Proteolytic properties of single-chain factor XII: A mechanism for triggering contact activation

Ivan Ivanov[1], Anton Matafonov[1], Mao-fu Sun[1], Qiufang Cheng[1], S. Kent Dickeson[1], Ingrid M. Verhamme[1], Jonas Emsley[2], and David Gailani[1]

[1]Department of Pathology, Microbiology and Immunology, Vanderbilt University Medical Center, Nashville, TN; [2]Centre for Biomolecular Sciences, School of Pharmacy, University of Nottingham, Nottingham, UK

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To whom correspondence should be addressed: David Gailani, Hematology/Oncology Division, Vanderbilt University, 777 Preston Research Building, 2220 Pierce Ave., Nashville, TN, USA, Tel. 615-936-1505; Fax 615-936-3853; E-mail: dave.gailani@vanderbilt.edu

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ABSTRACT

When blood is exposed to variety of artificial surfaces and biologic substances, the plasma proteins factor XII (FXII) and prekallikrein undergo reciprocal proteolytic conversion to the proteases αFXIIa and α-kallikrein by a process called contact activation. These enzymes contribute to host-defense responses including coagulation, inflammation, and fibrinolysis. The initiating event in contact activation is debated. To test the hypothesis that single-chain FXII expresses activity that could initiate contact activation, we prepared human FXII variants lacking the Arg353 cleavage site required for conversion to αFXIIa (FXII-R353A), or lacking the three known cleavage sites at Arg334, Arg343, and Arg353 (FXII-T, for "triple" mutant), and compared their properties to wild type αFXIIa. In the absence of a surface, FXII-R353A and FXII-T activate prekallikrein and cleave the tripeptide S-2302, demonstrating proteolytic activity. The activity is several orders of magnitude weaker than that of αFXIIa. Polyphosphate, an inducer of contact activation, enhances PK activation by FXII-T, and facilitates FXII-T activation of FXII and factor XI. In plasma, FXII-T and FXII-R353A, but not FXII lacking the active site serine residue (FXII-S544A), shortened the clotting time of FXII-deficient plasma and enhanced thrombin generation in a surface-dependent manner. The effect was not as strong as for wild type FXII. Our results support a model for induction of contact activation in which activity intrinsic to single-chain FXII initiates αFXIIa and α-kallikrein formation on a surface. αFXIIa, with support from α-kallikrein, subsequently accelerates contact activation and is responsible for the full procoagulant activity of FXII.

KEY POINTS

- The single chain form of Factor XII, a component of the plasma contact system, has proteolytic activity.
- Single-chain FXII activity suggests a mechanism for initiation of contact activation when blood is exposed to physiologic or artificial surfaces.

INTRODUCTION

The cascade/waterfall hypotheses proposed by Macfarlane and by Davie and Ratnoff in 1964 describe plasma coagulation as a series of proteolytic steps culminating in thrombin-mediated conversion of fibrinogen to fibrin [1,2]. Coagulation is initiated in these models by activation of factor XII (FXII) when blood is exposed to a suitable surface [1-3]. These investigators postulated that FXII undergoes changes upon surface binding that form or expose an active site. It is now clear that a variety of substances promote reciprocal proteolytic conversion of FXII and prekallikrein (PK) to the proteases α-factor XIIa (αFXIIa) and α-kallikrein by a process called contact activation [4-6]. αFXIIa then promotes coagulation by converting factor XI (FXI) to factor XIa (FXIa). While patients lacking FXII do not have a bleeding disorder [7], the protein contributes to pathologic coagulation particularly when blood is exposed to artificial surfaces, such as during cardiopulmonary bypass, extracorporeal membrane oxygenation, and after placement of intravascular devices [8-15]. In animal models, FXII contributes to thrombosis [16-20], with anionic polymers such as polyphosphate (Poly-P) and nucleic acids probably serving as counterparts to the non-biologic surfaces used to induce contact activation *in vitro* [17,21-25].

Despite progress in our understanding of the pathophysiology of contact activation, the processes that initiate FXII activation remain uncertain. Several triggering mechanisms have been proposed that are not mutually exclusive. Contact activation could be initiated by traces of αFXIIa, α-kallikrein, or other proteases that may always be present in plasma [26-28]. Alternatively, single-chain FXII may have activity, either intrinsically or after binding to a surface, that starts the process [29-31]. Here we show that a form of FXII that is not converted to αFXIIa has proteolytic activity, and that the activity is enhanced in the presence of Poly-P. The results suggest a mechanism for initiation of surface-induced coagulation that does not depend on pre-existing αFXIIa or α-kallikrein.

EXPERIMENTAL PROCEDURES

Materials. Normal plasma; Precision BioLogic. FXII-deficient plasma; George King Biomed. FXII, FXIIa, PK, α-kallikrein and corn trypsin inhibitor (CTI); Enzyme Research Lab. S-2302 (H-D-prolyl-L-phenylalanyl-L-arginine-p-nitroaniline); DiaPharma. PTT-A reagent; Diagnostica Stago. Synthetic Poly-P

(ILC Performance Products) of 60-100 unit chain length (not calcium saturated) was provided by Drs. Thomas Renné and Katrin Nikel (Karolinska Institute). Monoclonal IgGs to FXI (O1A6) [32], FXIIa (559C-X181-D06 [D06]) [33], and kallikrein (559A-M202-H03 [H03]) [33,34] have been described. Goat polyclonal IgGs against FXII, PK, and FXI were from Affinity Biologicals.

Recombinant Proteins. Schematic diagrams of human FXII, PK and FXI and their cleaved forms are shown in **Fig.1A.** A FXI cDNA with the active site serine replaced with alanine (FXI-S557A) was expressed in HEK293 cells using vector pJVCMV, purified by IgG affinity chromatography, and stored in 4 mM Tris-HCl 150 mM NaCl pH 7.4 [35]. FXII [36] and PK [37] cDNAs were introduced into pJVCMV and expressed in HEK293 cells under serum free conditions (**Supp. data 1**). In addition to wild type FXII (FXII-WT), variants were made with alanine replacing arginine at each known cleavage site (FXII-R334A, FXII-R343A or FXII-R353A) or all three cleavage sites (FXII-T for "triple" mutant), or replacing the active site serine (FXII-S544A). Wild type PK (PK-WT) and variants with alanine replacing arginine at the activation site (PK-R371A) or the active site serine (PK-S559A) were also prepared. FXII and PK were purified by anion exchange chromatography (**Fig.1B**) as described under **Supp. data 1**.

Chromogenic assays (continuous assay). Assays were conducted in microtiter plate wells coated with PEG-20,000 to prevent induction of contact activation. Activation of FXII (200 nM) and/or PK (200 nM) in 100 μL of standard buffer (20 mM HEPES, pH 7.4, 100 mM NaCl, 0.1% PEG-8000, 10 μM $ZnCl_2$) at 37 ºC. Reactions contained 200 μM S-2302 and changes in OD 405 nm were monitored on a microplate reader. Some reactions contained Poly-P (70 μM) or 10% PTT-A reagent. S-2302 (1.6 to 1000 μM) cleavage by 5 nM αFXIIa or 200 nM fXII-T was studied under similar conditions. Rates of ΔOD405 nm were converted to *p*-nitroanaline formed (extinction coefficient for *p*NA 9920 M^{-1} cm^{-1}). K_m and k_{cat} were determined by non-linear least squares fitting using Scientist Software (Micromath). ***(discontinuous assay).*** FXII activation (200 nM) with Poly-P (70 μM) or α-kallikrein (50 nM), and PK (12.5 to 600 nM) cleavage by αFXIIa (25 pM) or FXII-T (15 nM) were performed in standard buffer at 37 ºC. For FXII activation, reactions were stopped with Polybrene (0.1 mg/ml) with or without H03 (10 nM). For PK cleavage,

reactions were stopped with CTI (1.6 μM final). For all reactions, cleavage of 200 μM S-2302 (ΔOD405 nm) was monitored. Amounts of FXIIa or kallikrein formed were determined with standard curves prepared with pure protease.

Western blots. Reactions were conducted in polypropylene tubes coated with PEG-20,000. FXII (200 nM) with or without PK (200 nM), FXI (30 nM), or α-kallikrein (50 nM) was incubated at 37 ºC in standard buffer at 37 ºC. Some reactions contained 70 μM Poly-P or PTT-A reagent (25% final volume). At various times, aliquots were removed and mixed with SDS-sample buffer. Samples were size fractionated by SDS-PAGE (12% acrylamide) and transferred to nitrocellulose membranes. Blots of reduced proteins were probed with polyclonal IgG to FXII, PK, or FXI. Non-reducing blots were probed with antibodies that preferentially recognize FXIIa (D06) or kallikrein (H03). For all blots, detection was with an HRP-conjugated secondary antibody and chemiluminescence.

Clotting assays. FXII-deficient plasma (30 μL) was mixed with 30 μL FXII (400 nM) in PBS. PTT-A reagent (30 μL) was added followed by incubation for 5 min at 37 °C. 25 mM $CaCl_2$ (30 μL) was added, and time to clot formation was measured on an ST-4 Analyzer (Diagnostica Stago). Results were compared to those for normal plasma. Means ± 1 SD were compared by Mann-Whitney test.

Thrombin Generation. Thrombin generation was measured in 96-well polypropylene plates on a Fluoroskan Ascent analyzer as described [35]. FXII-deficient plasma was supplemented with FXII (200 nM), 415 μM Z-Gly-Gly-Arg-AMC, 1.6 μM CTI or vehicle, 20 ug/ml of IgG O1A6 or vehicle, and PTT-A reagent (13% final volume). Supplemented plasma (40 μl) was mixed with 10 μl of 20 mM HEPES pH 7.4, 100 mM $CaCl_2$, 6% BSA and fluorescence (excitation λ 390 nm, emission λ 460 nm) was monitored.

Carotid artery thrombosis model. Procedures with mice were approved by the Vanderbilt University Animal Care and Use Committee. FXII-deficient C57Bl/6 mice [16] were anesthesthetized with 50 mg/kg IP pentobarbital. The right common carotid artery was exposed and fitted with a Doppler probe (Model 0.5 VB, Transonic System). FXII (25 μg in 100 uL of PBS) was infused through the internal jugular vein. Five

minutes later two 1 x 1.5 mm filter papers (GB003, Schleicher & Schuell,) saturated with 5% $FeCl_3$ were applied to opposite sides of the artery for three min [18]. Flow was monitored for 30 min.

RESULTS

FXII autoactivation. FXII undergoes autocatalytic activation in the presence of several polyanions [17,21,26-29]. The Coomassie-stained reducing gel in **Fig.2A** shows the 80 kDa FXII polypeptide undergoing autocatalysis in the presence of Poly-P, forming the 50 kDa heavy and 30 kDa light chains of αFXIIa. The changes are accompanied by a rapid increase in amidolytic activity (**Fig.2B**). FXII is cleaved at up to three sites during activation (after Arg334, Arg343, or Arg353 **Fig.1A**). Cleavage after Arg353 is required for αFXIIa generation [36,38]. FXII species with Arg353 (FXII-WT, FXII-R334A. FXII-R343A) undergo autocatalysis in the presence of Poly-P, while species lacking Arg353 (FXII-R353A and FXII-T) do not (**Fig.2C**, middle column). Loss of the catalytic active site serine (Ser544) [36], as expected, also prevents autocatalysis (**Fig.2C**, middle column). Experiments examining cleavage of the tripeptide S-2302 support the western blot data, with amidolytic activity increasing in reactions with species that undergo conversion to αFXIIa, but not in reactions with FXII-R353A, FXII-T or FXII-S544A (**Fig.2D**). These data show that cleavage after Arg353 is required for full elaboration of FXII amidolytic activity.

FXII activation by α-kallikrein. During contact activation, α-kallikrein cleaves FXII after Arg353 forming αFXIIa [36,38]. Substitutions for Arg334 or Arg343 do not prevent this (**Fig.2C**, right column). α-kallikrein also cleaves FXII-R353A (**Fig.2C**, right column); however, this does not increase amidolytic activity (**Fig.2E**), indicating cleavage is after a residue other than 353. Because Arg353 replacement did not prevent FXII cleavage, we prepared FXII-T, which lacks all three known FXII cleavage sites. The failure of α-kallikrein to cleave FXII-T (**Fig.2C**, right column) indicates α-kallikrein cleaves FXII-R353A after Arg334 and/or Arg343. FXII-S544A, which has Arg353, is cleaved by α-kallikrein (**Fig.2C**, right column) but, as expected, also lacks amidolytic activity because of the absence of Ser544 (**Fig.2E**). We performed non-reducing western blots for reactions similar to those in the right hand column of **Fig.2C** using IgG D06, which preferentially recognizes fully formed FXIIa (**Supp. data 2**). Signals are detected

for forms of FXII that have arginine at position 353, but not form forms with alanine at this position. This confirms that when FXII-R353A is cleaved by kallikrein it does not assume a conformation similar to αFXIIa. Cumulatively, the results support the premise that full αFXIIa amidolytic activity requires cleavage after Arg353 and an active catalytic mechanism (i.e. Ser544).

Reciprocal FXII and PK activation. Mixing FXII and PK in the absence of a surface leads to cleavage of both proteins as revealed by reducing western blots (**Fig.3A**). Each protein undergoes more than one cleavage, but at least one involves the respective activation sites (after FXII Arg353 or PK Arg371) [38]. This is shown in the non-reducing blots in **Fig.3B**, which use antibodies that preferentially recognize fully formed FXIIa (D06) and kallikrein (H03) [33,34]. Taken as a whole, the reducing and non-reducing blots indicate FXII is converted first to αFXIIa (80 kDa) by cleavage after Arg353, then more slowly to βFXIIa (30 kDa) by cleavage after Arg334 (**Fig.1A**). PK is converted to α-kallikrein by cleavage after Arg371, and then β-kallikrein by cleavage after Arg140 (**Fig.1A**), both of which are 90 kDa proteins (**Fig.3B**) [37,38]. Mixing FXII-WT and PK-WT generates patterns similar to their plasma counterparts (**Fig.3C**).

Unexpectedly, forms of FXII that do not form αFXIIa (FXII-R353A and FXII-T) induce PK cleavage (**Fig.3C**). The results could not be accounted for by endogenous FXII production by HEK293 cells, nor by non-FXII constituents of conditioned media (**Supp. data 1**). Furthermore, PK-WT was not cleaved by FXII-S544A (**Fig.3C**), indicating cleavage requires recombinant FXII with an intact FXII catalytic apparatus. The variant PK-S559A, which lacks an active site serine, was also cleaved by FXII-R353A and FXII-T, indicating α-kallikrein activity is not needed for PK cleavage in reactions with FXII-R353A and FXII-T (**Supp. data 3**). These data show that single-chain FXII has proteolytic activity.

S-2302 cleavage by single-chain FXII. Single-chain FXII-WT cleaves S-2302 at a low rate (**Fig.4A**). This is usually interpreted as reflecting traces of contaminating αFXIIa, a possibility that cannot be ruled out. However, FXII-R353A and FXII-T also consistently demonstrated low-level amidolytic activity, while FXII-S544A does not (**Fig.4A**). S-2302 is cleaved by αFXIIa with K_m 100±10 μM and k_{cat} 700±20/min (**Fig.4B**), and by FXII-T with K_m 2700±3300 μM, k_{cat} 6.5±6.4/min (**Fig.4C**). The results indicate that

catalytic efficiency (k_{cat}/K_m) for S-2302 cleavage by FXII-T is about 3000-fold lower than for αFXIIa (**Fig.4I**), an estimate that should be considered an approximation, as the apparent K_m for the FXII-T-mediated reaction is greater than the achievable substrate concentration. While addition of silica-based PTT-A reagent (**Fig.4D**) or Poly-P (**Fig.2D**) increased amidolytic activity with FXII-WT because of conversion to αFXIIa, neither compound enhanced the amidolytic activity of FXII-R353A or FXII-T.

PK activation by single-chain FXII. In mixtures of FXII and PK, amidolytic activity reflects FXIIa and kallikrein generation, as both proteases cleave S-2302. This is shown in **Fig.4E** for mixtures of FXII-WT and PK-WT. Mixing PK with FXII-R353A or FXII-T also generates amidolytic activity (**Fig.4E**). As these FXII species cleave S-2302 poorly (**Fig.2E**) and do not form αFXIIa, the amidolytic activity must be largely due to PK conversion to kallikrein by cleavage after Arg371. In support of this, neither FXII-R353A nor FXII-T cleaved PK lacking Arg371 (PK-R371A, **Supp. data 4**). Reactions containing PK alone or PK mixed with FXII-R544S generate little amidolytic activity (**Fig.4E**), demonstrating the importance of a functional FXII catalytic domain. αFXIIa activates PK with K_m 0.17±0.03 μM and k_{cat} 44±4/min (**Fig.4F**), while FXII-T activates PK with K_m 0.80±0.06 μM and k_{cat} 0.0037±0.0002/min (**Fig.4G**). The catalytic efficiency (k_{cat}/K_m **Fig.4I**) of FXII-T-mediated PK activation is ~50,000-fold lower than for αFXIIa. Consistent with observations that polyanions accelerate reciprocal FXII/PK activation [31,33], PK activation by FXII-T is enhanced by Poly-P (**Fig.4H**).

Single-chain FXII catalyzes surface-dependent FXII activation. Neither FXII-T nor FXII-S544A undergo autocatalysis in the presence of Poly-P (**Fig.2C**), the former because it lacks cleavage sites and the later because it lacks catalytic activity. When these proteins are mixed in the presence of a silica-based aPTT reagent or Poly-P (**Fig.5A**) there is a time-dependent increase in a signal on western blots using IgG D06, indicating formation of a species with a catalytic domain with similar conformation to αFXIIa. Incubation of either protein alone with Poly-P does not produce a signal, consistent with results in **Fig.2C** (middle column). Of the two proteins, only FXII-S544A cleaved after Arg353 is recognized well by IgG D06 (**Supp. Data 2**). Therefore, the results are most consistent with surface-induced FXII-T cleavage of FXII-S544A

after Arg353. The results indicate that single-chain FXII can initiate FXII activation after the protein is bound to an activating surface.

Single-chain FXII catalyzes surface-dependent FXI activation. αFXIIa-mediated conversion of FXI to FXIa is a key step in contact activation-initiated coagulation. In the following experiments FXI-S557A is used instead of FXI because the latter undergoes autoactivation when exposed to polyanions [35] making it difficult to assess the FXII contribution to FXI activation. In the absence of Poly-P, mixing FXI-S557A with FXII-WT, FXII-T or FXII-S544A does not result in detectable FXI-S557A cleavage (**Fig.5B** top row). With Poly-P (**Fig.5B** bottom row), FXII-WT converts FXI-S557A to a two-chain species representing FXIa. This is consistent with FXII-WT undergoing autoactivation, and αFXIIa-WT then cleaving FXI-S557A. In the presence of Poly-P, FXII-T but not FXII-S544A cleaved FXI-S557A to the two-chain form, indicating that single-chain FXII possesses activity that activates FXI on a surface.

Single-chain FXII activity in plasma. In aPTT assays, the clotting time of normal and FXII-deficient plasma were 40±1 and 327±30 seconds, respectively. Adding forms of FXII that can be converted to αFXIIa substantially shortened the aPTT of FXII-deficient plasma (FXII-WT [40±2 sec], FXII-R334A [41±1 sec] or FXII-R343A [51±1 sec] **Fig.6A**). FXII-R353A and FXII-T, which do not form αFXIIa, also shortened the aPTT of FXII-deficient plasma (202±5 and 164±19 sec, respectively, $p<0.0001$ for both compared with FXII-deficient plasma), but not to the same extent as FXII-WT. FXII-S544A did not affect the clotting time significantly (302±6 sec, $p=0.2$). FXII-WT and plasma FXII support thrombin generation in FXII-deficient plasma supplemented with PTT-A reagent comparably (**Fig.6B**). FXII-T also supports thrombin generation, but the peak is smaller and delayed compared to FXII-WT (**Fig.6C**), while FXII-S544A did not support thrombin generation. The effects of FXII-WT and FXII-T on thrombin generation were blocked by the FXII inhibitor CTI and the FXIa inhibitor O1A6 (**Fig.6D**), showing that single-chain FXII promotes thrombin generation by activating FXI in the presence of a surface.

FXII and arterial thrombosis. FXII-deficient mice are protected from carotid artery thrombosis induced by application of $FeCl_3$ to the vessel exterior (**Fig.7**) [18]. Infusion of FXII-WT, as expected, restored

$FeCl_3$-induced carotid artery occlusion in FXII-deficient mice. In contrast, FXII-R353A, FXII-T and FXII-S544A failed to support thrombosis, with the exception of one of nine animals supplemented with FXII-T. These data are consistent with results from plasma the assays, and indicate that the relatively small amount of thrombin generated through the proteolytic activity of single-chain FXII is not sufficient to promote thrombosis in this model, and that αFXIIa is required for full elaboration of protease activity.

DISCUSSION

FXII becomes active when blood is exposed to a variety of biologic and non-biologic substances, however, the mechanism that initiates activation is debated. Given the range of surfaces that support contact activation, different processes may operate in different situations [39]. In 1976, Griffin and Cochrane raised the question of whether a proteolytic or non-proteolytic process best explained initial FXII activity [40]. Several groups proposed that FXII gains activity once surface-bound, and postulated that conversion to a two-chain form is not required in all circumstances [29-31]. Silverberg *et al.* [26,27] and Tans *et al.* [28] came to a different conclusion. In their opinions αFXIIa was the active form of FXII, and the sigmoidal progress curves for surface-dependent FXII autoactivation were most compatible with a process initiated by traces of αFXIIa in FXII preparations. However, recognizing the difficulty of eliminating other possibilities with assays based on plasma-derived FXII, Silverberg and Kaplan suggested that if single-chain FXII had activity, it is weak, and calculated a limit on its ability to cleave the tripeptide S-2302 at <4200-fold that of αFXIIa [27].

In vivo, polymeric anions may serve as counterparts to the non-biologic substances traditionally used to study contact activation. One of the best characterized is Poly-P, polymers of inorganic phosphate that produce procoagulant effects in plasma including induction of contact activation [17,21,25,41]. Engel *et al.* recently reported that FXII bound to Poly-P chains of the size released by platelets cleaves S-2302 and activates PK and FXI without undergo conversion to αFXIIa [31], indicating Poly-P-binding induces conformational changes in single-chain FXII that confer activity. A striking finding in their study, which conflicts with the data presented here, was that the amidolytic activities of αFXIIa and Poly-P-bound FXII

were comparable, suggesting conversion to αFXIIa is not necessary for full (or substantial) elaboration of protease activity. These experiments, and earlier work, used plasma FXII, making it difficult to exclude effects from αFXIIa, either as an initial contaminant or as a product generated during reactions. We addressed this issue with recombinant FXII that cannot be converted to αFXIIa. Our results show that single-chain FXII has activity and that Poly-P enhances that activity toward the macromolecular substrates PK, FXII and FXI. However, there are important differences between our results and those reported by Engel *et al.*, and they support different mechanisms for the role of cofactors such as Poly-P in FXII activity. Differences between recombinant and plasma-derived FXII, either as a consequence of the substitutions we introduced or subtler factors such as variance in post-translational modification, may partly explain the weaker activity we detected for single chain FXII, compared with the activity for plasma FXII reported by Engel *et al*. However, this would not explain the different behaviors observed for plasma FXII in the presence of Poly-P.

Members of the trypsin-like protease family are usually secreted as inactive single-chain zymogens that require internal proteolysis after Arg15 (chymotrypsin numbering) for activity [42]. Upon cleavage, the new catalytic domain N-terminus (residue 16) forms a salt-bridge with Asp194, creating a functional substrate recognition site. Consistent with this, we observed that increased rates of S-2302 cleavage by FXII (plasma or recombinant) in the presence of Poly-P are associated with conversion to αFXIIa. Arg353 in human FXII corresponds to chymotrypsin Arg15 [36,38], and replacing it blocks Poly-P-mediated FXII autocatalysis and the associated increase in amidolytic activity. These findings support the premise that polyanion-induced elaboration of full FXII activity involves cleavage after Arg353 [27,28]. Our data with plasma-derived and recombinant FXII-WT are in disagreement with those of Engel *et al.* on this point. Perhaps differences in preparative techniques for Poly-P, or methods for detecting FXIIa contribute to the discrepancy. We observed that Poly-P-based reactions behave differently depending on buffer conditions, which may be relevant. However, it is worth noting that forms of FXII that do not form αFXIIa did not reconstitute FXII-deficient mice in a thrombosis model in which FXII and Poly-P play prominent roles

[17,25]. This argues against a mechanism in which binding of a cofactor converts FXII to a fully or substantially active protease without cleavage after Arg353, and is consistent with the marked decrease in activity of the natural variant FXII Locarno, in which proline replaces Arg353 [43].

Single chain FXII-T cleaves S-2302 and activates PK without adding Poly-P or a surface, suggesting it has a low level of proteolytic activity when not surface-bound. Poly-P does, however, enhance PK activation, and facilitates FXII and FXI activation by FXII-T. There may be multiple effects that contribute to the mechanism involved. First, Poly-P-bound FXII-T may assume a conformation that enhances extended interaction with macromolecular substrates. It is also likely that the substrate (PK, FXII or FXI) is altered upon binding to Poly-P, exposing activation cleavage sites. Furthermore, the size dependence of the Poly-P-effect [41,44] points to facilitation of protease and substrate binding in proximity to each other through a bridging (template) mechanism.

There is precedent for plasma serine proteases having activity in their single-chain forms, a property referred to as low zymogenisity. Single chain tissue plasminogen activator (tPA) is 8-fold less active against tripeptide substrates and 20-50-fold less active towards plasminogen than two-chain tPA [45], but in the presence of fibrin both forms cleave plasminogen similarly. Renatus *et al.* proposed that a salt bridge between Lys156 and Asp194 in tPA (**Fig8A**) not found in most trypsin-like proteases contributes to formation of a functional active site in the single-chain protein [46]. Single-chain urokinase, also has activity toward plasminogen in the presence of fibrin and, like tPA, has lysine at position 156 [47]. Gln156 in FXII cannot form a stabilizing salt bridge with Asp194, but it may provide weak stabilization through hydrogen bonding interactions. In addition to forming a salt bridge with Lys156, the carboxylate group of Asp194 in tPA forms hydrogen bonds with the main-chain nitrogen atoms of Gly142 and Cys191 (**Fig.8A**). A homology model of FXII-T based on the tPA structure reveals that the FXII Asp194 carboxylate can form the same hydrogen bonds (**Fig.8B**) [48]. Furthermore, because of additional hydrogen bond with the side-chain of Gln156, there is no impediment to oxyanion hole formation. Gln156 would be less effective at stabilizing Asp194 in FXII-T than the N-terminal Val16 in αFXIIa, consistent with the lower catalytic

activity of FXII-T. Recently the crystal structure of FXII catalytic domain with Arg-Ser blocking the N-terminus (FXIIc) was reported [49]. FXIIc has a zymogen-like conformation, lacking the oxyanion hole, and Asp194 interacts with Arg73 in a configuration similar to the zymogen triad described for chymotrypsinogen (**Fig. 8C**) [50,51]. This suggests a complex interplay of factors, including conformational changes within FXII induced by interactions with substrates, determine formation of a productive FXII-T enzyme-substrate complex, as has been proposed for other members of the trypsin-like protease family [52].

Our results support a model for contact activation that does not require pre-existing αFXIIa or α-kallikrein. Single-chain FXII would catalyze initial FXII and PK conversion to αFXIIa and α-kallikrein upon surface exposure. The relatively low activity of single-chain FXII suggests that it is αFXIIa and α-kallikrein that are responsible for subsequent acceleration of contact activation. This is consistent with the observation that progress curves for FXII autoactivation fit models based on initiation by traces of αFXIIa [27,28]. Single-chain FXII activity is relevant early in contact activation when αFXIIa concentration is low, but is progressively less important as αFXIIa accumulates. Finally, our plasma-based studies indicate that while surface-bound single-chain FXII activates FXI, αFXIIa is a considerably better activator, supporting the premise that the procoagulant and prothrombotic activities of FXII are mediated largely, if not exclusively, through αFXIIa. The observation that single-chain FXII activates PK has interesting implications. While the reaction is several orders of magnitude less efficient than for αFXIIa activation of PK, the plasma PK concentration (~600 nM) is near K_m for fluid phase activation by FXII-T. Furthermore, under normal conditions the plasma concentration of FXII (~400 nM) is probably several orders of magnitude higher than that of αFXIIa. This suggests that the relatively high plasma FXII level could compensate for the weak specific activity of single-chain FXII toward PK. Given this, single-chain FXII activity may contribute to the basal reciprocal turnover of FXII and PK observed *in vivo* [19].

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AUTHORSHIP AND CONFLICT-OF-INTEREST STATEMENTS

I.I. and A.M. conducted the in vitro experiments and contributed to writing the manuscript. M-f.S. expressed, purified and characterized recombinant proteins. Q.C. designed and performed mouse experiments, S.K.D. designed recombinant FXII variants and contributed to the manuscript, and I.M.V. performed kinetic analysis and contributed to manuscript writing. J.E. performed structural analysis and contributed to writing the manuscript. D.G. oversaw the project and writing of the manuscript.

D.G. is a consultant, and has received consultant fees, for several pharmaceutical companies (Aronora, Bayer, Dyax, Ionis, Merck, Novartis, Ono) with an interest in inhibition of contact activation proteases for therapeutic purposes. The other authors do not have conflicts of interest to report.

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FIGURE LEGENDS

Figure 1. Recombinant Contact Factors. (A) Schematic diagrams of contact factors showing non-catalytic (white boxes) and catalytic (gray boxes) domains. Positions of active site serine residues are indicated by black bars. Sites of proteolysis during activation are indicated by arrows, with black arrows indicating sites of cleavage required for full protease activity. FXII is an 80 kDa polypeptide that may be cleaved at three locations. Cleavage after Arg353 converts FXII to αFXIIa. Cleavage of αFXIIa after Arg334 separates the non-catalytic and catalytic domains, forming βFXIIa. The importance of cleavage after Arg343 is not clear. The FXII non-catalytic domains are the fibronectin type 2 (F2), epidermal growth factor (EGF), fibronectin type 1 (F1), and kringle (K) domains, and a proline–rich region (PRR). PK is a 93 kDa polypeptide that is cleaved after Arg371 to form α-kallikrein (α-Kal). A second cleavage after Arg140 produces β-kallikrein (β-Kal). FXI is a homodimer of 80 kDa polypeptides. It is converted to FXIa by cleavage after Arg369. The non-catalytic portions of PK and FXI contain four apple domains, designated A1 to A4. **(B)** Coomassie blue-stained non-reducing SDS-PAGE of purified FXII (left panel) and PK (right panel) containing ~2 μg samples per lane. Positions of molecular mass standards in kilodaltons are shown to the left of the images.

Figure 2. FXII autoactivation and activation by kallikrein. (A) Plasma FXII (200 nM) was incubated in standard buffer without Poly-P (–Poly-P) or presence of 70 μM Poly-P (+ Poly-P). At the indicated times, samples were removed from the reaction into reducing sample buffer, size fractionated on a 10% polyacrylamide-SDS gel and stained with Coomassie Blue. Positions of standard for single chain FXII and the heavy chain (HC) and light chain (LC) of αFXIIa are shown at the right of each panel. **(B)** Chromogenic substrate assay for FXIIa activity for the fractions shown in the gel in panel **A**. Reactions were run in the presence (△) or absence (◆) of 70 μM Poly-P. **(C)** Recombinant FXII species (200 nM) incubated in the absence of an activator (control - left column), in the presence of 70 μM Poly-P (center column), or in the presence of 50 nM α-kallikrein without a surface (right column). At the indicated times, samples were removed into reducing sample buffer. Samples were size fractionated by SDS-PAGE, followed by western blot analysis with a polyclonal anti-human FXII IgG. For panels **A** and **C**, positions of standards for FXII

and the heavy chain (HC) and light chain (LC) of αFXIIa are indicated at the right of each image. **(D)** Recombinant FXII species (200 nM) were incubated in the presence of 70 μM Poly-P and 200 μM S-2302. Changes in OD 405 nm were continuously monitored on a microplate reader. **(E)** FXII (200 nM) was incubated with 50 nM α-kallikrein for 120 min at 37 °C in the absence of a surface. Kallikrein was inhibited with IgG H03 and FXIIa cleavage of S-2302 (200 μM) was measured.

Figure 3. Reciprocal activation of FXII and PK in the absence of a surface. (A to **C)** FXII (200 nM) and PK (200 nM) species were incubated at 37 °C. At indicated time points, samples were removed into reducing **(A** and **C)** or non-reducing **(B)** sample buffer, size fractionated by SDS-PAGE and analyzed by western blot using **(A** and **C** polyclonal IgG to FXII (XII) or PK**)** or **(B)** monoclonal IgGs that preferentially recognize the activated forms FXIIa (D06) and kallikrein (H03). **(A** and **B)** Reciprocal activation of plasma FXII and PK. **(C)** activation of PK-WT by recombinant FXII species. For panels **A** and **C**, positions of standards for FXII (XII) and the heavy chain (HC) and light chain (LC) of FXIIa; and standards for PK (PK), the heavy chain (HC) and light chain (LC) of α-kallikrein, and a fragment of the heavy chain of β-kallikrein (β) are indicated at the right of each image. For panel **B**, positions of standards for αFXIIa, βFXIIa, α-kallikrein (α-kal) and β-kallikrein (β-kal) are indicated on the right.

Figure 4. Cleavage of S-2302 and PK by FXII species. (A) 200 nM FXII-WT (WT), FXII-R353A , FXII-T (T) or FXII-S544A were incubated with 200 μM S-2302 in standard buffer in the absence of a surface. Continuous formation of amidolytic activity was monitored at 405 nm. **(B** and **C)** Varying concentration of S-2302 (1.6-1000 μM) were incubated with **(B)** 5 nM αFXIIa or **(C)** 200 nM FXII-T in the absence of a surface. Changes in OD 405 nm/min were measured on a plate reader and converted to *p*NA generated per minute. Each point is a mean ± one SD for three separate experiments. **(D)** Same as reactions described in panel **A** except that incubations were conducted in the presence of a surface (PTT-A reagent, 10% final volume). Note that PTT-A reagent only increases the amidolytic activity of WT-FXII, compared to reactions in panel **A** because, of the four FXII species tested, it is the only one that is cleaved after Arg353 and has an active site serine at residue 544. **(E)** PK-WT (200 nM) was incubated with 200 nM FXII-WT,

FXII-R353A, FXII-T, FXII-S544A or control vehicle (C). Generation of kallikrein (or kallikrein and αFXIIa in the case of FXII-WT) was continuously monitored by cleavage of S-2302 (200 μM). **(F** and **G)** Varying concentration of PK were incubated with **(F)** 25 pM αFXIIa or **(C)** 15 nM FXII-T in the absence of a surface. Kallikrein generation was determined by measuring the rate of S-2302 (200 μM) cleavage. Each point is a mean ± one SD for three separate experiments. **(H)** Generation of kallikrein from PK-WT (200 nM) was followed by continuous monitoring of S-2302 (200 μM) cleavage in the presence or absence of a surface (70 μM Poly-P), and in the presence or absence of 200 nM FXII-T (T). **(I)** Kinetic parameters for cleavage of S-2302 and PK by αFXIIa or FXII-T determined from the curves in panels **B**, **C**, **F** and **G**.

Figure 5. Activation of FXII and FXI by single chain FXII. (A) Western blots of a mixture of FXII-T (200 nM) and FXII-S544A (200 nM) in the absence of a surface (No Surface), or in the presence of PTT-A reagent (25% final volume) or Poly-P (70 μM). At indicated times samples were removed into non-reducing sample buffer, size fractionated by SDS-PAGE and analyzed by western blot using IgG D06 which recognizes formation of the FXIIa active site. The bottom row shows results for FXII-S544A and FXII-T incubated separately with 70 μM Poly-P. **(B)** FXI-S557A (30 nM) was incubated with 200 nM FXII-WT, FXII-T or FXII-S544A in the absence (top row) or presence (bottom row) of 70 μM Poly-P. At indicated times samples were removed into non-reducing sample buffer, size fractionated by SDS-PAGE and analyzed by western blot using a goat-anti-human FXI polyclonal IgG.

Figure 6. Surface-initiated clotting and thrombin generation in human plasma. (A) Clotting times in an aPTT assay for pooled normal plasma (PNP), FXII-deficient plasma (FXII DP) or FXII-deficient plasma supplemented with recombinant FXII species (FXII added to FXII DP). Each symbol indicates one clotting time and the horizontal bars indicate means for each group ± 1 SD. **(B)** Thrombin generation in FXII-deficient plasma supplemented with PTT-A reagent in the absence of FXII (No XII dotted line) or in the presence of plasma FXII (dashed line) or FXII-WT (solid line). **(C)** Thrombin generation in FXII-deficient plasma supplemented with PTT-A reagent and FXII-WT (solid line), FXII-T (dashed line) or FXII-S544A

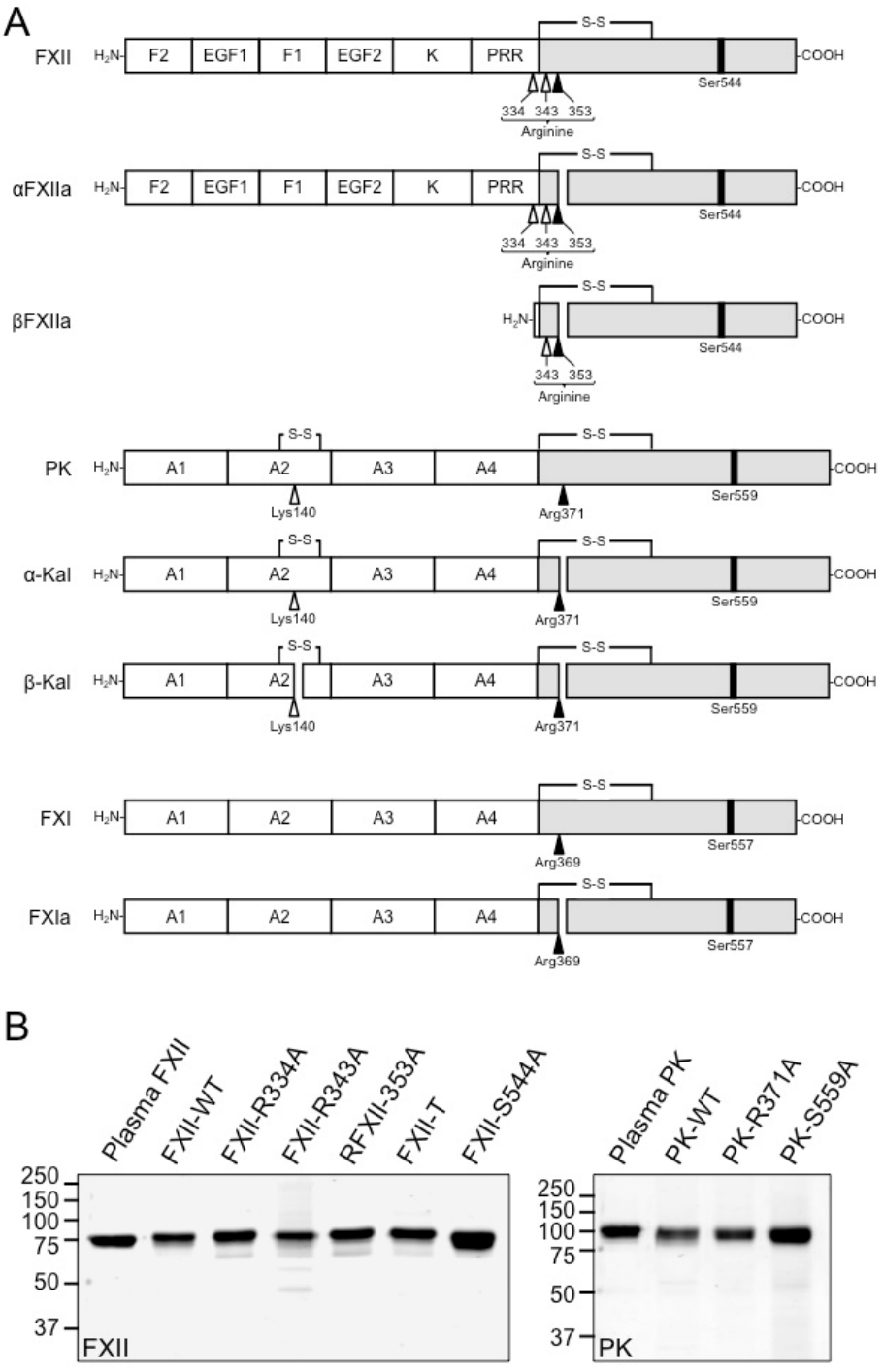
(dotted line). **(D)** Reactions for FXII-WT and FXII-T shown in panel **C** run in the presence of CTI or anti-FXI IgG 01A6. For all panels, results represent means for three runs.

Figure 7. Mouse carotid artery thrombosis model. FXII-deficient C57Bl/6 mice were infused with 100 µl of PBS (C) or 100 µl of PBS containing 25 µg of FXII-WT, FXII-R353A, FXII-T or FXII-S544A. Thrombus formation was induced by application of two pads saturated with 5% $FeCl_3$ to opposite sides of the carotid artery for three min. Flow through the artery was recorded for 30 minutes. The percent of animals with occluded arteries 30 minutes after $FeCl_3$ application are shown in the bar graph (n = 9 for each bar). Representative plasma samples from test mice were analyzed by western blot to make certain that FXII was still in the circulation at the end of the study. Abbreviations: C – FXII control; NP – normal mouse plasma; DP – FXII deficient mouse plasma. Each number indicates a separate animal.

Figure 8. Comparison of tPA and FXII structures. Shown are stick diagrams of S1 pocket structures with hydrogen bonds and electrostatic interactions shown as dotted lines (purple). In panels **A** and **B** the position of the oxyanion hole is indicated by the juxtaposed blue spheres that represent the nitrogen atoms of Ser195 and Gly193. **(A)** Single-chain tPA active S1 pocket crystal structure (pdb:1BDA) is shown with Asp194 stabilized by the salt bridge formed with Lys156 (Indicated by + and – symbols), and also by hydrogen bonds with the main-chain nitrogens of Gly142 and Cys191. The cyan stick figure represents the side-chain of the arginine P1 residue of the tPA inhibitor dansyl-Glu-Gly-Arg-chloromethylketone. **(B)** Homology model (SWISS-MODEL [48]) of the S1 pocket of FXII-T based on the tPA crystal structure where Gln156 forms a hydrogen bond to the Asp194 carboxylate group. The side-chain shown in cyan represents the P1 arginine of a substrate (PK or FXI). **(C)** Crystal structure (pdb:XDE) showing the inactive zymogen conformation of FXIIc where the oxyanion hole is absent (all figures prepared with PyMOL Molecular Graphics System, Version 1.8 Schrödinger, LLC).

FIGURES

Figure 1.



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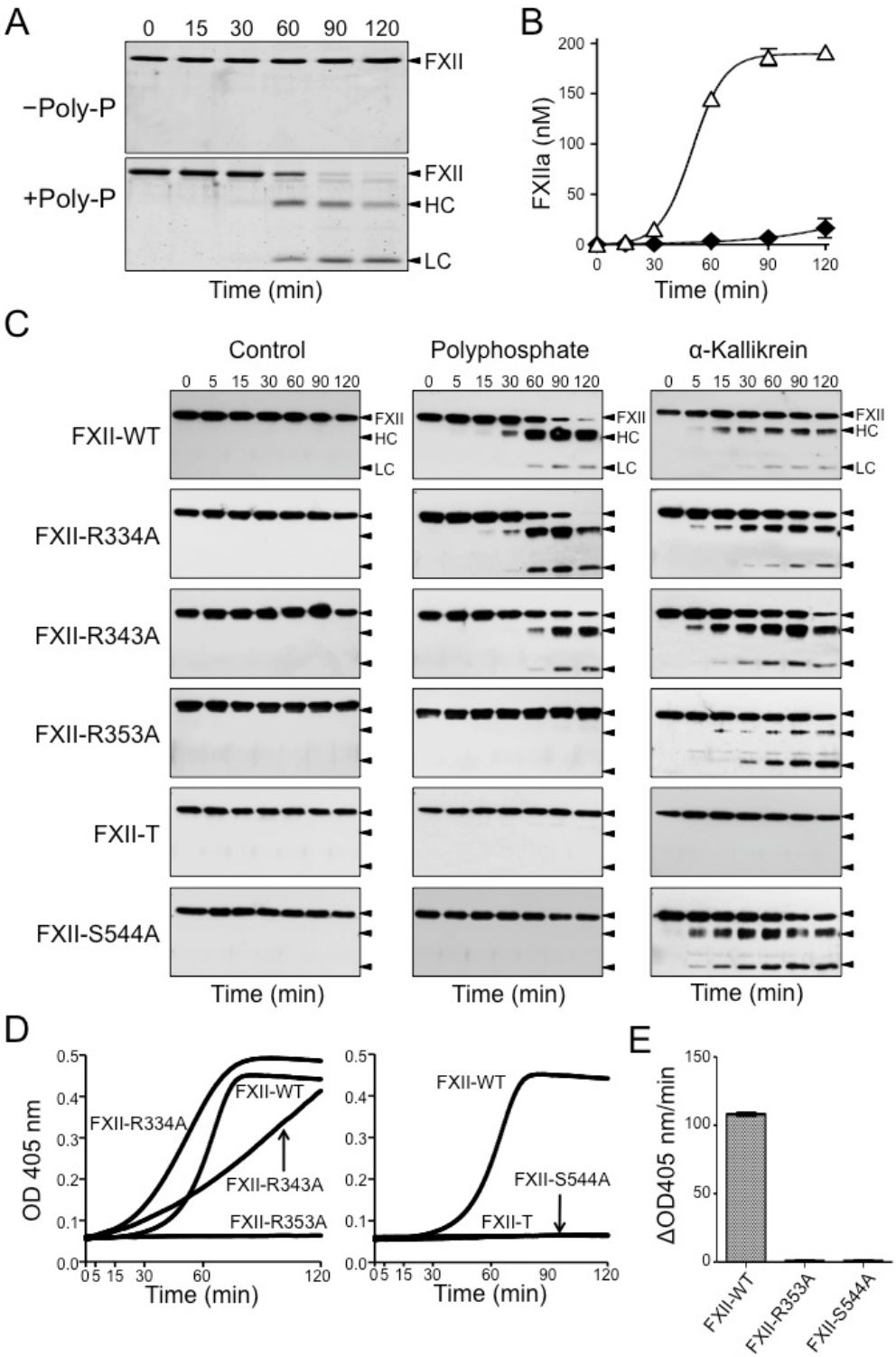
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FXII
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FXIIa (nM)
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100
50
0
0 30 60 90 120
Time (min)
C
Control
Polyphosphate
α-Kallikrein
0 5 15 30 60 90 120
FXII-WT
FXII-R334A
FXII-R343A
FXII-R353A
FXII-T
FXII-S544A
FXII
HC
LC
Time (min)
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FXII-R334A
FXII-R343A
FXII-R353A
FXII-S544A
FXII-T
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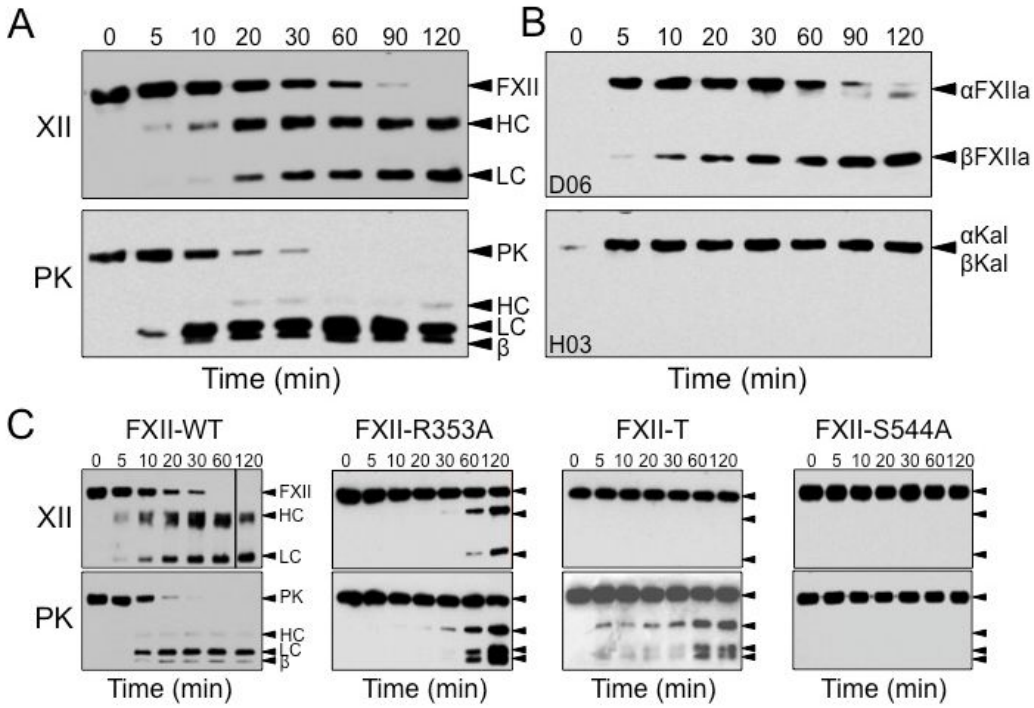
FXII-R353A
FXII-S544A

Figure 3.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H03
Time (min)
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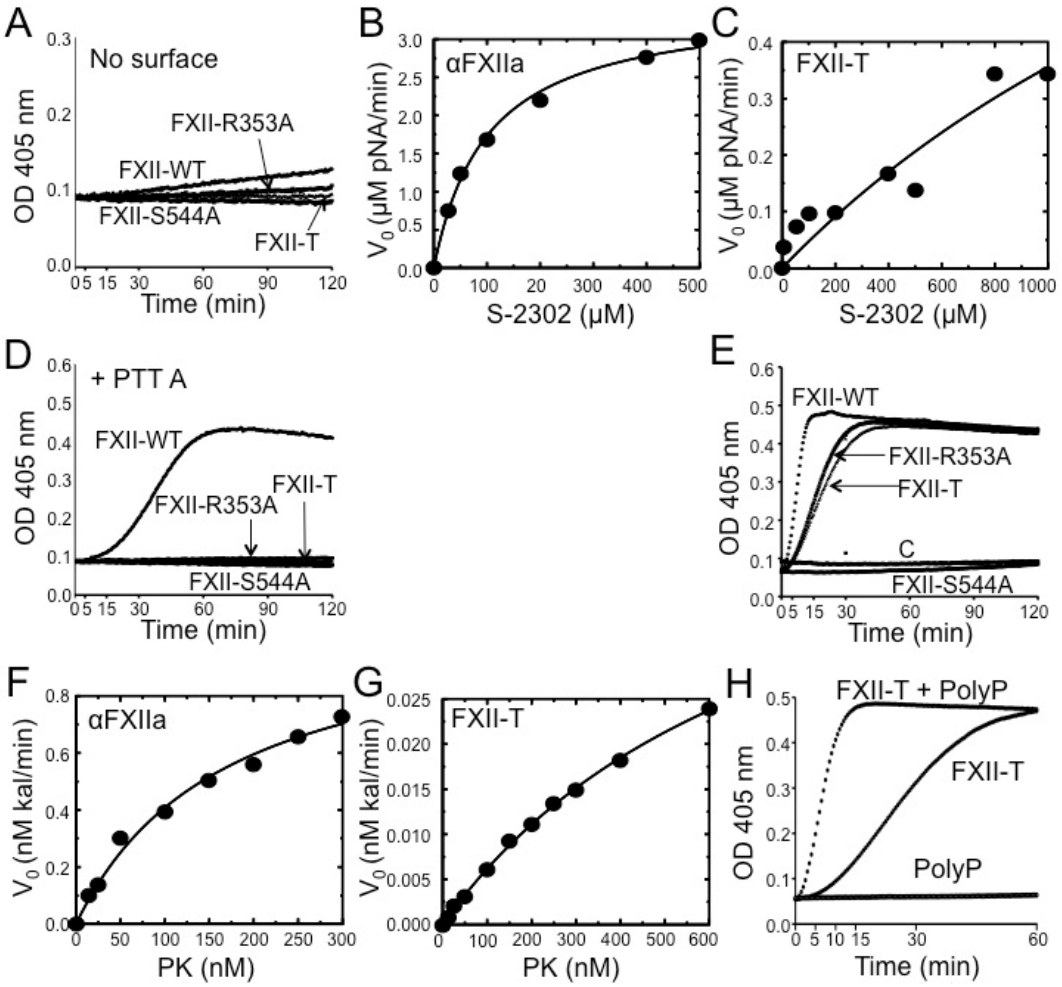
Time (min)

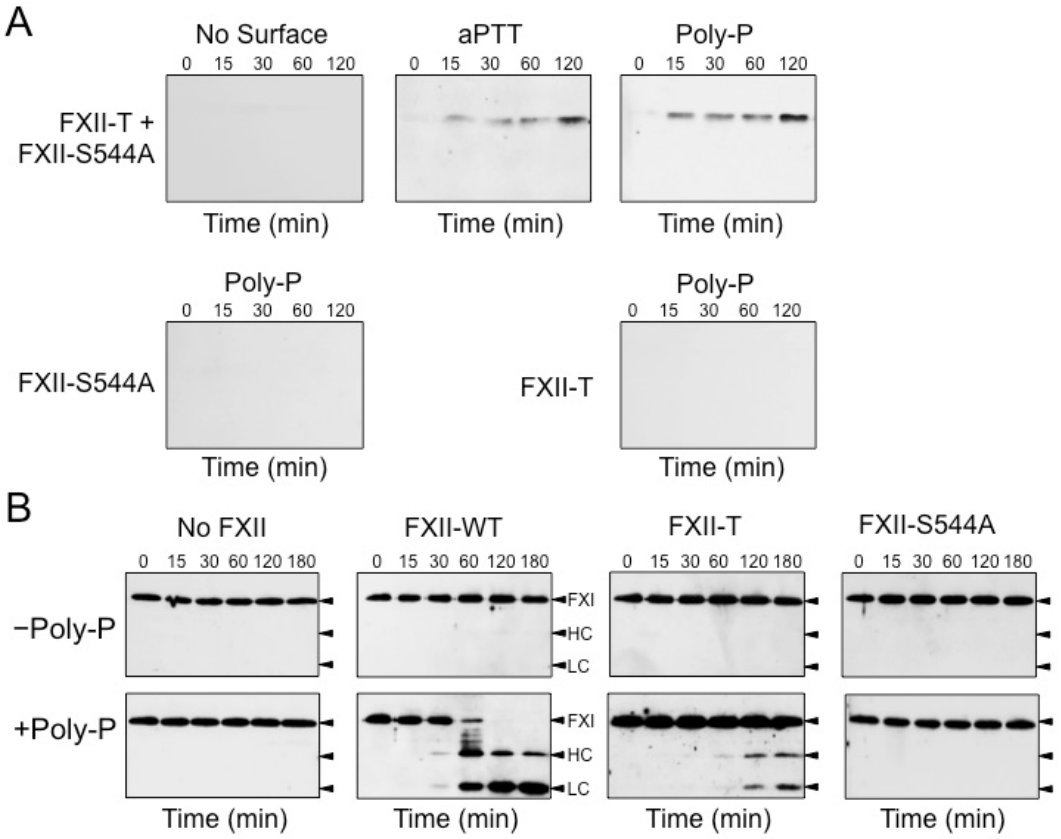
Figure 4.



I Kinetic parameters for cleavage of S-2302 and activation of PK by FXII

Protease	Substrate	K_m (μM)	K_{cat} (min^{-1})	k_{cat}/K_m ($\mu M^{-1}.min^{-1}$)
FXIIa	S-2302	100 ± 10	700 ± 20	7
	Prekallikrein	0.17 ± 0.03	44 ± 4	260
FXII-T	S-2302	2700 ± 3300	6.5 ± 6.4	0.0024
	Prekallikrein	0.80 ± 0.06	0.0037 ± 0.0002	0.0046

Figure 5.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FXII-T +
FXII-S544A
Time (min)
Poly-P
FXII-S544A
FXII-T
B
No FXII
FXII-WT
FXII-T
FXII-S544A
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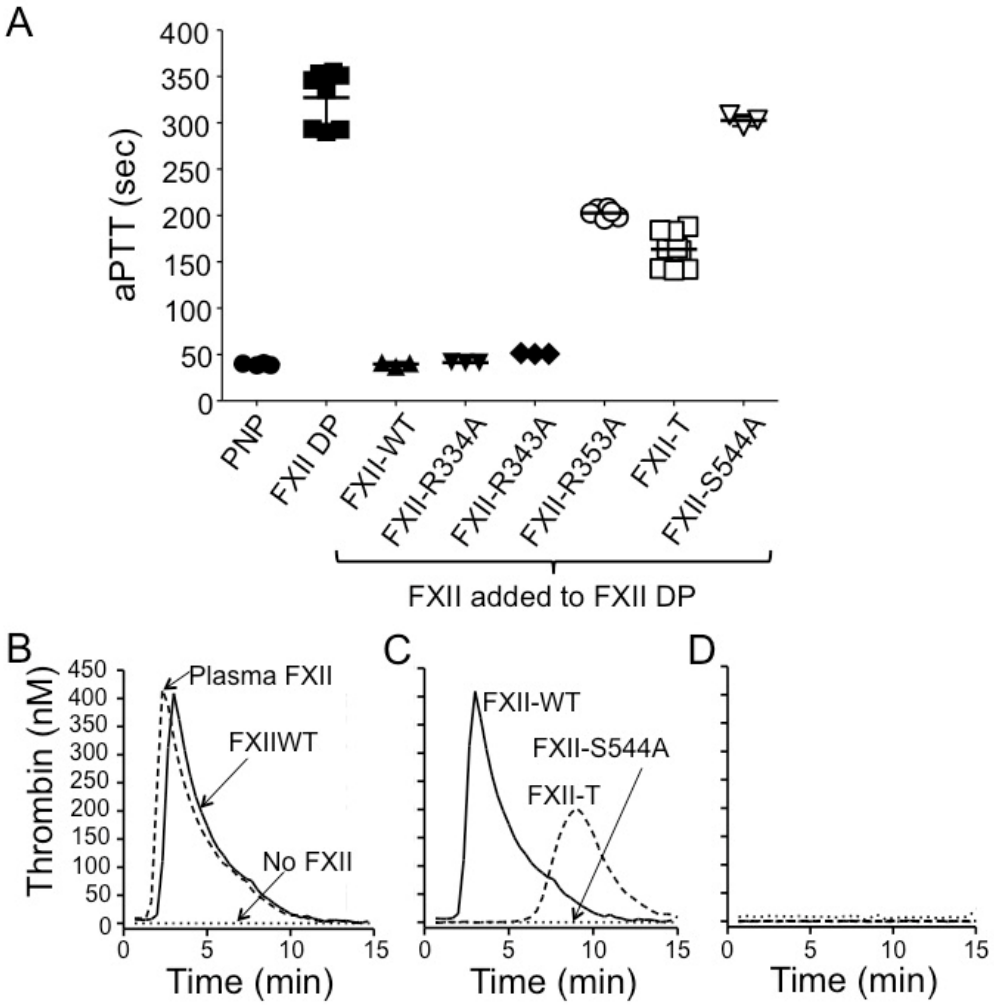
+Poly-P
FXI
HC
LC
Time (min)

Figure 6.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100
50
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FXII-R343A
FXII-R353A
FXII-T
FXII-S544A
FXII added to FXII DP
B
Thrombin (nM)
450
400
350
300
250
200
150
100
50
0
Plasma FXII
FXIIWT
No FXII
0
5
10
15
Time (min)
C
FXII-WT
FXII-S544A
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0
5
10
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Time (min)
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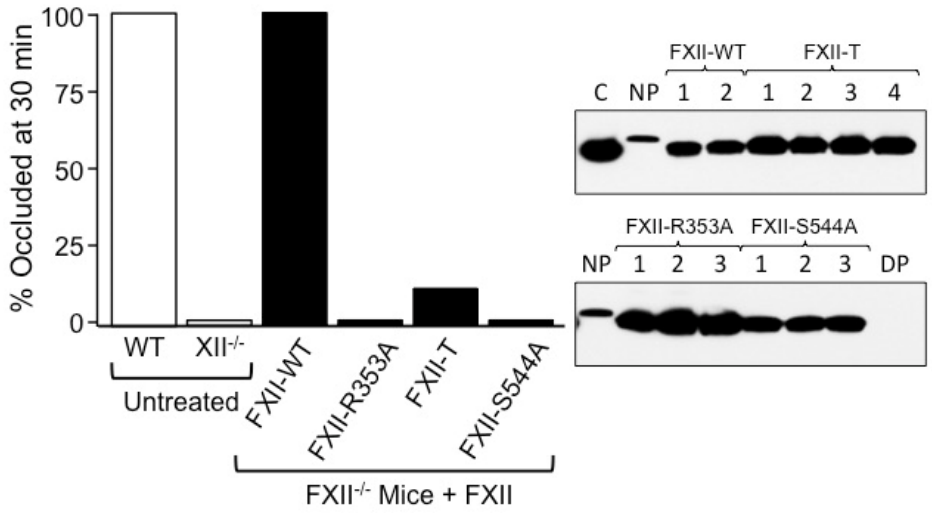
0
5
10
15
Time (min)

Figure 7.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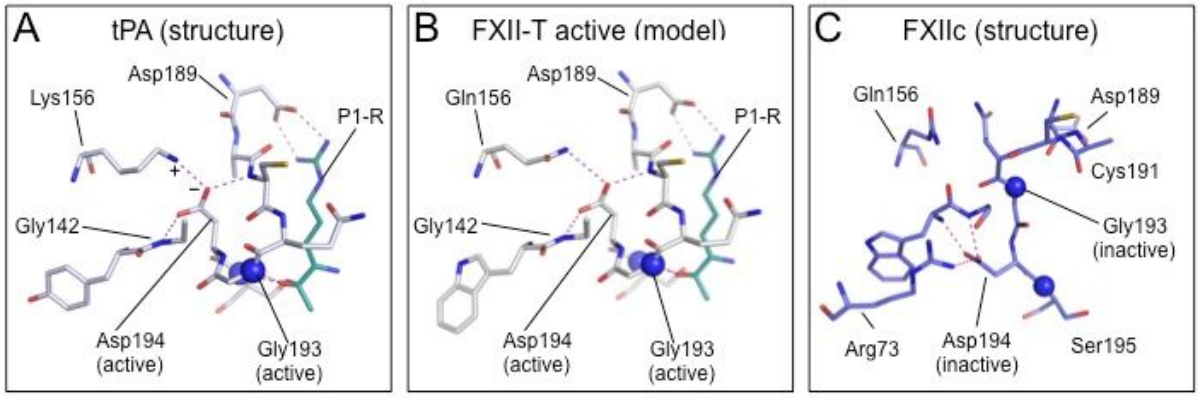
FXII-R353A FXII-S544A
NP 1 2 3 1 2 3 DP

Figure 8.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