants only displayed a slow and moderate increase in transglutaminase activity over time. On the contrary, at high Ca^{2+} concentration (Fig. 4B), there was no difference between the FXIII-A Pro36 and Ser36 variants, both exhibited a rapid increase in transglutaminase activity resulting in a similarly high activity level, independent of the presence or absence of thrombin. Even when thrombin concentrations were further increased up to 20-fold (Fig. 4C), no significant transglutaminase activity was induced in the mutant FXIII-A Ser36 variant. Cell lysates from untransfected cells did not show any transglutaminase activity (data not shown). Our results demonstrated that the Pro36Ser mutation prevents proteolytic FXIII-A activation by thrombin. At the same time, the mutant FXIII-A Ser36 protein can be activated in a non-proteolytic way at a high Ca^{2+} concentration and then displays normal FXIII activity. In order to prove that the proteolytic cleavage of the Ser36 mutant by thrombin is abolished, we assessed the cleavage and release of the activation peptide by gel electrophoresis and AP-FXIII ELISA. These experiments and their confirmatory results are provided in the supplementary material.

NMR analysis of AP-FXIII - thrombin interactions

Next, we aimed at investigating the interactions between thrombin and FXIII-A wild-type Pro36 and mutant Ser36 peptides by NMR. One dimensional proton spectra were acquired for the AP-FXIII sequences free in solution and complexed with thrombin. Proton (^1H) peak

broadening could be detected for both AP-FXIII (28–37) Pro36 and AP-FXIII (28–37) Ser36 in the presence of thrombin. Spectral regions that highlight the peptide backbone amide protons (NH) (7.8 – 8.4 ppm), the side chain amide protons of glutamine (7.4 and 6.7 ppm), and the side chain εNH of arginine (7.05 ppm) are shown in Fig. 5.

For the AP-FXIII (28–37) Ser36 sequence, proton line broadening could be observed for both the amide backbone NH and the side chain εNH of Arg37 (Fig. 5A, B). Moderate line broadening could be observed for the backbone amide protons of Val34 and Val35. Little if any line broadening was detected for the amide proton of Ser36. The results obtained with the AP-FXIII Ser36 sequence were compared with those for the wild-type AP-FXIII sequence containing a stabilising Pro36 residue. For this AP-FXIII sequence, substantial line broadening was observed for the backbone amide protons of Gly33 through Arg37 (Fig. 5C, D). Once again extensive line broadening was observed for the side chain εNH of Arg37. Moderate broadening effects were detected for the proline Cβ-proton (data not shown). For both the mutant AP-FXIII Ser36 and the wild-type AP-FXIII Pro36 sequences, no line broadening was visible for the Thr28 to Glu30 region indicating that this N-terminal portion of the peptides makes only very limited contact with the thrombin surface.

Discussion

Among transglutaminases, the activation peptide of FXIII-A (AP-FXIII) is a special feature with several functions and has been at the centre of FXIII research for a long time.[23] In particular, the description of the Val34Leu polymorphism and its beneficial effect on myocardial infarction[24] have raised a lot of interest. Since then, the effects of individual amino acids of AP-FXIII around the thrombin cleavage site on protein conformation, interactions with thrombin, and efficiency of FXIII activation have been studied.[25–27,10] However, the role of Pro36, an amino acid with unique properties which is located just one position upstream from the cleavage site, has never been investigated using mutant variants. Duval et al. have recently attempted to express a Pro36Ala variant which failed to transform and express in *E. coli*.[28] We have addressed the role of Pro36 when we came across a patient with heterozygous congenital FXIII deficiency who had a missense mutation at this very position 36, replacing the putatively important proline with a serine. This case served as a perfect *in vivo* model in our study on the role of Pro36.

The AP-FXIII ^{34}VVPR^{37} sequence corresponds to the P_4-P_1 residues known to be critical anchoring points to the thrombin active site region.[29,30,25,26] The Arg37 at the P_1 residue position sits within the catalytic cleft and is stabilised via a salt bridge with thrombin Asp189. In the native sequence, the P_2 residue is often a proline which interacts effectively with the thrombin surface below the β-insertion loop.[18,8] With APFXIII Pro36Ser, the beneficial contact site from the proline is lost. The Arg37 at the P_1 position still makes strong interactions with the active site cleft – as seen in our NMR analysis - but now the P_4-P_2 residues (^{34}VVS^{36}) appear to be less well positioned to contact the neighbouring thrombin active site region.

These structural considerations and NMR data on the altered interaction with thrombin also provide an explanation for the observed inability of the mutant FXIII-A Ser36 to be

activated by thrombin. We could show that the Ser36 mutant retains full transglutaminase activity when activated non-proteolytically with high Ca^{2+} concentrations, but it cannot be activated via the proteolytic pathway that involves cleavage by thrombin. This was confirmed by showing the abolished release of AP-FXIII (supplementary material). In our experiments, we did see some slow activation of both wild-type Pro36 and mutant Ser36 FXIII-A at the standard Ca^{2+} concentration of 10 mM over the course of the experiment (Figs. 4A and 4C), but this occurred also in case of the wild-type FXIII-A in the absence of thrombin. Therefore, this slow and low level activation is not due to thrombin activation, but rather due to non-proteolytic Ca^{2+} activation, as it has been shown that 2 mM Ca^{2+} is enough to activate (cellular) FXIII-A_2 when the regulatory effect of the FXIII B-subunit is absent.[6] Interestingly, at the high Ca^{2+} concentration of 50 mM (Fig. 4B), thrombin has no additional effect on the activation of wild-type FXIII-A, and on the contrary, activation of wild-type FXIII-A in the presence of thrombin was even delayed (increasing only at 30 min) at high Ca^{2+} when compared with the standard Ca^{2+} concentration where wild-type FXIII-A activation increased at 20 min (Fig. 4A). This could be explained by an inhibiting effect of high Ca^{2+} concentration on thrombin amidolytic activity that has been reported before.[31]

While the activation of the Pro36Ser FXIII mutant was affected, its expression was normal both *in vitro* in CHO cells and *in vivo* in our patient with heterozygous congenital FXIII deficiency. This confirms a rare case of FXIII-A type II deficiency that is characterised by the presence of a functionally defective FXIII-A protein, in contrast to FXIII-A type I deficiency where the mutant protein is completely absent from the patient's plasma.[22] More than a hundred mutations in the *F13A* gene causing FXIII-A deficiency have been described so far[32], but there have been only three mutations reported to cause a type II deficiency. The first mutation was a homozyogus insertion of four bases in exon 14 just before Pro675 leading to a frame-shift and a stop codon occurring seven residues after the insertion and possibly a truncated protein.[33] While FXIII activity was severely reduced, antigen levels at the lower end of the normal range were detected in the patient, but the presence of a functionally defective protein was not confirmed by *in vitro* cell expression studies. Two heterozygous mutations directly at the thrombin cleavage site, Arg37Gln and Arg37Pro, were expressed and were confirmed to lead to a FXIII-A type II deficiency with mildly decreased to normal antigen levels but severely decreased FXIII activity.[34] It is not surprising, that FXIII activation is inhibited when the thrombin cleavage site itself is abolished by mutation. In our case, however, mutation of the neighbouring amino acid had a similar deleterious effect.

Patients with heterozygous FXIII deficiency have around 50% of normal FXIII-A subunit antigen and activity levels, and they usually do not show any clinical signs of bleeding under normal circumstances. However, in situations such as trauma, surgery, or pregnancy, FXIII-A levels can drop significantly into a range with increased risk of bleeding and severe bleeding complications in heterozygous individuals in such haemostatic stress situations have been reported in the literature by others and us [35–39]. Thus, heterozygous FXIII-A deficiency leads to a phenotype with increased risk of bleeding under medical conditions involving a haemostatic challenge. This is exactly what we observed with our patient. She has suffered from bleeding complications following triggers such as menorrhoea, pregnancy loss, or childbirth. Despite the fact that normal FXIII-A antigen levels are present in our

patient, half of her FXIII cannot be activated, therefore her phenotype is fully comparable with the reported cases of heterozygous FXIII deficiency, and her severe pathology is typical for heterozygous FXIII deficiency.

In summary, we have shown here the crucial role of Pro36 in substrate recognition and FXIII activation. The characterisation of a novel mutation, Pro36Ser, leading to a rare FXIII-A type II deficiency, represented a unique opportunity to study the role of Pro36 and back the results with a perfect *in vivo* model. The mutant protein FXIII-A Ser36 is normally expressed, shows normal FXIII transglutaminase activity when induced by high Ca^{2+} concentration, but it cannot be proteolytically activated by thrombin. Our results contribute to the understanding of structure-function relationship in FXIII.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

This work was supported by grants from the Swiss National Science Foundation (Grant 310030_169220; awarded to HPK and VS) and the National Institutes of Health (Grant R15 HL120068; awarded to MCM). The authors would like to thank Ms Allison Davidson (Maurer group) for preparing the FXIII-A cleavage gel (supplementary Fig. S1).

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Summary Table

What is known about this topic?

- The activation peptide of blood coagulation factor XIII (AP-FXIII) has important functions in stabilising the FXIII-A_2 dimer and regulating FXIII activation.
- For proteolytic cleavage of AP-FXIII during FXIII activation, the FXIII-A cleavage site at Arg37-Gly38 must be recognised by thrombin.
- Several amino acids of AP-FXIII near the thrombin cleavage site have been mutated to study their effects on substrate recognition by thrombin and FXIII activation. However, the role of Pro36 has never been specifically investigated.

What does this paper add?

- Proline 36 of the FXIII activation peptide is crucial for substrate recognition by thrombin and proteolytic FXIII activation.
- A Pro36Ser mutation in the FXIII-A gene gives rise to a mutant protein that is expressed but can not be activated by thrombin, while it exhibits normal FXIII activity when activated non-proteolytically by high Ca2+ concentration.
- The Pro36Ser mutation causes a rare case of congenital FXIII-A type II deficiency.

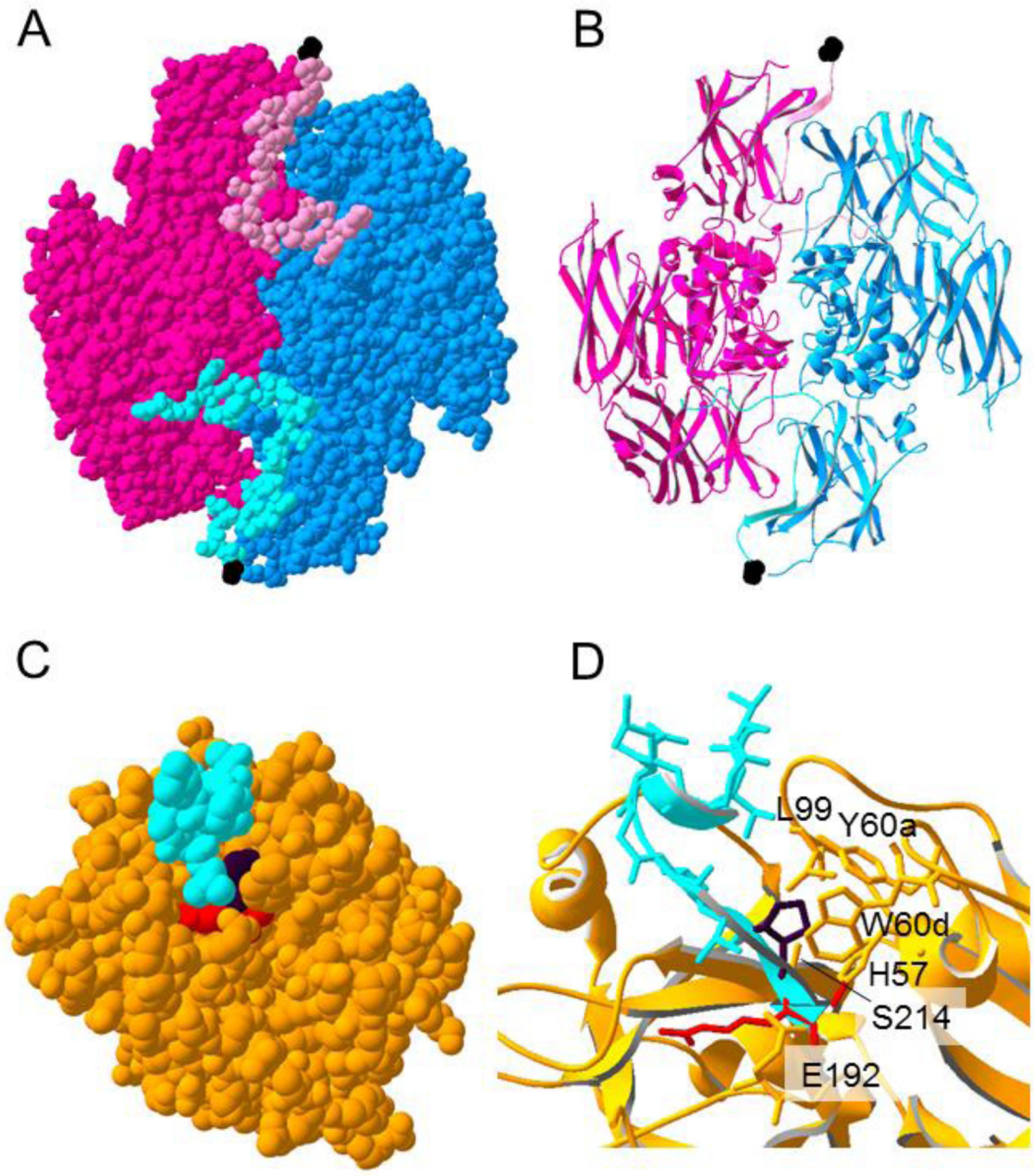

Fig. 1. Localisation of Pro36 in the FXIII-A molecule and in complex with thrombin.

The FXIII-A_2 dimer is depicted as space-filled model (A) and ribbon model (B) based on the crystal structure (PDB-ID 1F13). One FXIII-A monomer is coloured in pink with its activation peptide in rose (with amino acids 1–4 and 37–40 missing), the other monomer is coloured in blue with its activation peptide in turquois (with amino acids 1–5 and 37–38 missing). The loop containing Pro36, with Pro36 coloured in black, is protruding from the molecule. In the complex of the AP-FXIII (28–37) with thrombin (PDB-ID 1DE7), shown in panels C and D, AP-FXIII (28–35) is coloured in turquois, Pro36 in black, Arg37-Gly38 in red, and thrombin in orange. Pro36 is extending into the thrombin molecule (C), it forms a

hydrogen bond with E192 and interacts with L99, Y60a, W60d, H57, and S214 via van der Waals interactions (D).

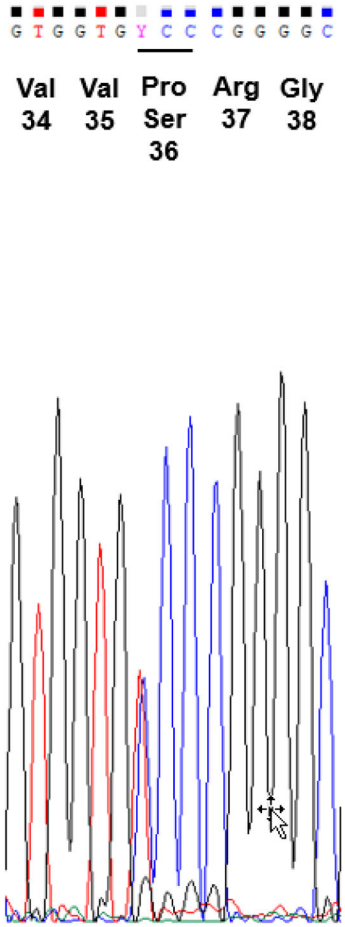

Fig. 2. Sequence analysis.

Chromatogram obtained from Sanger sequencing showing a heterozygous c.109C>T mutation (numbering started with ATG start codon) coding for a proline to serine exchange at position 36 in the mature FXIII-A protein.

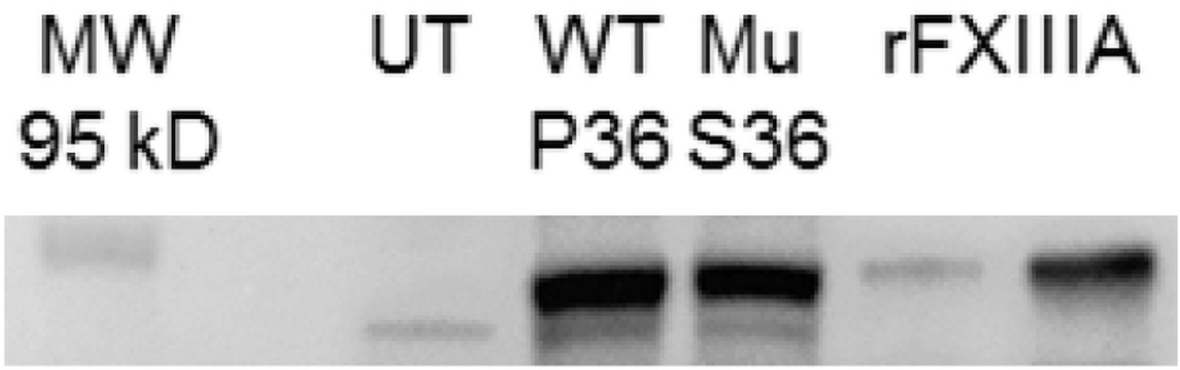

Fig. 3. Expression of wild-type and mutant FXIII-A Pro36Ser.

Western blot developed with a monoclonal anti-FXIII-A antibody. From left to right: protein marker band of 95 kDa (MW), lysate from untransfected CHO cells (UT) as negative control, lysates from CHO cells transfected with wild-type FXIII-A Pro36 (WT P36) or mutant FXIII-A Ser36 (Mu S36), commercially available rFXIII-A (Zedira) as positive control (15 ng and 50 ng loaded).

Figure 4A

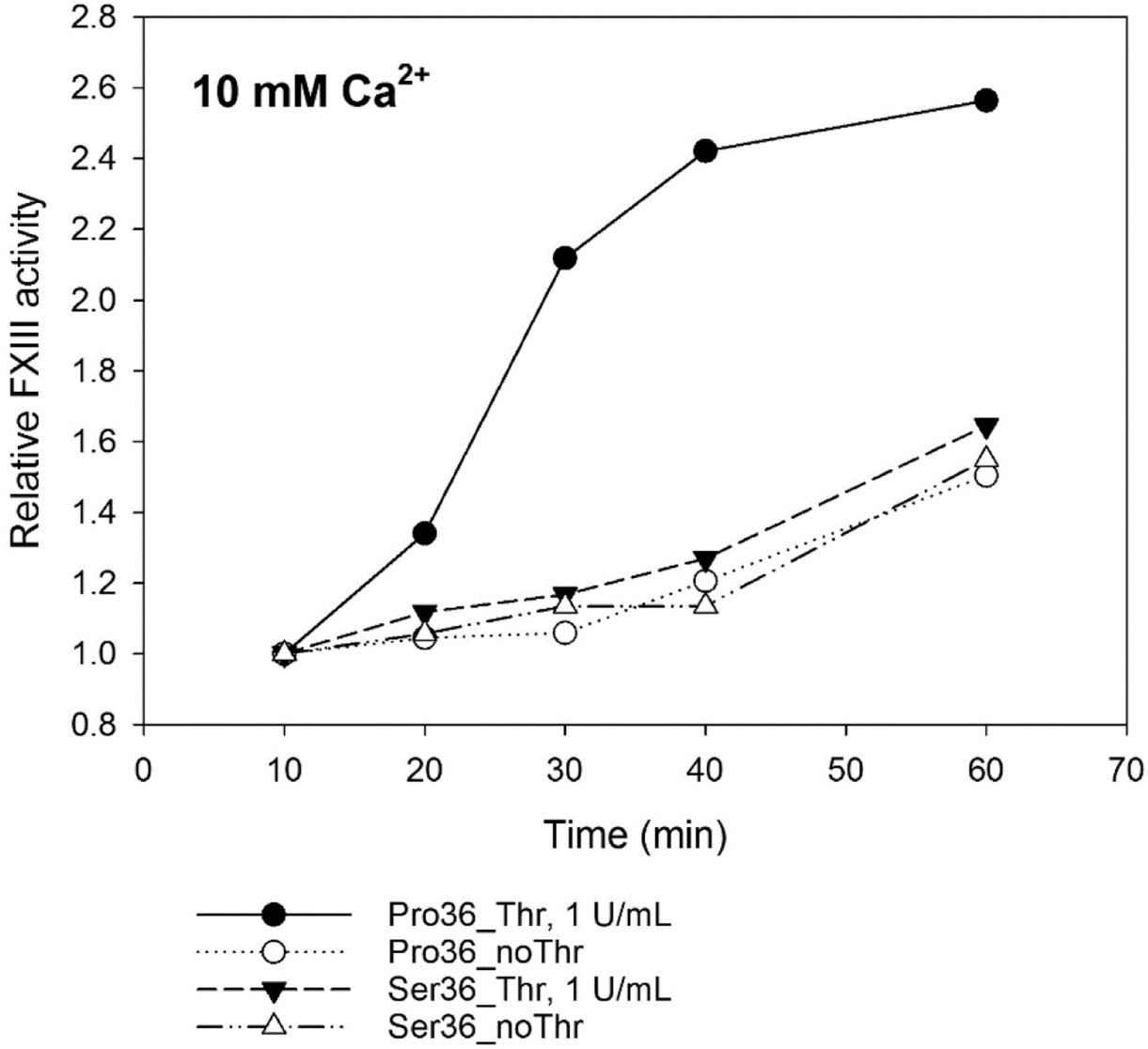

Figure 4B

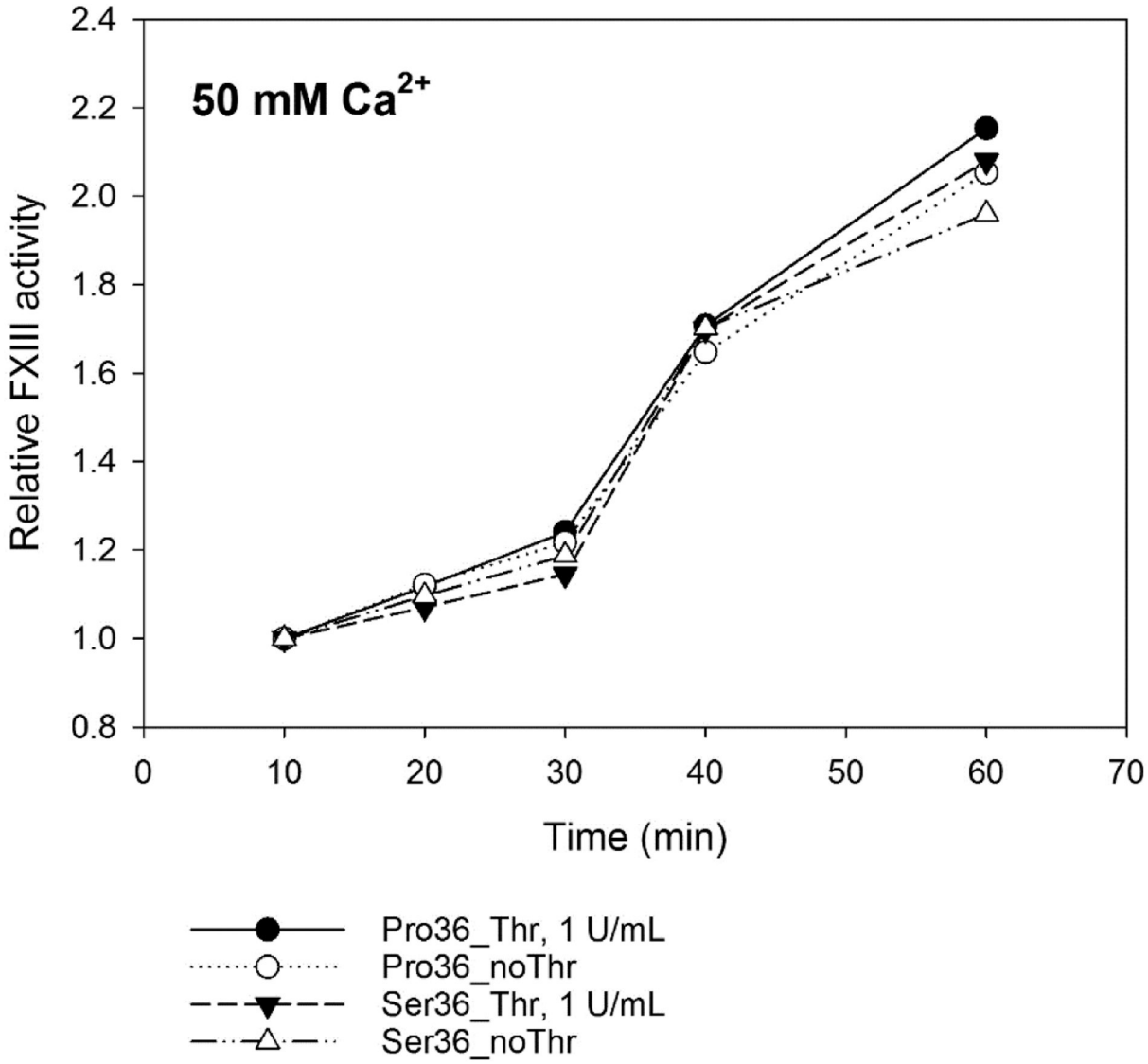

Figure 4C

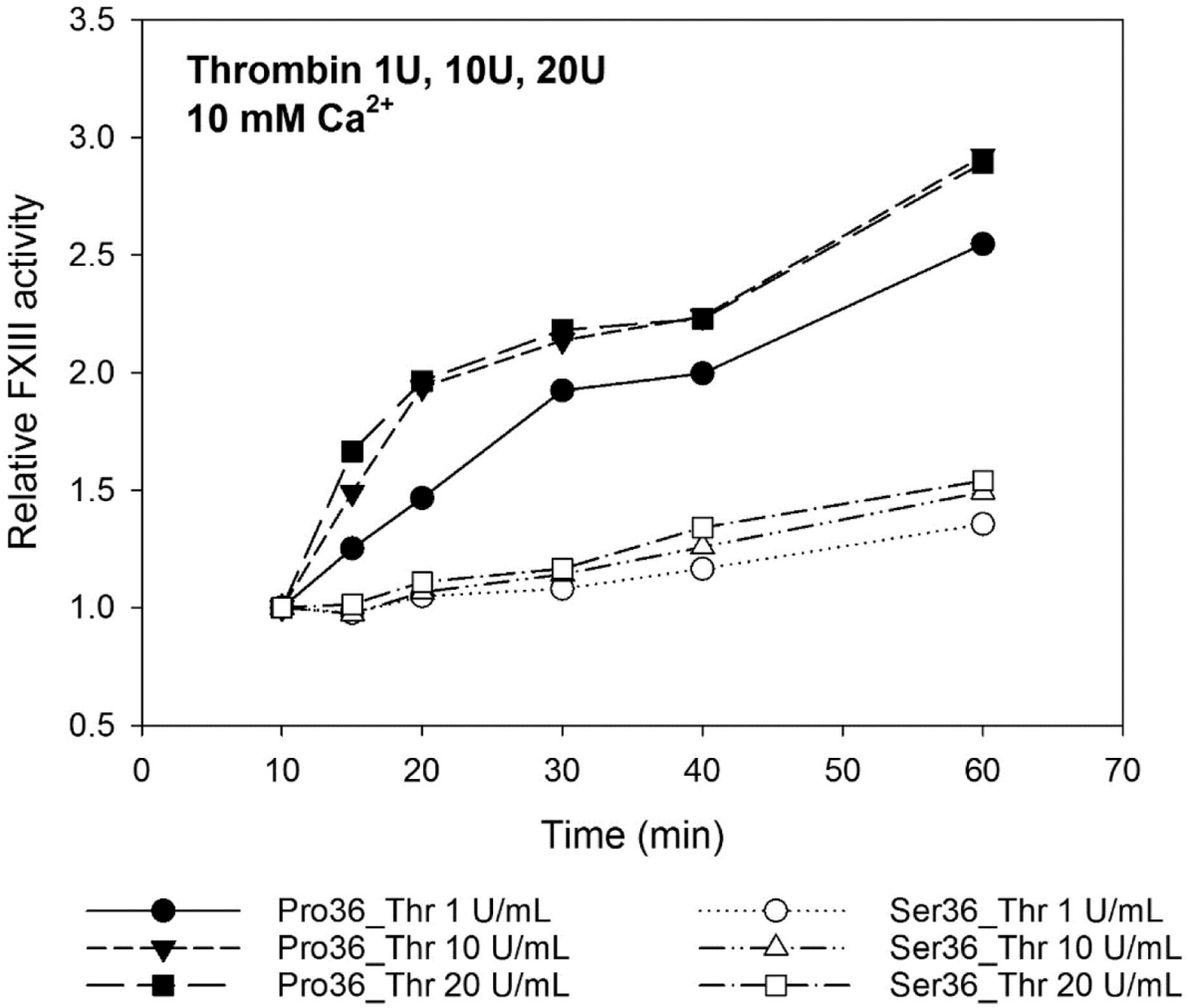

Fig. 4. FXIII activity induced by thrombin and/or Ca^{2+}.

An incorporation assay was performed without/with 1U/ml thrombin (indicated as noThr or Thr, respectively) in the presence of (A) a standard 10 mM Ca^{2+} concentration or (B) a high 50 mM Ca^{2+} concentration. Wild-type FXIII-A Pro36 is depicted as circles, mutant Ser36 as triangles. (C) Increasing thrombin concentrations were used with standard 10 mM Ca^{2+} concentration. Wild-type FXIII-A Pro36 is depicted as filled symbols, mutant Ser36 as empty symbols. FXIII activity is expressed relative to the first time point. Data shown are mean values from four experiments.

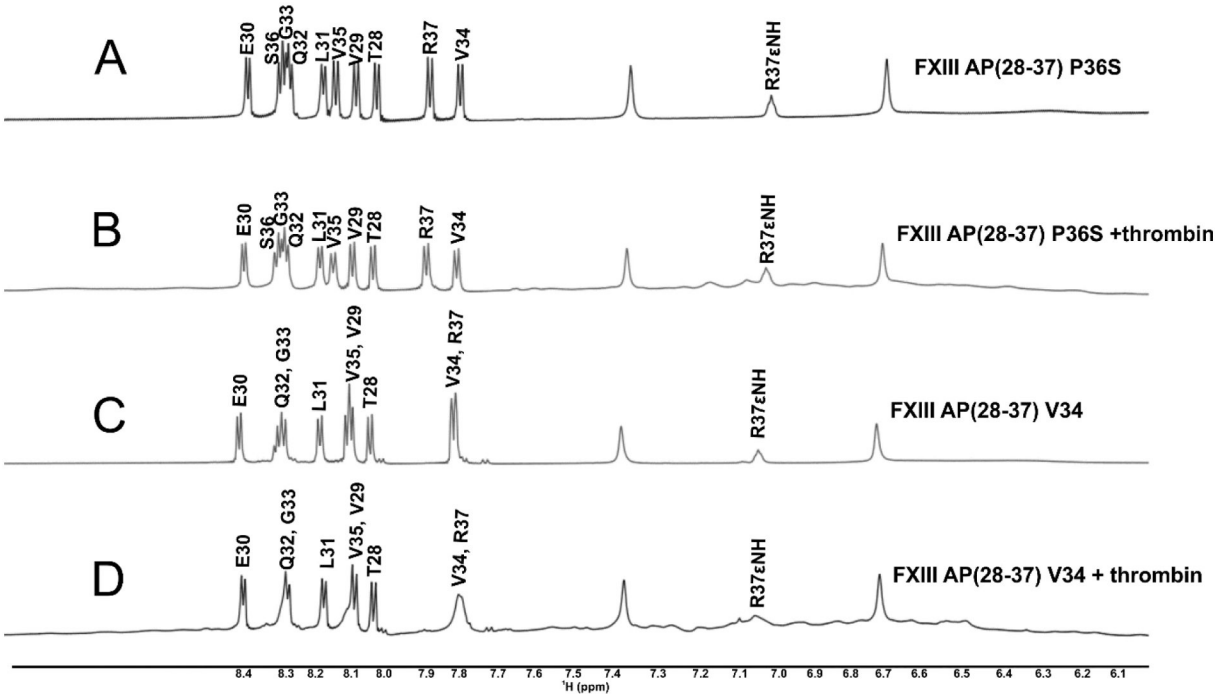

Fig. 5. NMR analysis of AP-FXIII – thrombin interactions.

1D line broadening studies of peptides corresponding to the AP-FXIII (28–37) sequence. (A) 1mM AP-FXIII (28–37) Ser36 (mutant). (B) 1.5 mM AP-FXIII (28–37) Ser36 in complex with thrombin. (C) 1.2 mM AP-FXIII (28–37) Pro36 (wild-type). (D) 1.5 mM AP-FXIII (28–37) Pro36 in complex with thrombin.