Factor XI Contributes to Thrombin Generation in the Absence of Factor XII.

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ABSTRACT

During surface-initiated blood coagulation in vitro, activated factor XII (fXIIa) converts factor XI (fXI) to fXIIa. While fXI deficiency is associated with a hemorrhagic disorder, factor XII deficiency is not, suggesting fXI can be activated by other mechanisms in vivo. Thrombin activates fXI, and several studies suggest that fXI promotes coagulation independent of fXII. However, a recent study failed to find evidence for fXII-independent activation of fXI in plasma. Using plasma in which fXII is either inhibited or absent, we show that fXI contributes to plasma thrombin generation when coagulation is initiated with low concentrations of tissue factor, factor Xa or α-thrombin. The results could not be accounted for by fXIIa contamination of the plasma systems. Replacing fXI with recombinant fXI that activates factor IX poorly, or fXI that is activated poorly by thrombin, reduced thrombin generation. An antibody that blocks fXIIa activation of factor IX reduced thrombin generation, however, an antibody that specifically interferes with fXI activation by fXIIa did not. The results support a model in which fXI is activated by thrombin or another protease generated early in coagulation, with the resulting fXIIa contributing to sustained thrombin generation through activation of factor IX.
INTRODUCTION

The plasmas of placental and marsupial mammals contain factor XI (fXI), the zymogen of a protease (fXla) that contributes to fibrin formation and stability through activation of factor IX. Congenital factor XI deficiency is associated with a variable trauma-induced bleeding disorder in humans and other species. The mechanism by which fXI is converted to fXla during blood coagulation has been a topic of recent debate. When blood is exposed to a surface in vitro, the process of contact activation converts factor XII (fXII) to the protease fXIIa, which then activates fXI. Substances such as RNA, polyphosphates, and collagen induce pathologic coagulation in mice in a fXII-dependent manner, and may represent physiologic surfaces for fXII activation. However, the contribution of fXIIa-mediated fXI activation to normal hemostasis is unclear, as fXII deficiency, unlike fXI deficiency, is not associated with abnormal bleeding in any species in which it has been identified. This key observation supports hypotheses proposing that fXI is either activated during hemostasis by a protease other than fXIIa, or that auxiliary mechanisms for fXI activation compensate in the absence of fXII.

Candidates for fXI activating proteases include α-thrombin, meizothrombin, and fXIIa (autoactivation). Thrombin has received much attention in this regard. Several laboratories have presented evidence suggesting that a protease generated early in coagulation, such as thrombin, converts fXI to fXla. This hypothesis has been challenged by a recent study that did not find evidence for fXI activation in thrombin or tissue factor (TF) stimulated plasma in the absence of fXII. This work also showed that the process of preparing plasma can generate fXIIa, giving the false impression in subsequent assays that fXIIa-independent fXI activation has occurred. These observations have been presented in support of a hypothesis, also proposed by other investigators, that normal hemostasis in fXII deficiency reflects loss...
of fXIIa-initiated processes such as fibrinolysis that negate the propensity to bleed from simultaneous loss of fXI activation. Here, we present results of studies on the contribution of factor XII-independent fXI activation to thrombin generation in plasma, using systems designed to limit fXIIa contamination and its effects. The results show that fXI can be activated by a fXII-independent mechanisms in a plasma environment.

METHODS

Reagents: FXII-deficient plasma was from George King (Overland Park, KS). FXI, fXIIa, factor Xa, α-thrombin, and fXI-deficient plasma were from Haematologic Technologies (Essex Junction, VT). Recombi-plastin TF was from Instrument Laboratories (Lexington, MA). FXIIa and corn-trypsin-inhibitor (CTI) were from Enzyme Research (South Bend, IN). Lepirudin was from Bayer (Tarrytown, NY). S-2366 (L-pyro-glutamyl-L-prolyl-L-arginine-p-nitroanilide) was from DiaPharma (West Chester, OH). Z-Gly-Gly-Arg-AMC was from Bachem (King-of-Prussia, PA). Dioleoylphosphatidylcholine:dioleoylphosphatidylserine (7:3 w/w) was from Avanti Polar Lipids (Alabaster, AL). STA PTT Automate 5 reagent was from Diagnostic Stago (Asnières sur Seine, France). Bovine serum albumin (BSA), rabbit brain cephalin (RBC) and diisopropylfluorophosphate (DFP) were from Sigma (St. Louis).

Expression and Purification of Recombinant Factor XI.

Recombinant human fXI was expressed in HEK 293 cells, as described.26 cDNAs expressed were for (1) wild type fXI (fXlWT), (2) fXI with Lys83 and Gln84 replaced with alanine (fXI-Ala83-84), (3) fXI with Ser195, Asn196, and Ile197 replaced with alanine (fXI-Ala195-197), and (4) fXI with Ser557 replaced with alanine (fXI-Ala557). FXI was purified from conditioned media (Cellgro Complete, Mediatech, Herndon, VA) by chromatography using anti-
human factor XI-IgG 1G5.12.26 Protein was eluted with 2 M sodium thiocyanate in 50 mM Tris-HCl pH 7.5, 100 mM NaCl (Tris/NaCl), concentrated by ultrafiltration, dialyzed against Tris/NaCl, and stored at -80 °C. FXI (~200 µg/ml) was converted to fXIIa by incubation with 2 µg/ml FXIIa at 37 °C. Factor XIa was separated from factor XIIa by reapplying it to the 1G5.12 affinity column and eluting as described above.

**Characterization of recombinant factor XI.**

Serial 1:2 dilutions of fXI (65 µl), starting at 5 µg/ml, in 50 mM Tris-HCl pH 7.4, 100 mM NaCl, 0.1% BSA (TBSA) were mixed with equal volumes of fXI-deficient plasma and STA PTT Automate 5 reagent, and incubated for five min at 37 °C. After incubation, 65 µl of 25 mM CaCl₂ was added, and time to clot formation determined on a Dataclot II fibrometer (Helena Laboratories, Beaumont, TX). Results for 5 µg/ml fXI WT was designated 100% activity. The specific activity of FXI WT was similar to plasma fXI (~200 units/mg, with one unit representing the fXI activity in an ml of normal plasma). Activities of fXIa in plasma were compared by adding 65 µl of serial dilutions of protease to equal volumes of fXI deficient plasma and RBC. After 30 secs, 65 µl of 25 mM CaCl₂ was added and time to clot formation determined. Activities were determined relative to 5 µg/ml fXI WT (assigned a value of 100%).

FXI WT and fXII83-84 (25 nM) were incubated with 5 nM fXIIa or 15 nM α-thrombin at 37°C. At intervals, 50 µl aliquots were removed and supplemented with 750 µM CTI (for fXIIa) or 150 µM lepirudin (for α-thrombin). Samples were mixed with equal volumes of TBSA containing 1 mM S-2366 and changes in OD 405 nm were followed on a SpectraMAX 340 microtiter plate reader (Molecular Devices, Sunnyvale, CA). In some reactions, monoclonal antibodies to fXI (see below) were included.
**Characterization of murine anti-fXI monoclonal antibodies O1A6 and 14E11**

Murine IgG O1A6 was raised against human fXI. The antibody prolongs the clotting time of human plasma (IC$_{99}$ of ~10 nM) in a partial thromboplastin time (PTT) assay. IgG 14E11 was raised against murine fXI in a fXI deficient mouse, and prolongs the PTT of mouse and human plasma. Recombinant human fXI, prekallikrein (PK), and fXI/PK chimeras have been described. Western blots of these recombinant proteins size-fractionated on 10% polyacrylamide-SDS gels were performed using O1A6 or 14E11 as the primary antibody and chemiluminescence for detection. The effect of O1A6 and 14E11 on fXI activation was tested using the chromogenic assay described above.

**Preparation of human platelets.**

Blood was drawn from healthy volunteers into a one-tenth volume of acid citrate dextrose anticoagulant, followed by sedimentation at 200g for 20 min at RT. These experiments were approved by the Vanderbilt University institutional review board and informed consent was obtained in accordance with the Declaration of Helsinki. Platelet rich plasma (PRP) was removed from the pellet. Platelets were pelleted in the presence of 1U/ml VII grade Apyrase (Sigma) at 800g for 20 min, resuspended in Tyrode’s buffer (15mM Hepes pH 6.5, 125mM NaCl, 2.7mM KCl, 1 mM MgCl2, 0.4 mM NaH2PO4, 5.6 mM dextrose, 0.35% BSA), and passed over a Sepharose 4B (Sigma) size exclusion column. After pelleting at 800g for 20 min, platelets were resuspended in Tyrode’s buffer pH 7.4, and counted on a Hemavet HV950FS multispecies hematology instrument (Drew Scientific, Oxford, CT).

**Thrombin Generation Assay.**
Thrombin generation in plasma was measured by following cleavage of the fluorogenic substrate Z-Gly-Gly-Arg-AMC at 37 °C on a Thrombinscope® (Maastricht, The Netherlands), with thrombin-α2-macroglobulin calibrators supplied by the manufacturer. Studies were performed in 96-well plates (Immulon 2HB, Thermo Scientific, Waltham, MA) coated with PEG 20,000. Prior to use, all plasma and recombinant fXI preparations (0.7-1.9 μM in Tris/NaCl) were treated with a 1000-fold molar excess of DFP for 30 min at RT, followed by dialysis against Tris/NaCl. To avoid variation due to differences between donor plasmas, all experiments were performed on single lots of fXI- or fXII-deficient plasma containing 50 μg/ml CTI and 415 μM Z-Gly-Gly-Arg-AMC. FXI-deficient plasma was supplemented with fXI (30 nM) or vehicle. FXII-deficient plasma was supplemented with O1A6 or 14E11 IgG (300 nM) or vehicle 30 minutes before use. Addition of supplements diluted the plasma <10%.

Supplemented plasma (80 μl) was mixed with 20 μl Tyrode buffer pH 7.4 containing PC:PS vesicles (30 μM) or gel-filtered platelets (600,000/mm3), and either TF (0.96-9.6 pM), α-thrombin (30-300 nM), factor Xa (36-180 pM) or fXIIa (0.6-6 nM). Final concentrations are 5 μM PC:PS vesicles, 100,000/mm3 platelets, 0.16-1.6 pM TF, 5-50 nM α-thrombin, 6-30 pM factor Xa, or 0.1-1 nM fXIIa. For controls, 80 μl supplemented plasma was mixed with 20 μl calibrator. Reactions were initiated by adding 20 μl of 20 mM HEPES pH 7.4, 100 mM CaCl2, 6% BSA, and fluorescence was monitored (excitation λ 390 nM, emission λ 460 nM). Each set of conditions was run at least two times in triplicate. Thrombin generation was determined using Thrombinscope Analysis software, version 3.0. The area under the thrombin generation curves is referred to as the Endogenous Thrombin Potential (ETP).
RESULTS

FXI-dependent thrombin generation in FXI-deficient plasma supplemented with plasma FXI.

The contribution of fXII to coagulation was assessed by measuring thrombin-mediated cleavage of a fluorogenic substrate, as described by Hemker et al.\textsuperscript{31,32} Initial studies were performed in fXII-deficient plasma supplemented with CTI, a trypsin inhibitor that selectively binds and inhibits fXI and fXIa in plasma.\textsuperscript{33} Different sources of fXII-deficient plasma were tested, including plasma from a patient homozygous for a null mutation in the factor XI gene. All plasmas tested gave similar results, and subsequent studies used a single source of plasma. FXI was added immediately prior to addition of calcium and an initiator of coagulation (TF, factor Xa, or α-thrombin). In the absence of CTI, thrombin generation was detected in some (but not all) reactions in the absence of an initiator. This is consistent with fXI activation by fXIa. Also, some fXI preparations promoted thrombin generation in the absence of an initiator, even with CTI present, consistent with fXIa contamination of fXI. To address this, fXI preparations were treated with DFP, which irreversibly inhibits fXIa. When DFP-treated fXI was added to fXI-deficient plasma with CTI, thrombin generation was not observed after recalcification in the absence of an initiator over a two hour period. Based on these results, CTI was included in all reactions, and all preparations of fXI were treated with DFP prior to use.

In published studies, thrombin generation did not require fXI when coagulation was initiated by \( \geq 5 \) pM TF.\textsuperscript{18,20,21} In our system, the contribution of fXI to thrombin generation was observed with TF < 1.6 pM (Fig. 1A and 1B). With 0.23 pM TF, thrombin generation was significantly greater in the presence of fXI than in its absence (Fig. 1C, ETP 810 ± 174 and 180 ± 20 nM, respectively), while results were similar with 1.6 pM TF (ETP 1160 ± 79 and 1131 ±
78 nM). Similarly, thrombin generation occurred earlier, and was greater, in the presence of fXI when reactions were initiated with 6 pM, but not 30 pM, factor Xa (Fig. 1D). These findings suggest fXI is activated by a protease generated after addition of TF to plasma. This protease may be thrombin, as 5 nM α-thrombin promoted fXI-dependent thrombin generation (Fig. 1E). At this concentration, α-thrombin does not cleave the fluorogenic substrate appreciably, and the observed signal is due to activation of endogenous prothrombin.

Sensitivity of the thrombin generation assay to fXIIa.

Experiments were conducted to determine the sensitivity of the thrombin generation assay to fXIIa, and to specifically address the possibility that small residual amounts of fXIIa in the fXI preparations were influencing the results. In fXI-deficient plasma, robust thrombin generation occurred after addition of fXIIa to a final concentration of 30 pM (0.1% of plasma fXI concentration) (Fig. 2A). Late thrombin generation was detectable in some, but not all, experiments with 3 pM fXIIa, and was not observed with 0.3 pM fXIIa (Fig. 2A). If we accept the premise that fXIIa contamination of fXI explains the apparent fXII-independent contribution of fXI reported in the literature, then our fXI preparations would need to be contaminated with sufficient fXIIa to produce a final plasma concentration in excess of 3 pM. This seems unlikely, as no preparation of DFP-treated fXI promoted thrombin generation after plasma recalcification over a two hour period (see above).

Next we tested the sensitivity of the assay to fXIIa in the presence of a normal plasma concentration of fXI. In contrast to the results in the absence of fXI, 0.3 pM fXIIa reproducibly initiated thrombin generation (Fig. 2B, ETP 441 nM), comparable to results with initiation by 5 nM α-thrombin (Fig. 1E, ETP 549 nM). Given the results above, there is insufficient fXIIa contamination from the fXI preparation in this system to account for the result. That thrombin
generation occurs with fXIIa as an initiator at a concentration (0.3 pM) more than 10-fold below that needed to trigger thrombin generation (> 3 pM) strongly indicates that fXIIa is generated after addition of the initiator. Again, note that no thrombin generation was detected in the absence of the 0.3 pM fXIIa initiator, despite the presence of a physiologic fXI concentration in the plasma. The time to peak thrombin generation was longer with 0.3 pM fXIIa as an initiator than with TF or α-thrombin (Fig. 1C, 1E and 2B). This is consistent with the fXIIa initiator generating a small amount of thrombin through factor IX activation, with thrombin subsequently converting fXI to fXIIa, promoting a subsequent larger burst of thrombin formation.

Recombinant wild type factor XI and platelets in the thrombin generation assay.

Previously, we showed that recombinant wild type fXI (fXI WT) and plasma fXI have similar activities in a variety of assays, including a PTT assay (specific activity ~200 units/mg). Peak thrombin generation induced by TF was consistently higher in the presence of fXI WT than plasma fXI (Fig. 3A and 1C), and total thrombin formed was moderately greater (ETP 1267 ± 20 vs. 810 ± 174 nM, respectively). FXI WT is activated faster than plasma fXI in purified systems (unpublished data), perhaps due to differences in glycosylation. This may explain the differences in the shapes of the thrombin generation curves. While platelets have been reported to enhance fXII-independent fXI activation, in our system thrombin generation was similar in the presence of phospholipid or human platelets (Fig. 3A and B, ETP 764 and 699 nM, respectively). FXI binds to platelet glycoprotein 1bα (GP1bα). An anti-GP1b antibody did not affect thrombin generation (data not shown), again indicating that platelets are not required for fXI activation or for factor IX activation by fXIIa in this system.
Recombinant factor XI variants in the thrombin generation assay.

The contribution of fXIIa to hemostasis is mediated largely, if not exclusively, through factor IX activation. To confirm the importance of factor IX activation in our system, fXI deficient plasma was supplemented with recombinant fXI, followed by addition of TF. Two fXI variants that activate factor IX poorly were compared to fXI WT. FXI195-197 is activated normally, but has a low affinity for factor IX due to mutations in a putative factor IX binding site.26 Activated fXI-Ala557 has normal affinity for factor IX but lacks catalytic activity due to replacement of the active site serine.35 Both variants have low specific activity (< 5% of fXI WT) in the zymogen (fXI) and activated (fXIIa) forms in plasma clotting assays, and both supported thrombin generation poorly (ETP < 100 nM) (Fig. 3A).

The results so far indicate that thrombin generation in this system requires fXI to be activated in a fXII-independent manner, with fXIIa subsequently activating factor IX. Previously, an α-thrombin binding site was identified in the fXI A1 domain.36 Using a saturation mutagenesis approach, we determined that replacing fXI Lys83 and Gln83 with alanine reduces affinity for α-thrombin 100-fold (data not shown). In a PTT assay, fXI-Ala83-84 preparations exhibited 100-150% of the specific activity (200-300 units/mg) of fXI WT (200 units/mg), while fXIIa-Ala83-84 has 70-100% of the activity of fXIIa WT. These studies show that fXI-Ala83-84 is activated by fXIIa, and subsequently activates factor IX in plasma. In a purified system, α-thrombin activates fXI-Ala83-84 at ~10% of the rate of fXI WT, while fXIIa activates fXI-Ala83-84 at ~65% of the rate of fXI WT (Fig. 3C). In thrombin generation assays initiated with 5 nM α-thrombin, fXI-Ala83-84 supported thrombin generation poorly (Fig. 3D), consistent with the premise that thrombin activates fXI in this system.
Thrombin generation in XII deficient plasma triggered by TF or thrombin.

We verified the results of the previous experiments in a second system using fXII deficient plasma, where endogenous fXI has not been exposed to fXIIa, effectively preventing contact activation-mediated generation of fXIIa during plasma preparation. The PTT of this plasma was > 200 seconds, and fXII cross-reactive material was not detected with a sensitive ELISA. Exogenous fXI is not used in this system, and the anti-fXI IgG O1A6 was used to generate the equivalent of a fXI deficient state when required.27 Chimeras of fXI and the related protein PK were used to localize the O1A6 binding site to the fXI A3 domain (Fig. 4A).29 Subsequent studies (not shown) indicated that O1A6 blocks access to residues required for factor IX binding to fXIIa.26 This system addresses the argument that supplementation of plasma with exogenous fXI may result in non-physiologic interactions of fXI with some of its binding partners.9

Thrombin generation was observed in fXII-deficient plasma when coagulation was initiated with 0.23 pM TF (Fig. 5A) or 5 nM α-thrombin (Fig. 5B), and was reduced when O1A6 was included in the reactions. Similar results were obtained by depleting fXII-deficient plasma of fXI by antibody-chromatography (data not shown).37 It is interesting to note that when a larger amount of α-thrombin (50 nM) was used as an initiator, a fXI-dependent component of thrombin generation was still observed. In Fig. 5B the initial signal abutting the ordinate is due to cleavage of fluorogenic substrate by the initiator. Note the subsequent peak of thrombin generation, and its absence when O1A6 is included in the reaction. This contrasts with results when TF and fXa were used as initiators, where the fXI-dependence of thrombin generation disappeared at higher
initiator concentrations (Fig. 1C and 1D), and is consistent with thrombin being a fXI activator in this system, as suggested by an earlier study.\textsuperscript{37}

Finally, we tested the sensitivity of the fXII-deficient system to fXIIa. Interestingly, while subpicomolar fXIa stimulated thrombin generation (see above), 0.1 to 1 nM fXIIa was required to generate similar results (Fig. 5C). As expected, addition of CTI blocked fXIIa-initiated thrombin generation. Antibody 14E11 binds to the fXI A2 domain (Fig. 4B), prolongs the PTT of human plasma, and immunoprecipitates fXI from plasma (data not shown). When fXIa was incubated with 14E11 at RT for 30 minutes, its specific activity was not affected in a clotting assay, indicating 14E11 interferes with fXI activation in plasma, but not subsequent fXIa activity. Consistent with this, in a purified system, 14E11 partially inhibited fXI activation by fXIIa but not activation by $\alpha$-thrombin (Fig. 4C). In the thrombin generation assay, 14E11 significantly reduced fXIIa-initiated thrombin generation (Fig. 5C), but did not affect thrombin generation triggered by TF (not shown) or $\alpha$-thrombin (Fig. 5D). This indicates that 14E11 interferes with a specific pathway for fXI activation (through factor XIIa) that is not required for fXI-dependent thrombin generation in assays triggered by TF or $\alpha$-thrombin.
DISCUSSION

Establishing the physiologic mechanism by which fXI is activated during hemostasis has been difficult, and remains controversial. During contact activation-initiated clotting of plasma in vitro, such as in the PTT assay, factors XII, XI and IX are activated in sequence. This series of reactions may also occur in vivo. Studies with fXII-deficient mice indicate that a factor XII-initiated process contributes to arterial thrombosis and central nervous system ischemia-reperfusion injury in a manner that is dependent on fXI. However, when the bleeding diatheses associated with deficiencies of factors XII, XI and IX are considered, it is clear that hemostasis in vivo must either be different, or more complex. Factor VIIa/TF activates factor IX, explaining the severe bleeding in factor IX deficiency relative to the milder disorder in fXI deficiency. Similarly, fXI activation by a protease other than fXIIa would explain the different phenotypes of fXI and fXII deficiency.

A body of work supports a model in which fXI contributes to coagulation in the absence of fXII. von dem Borne et al. showed that clot resistance to fibrinolysis depends on fXI in plasma treated with low concentrations of thrombin or TF. As with thrombin generation in the present study, clot degradation was prevented by picomolar fXIIa. This study used fXII-deficient plasma depleted of fXI, and fXIIa contamination was considered unlikely to account for the anti-fibrinolytic effect. This group also showed that a stable form of the thrombin precursor meizothrombin promoted fXI-dependent resistance to fibrinolysis, consistent with fXI activation by a product of prothrombin activation. Cawthern et al. and Keularts et al. showed that fXI
enhanced thrombin generation in blood and plasma, respectively, when coagulation was initiated with \( \leq 5 \) pM TF.

Recently, using a capture assay for fXIIa in complex with the plasma serpin C1-inhibitor (C1-INH), Pedicord et al. did not detect fXIIa generation when thrombin or TF was added to plasma.\(^9\) In this study, plasma was prepared from the blood of healthy normal donors collected into CTI. FXIa was detected only if CTI was omitted from the collection process, and an anti-fXI antibody blocked a general marker of coagulation (chromogenic substrate cleavage) only in plasma prepared without CTI. It was concluded, justifiably, that fXI was activated by fXIIa during plasma preparation, and it was postulated that prior studies did not take adequate steps to account for fXIIa contamination, leading to the erroneous conclusion that fXI activation had occurred independent of fXII. We addressed this issue using plasma systems designed to reduce the effects of fXIIa contamination on thrombin generation by avoiding exposure of fXI to fXIIa. One system used fXI-deficient plasma containing CTI to which DFP-treated fXI was added, while the second used fXII deficient plasma in which endogenous fXI has never been exposed to fXIIa. Thrombin generation did not occur in either system with recalcification in the absence of an initiator of thrombin generation (TF or fXa), or \( \alpha \)-thrombin itself. While this showed that contaminating fXIIa, if present, was below a threshold for initiating thrombin generation, it did not completely rule out the possibility that traces of fXIIa could synergize with an initiator to enhance thrombin generation.

To address this, we tested the sensitivity of the thrombin generation assay to fXIIa, and it is important to emphasize these results. Thrombin generation was weak after addition of 3.0 pM fXIIa to fXI deficient plasma, but was substantial with 0.3 pM fXIIa in plasma containing fXII. If the latter result was due to contamination of fXI with sufficient fXIIa to promote thrombin
generation (>3.0 pM), the 0.3 pM fXIIa trigger should not have been required. This was not observed. That a fraction of the fXIIa needed to trigger thrombin generation in the absence of fXI, induced thrombin generation in its presence, strongly indicates fXIIa is generated after addition of the fXII initiator. That this process does not involve fXIIa is demonstrated by the results with fXII deficient plasma, and by the observation that an antibody that interferes with fXIIa activation of fXI did not affect the contribution of fXI to thrombin generation initiated by TF or α-thrombin. We also tested the hypothesis that a thrombin-initiated feedback loop involving activation of fXI was required for activation of factor IX. In thrombin- or TF-treated plasma, fXIIa activation of factor IX is required, as disrupting or blocking a factor IX-binding site on fXIIa prevented thrombin generation. Furthermore, fXI-Ala83-84, which is activated slowly by thrombin, supported thrombin generation poorly.

Any consideration of fXI activation in plasma must take into account the substantial literature on the effects of platelets on this reaction. Previous studies suggested that platelets enhance fXI activation by thrombin through a GP1b-dependent mechanism, although subsequent work could not confirm this. Oliver et al. observed that fXI activation was enhanced by platelets, and Wielders et al. showed that thrombin initiates and propagates thrombin generation in CTI treated plasma only when fXI and platelets are present. These data are at odds with our results, possibly because we used phospholipid vesicles to supplement plasma. In our hands, phospholipids and platelets behave similarly, and platelet-dependence in previous studies may have been related to varying levels of phospholipids in different systems.

There are several possible explanations for the discrepancies between our results and those of Pedicord et al. The amount of fXIa needed to promote thrombin generation appears to be very small, and may be below the detection limit of the fXIa-C1-INH capture assay (~5 pM).
Also, fXIIa forms complexes with several plasma inhibitors,\textsuperscript{42} and it is not clear that measuring a single fXIIa-inhibitor complex provides a complete picture of fXI activation in all systems. Furthermore, it is not certain that all fXIIa is completely inhibited during the course of the experiments, and some portion of the protease would, therefore, not be detectable by the serpin-capture technique. While fXIIa is a homodimer with two active sites,\textsuperscript{2-4} fXI activation in solution and in plasma proceeds through an intermediate with one active site (1/2-fXIIa) that can activate factor IX.\textsuperscript{43} Given the small amount of fXI that is likely to be activated in our assays, it is possible that 1/2-fXIIa is a major species. The plasma half-life of 1/2-fXIIa and its interaction with serpins have not been studied, and this protease may not be detected well by the C1-INH capture assay, which was calibrated with fully activated fXIIa.

Finally, when considering factor XI activation, it is illustrative to consider the process in the context of vertebrate evolution. FXI is the most recent addition to vertebrate plasma coagulation, probably making its appearance in marsupial mammals. In comparison, the vitamin K-dependent coagulation proteases and factor XII were present in early land vertebrates (amphibians).\textsuperscript{1} However, a gene for a protein of unknown function that is clearly ancestral to fXI and its homolog PK (another fXIIa substrate) also first appears in amphibians.\textsuperscript{1} It is likely that all terrestrial non-mammalian vertebrates have this fXI/PK predecessor. As the fXI/PK predecessor and fXII both made their debuts in early tetrapods,\textsuperscript{1} fXIIa may well be an activator of the fXI/PK predecessor, retaining the capacity to activate fXI and PK after the relatively recent duplication event responsible for generating the genes for these proteins from the predecessor gene. Given these observations, it is interesting to note that fXII expression has been lost in at least two vertebrate lineages. Cetaceans (whales, porpoises, and dolphins) share a common terrestrial ancestor, and lack fXII as a result of a point mutation that inactivated the fXII gene.\textsuperscript{44,45} Despite
this, there is no evidence of deterioration of the fXI gene,\textsuperscript{44} indicating its product remains under selection pressure because it provides an adaptive function. Similarly, birds lack fXII,\textsuperscript{46-48} but have an intact gene for the fXI/PK predecessor.\textsuperscript{1} Here, there is convincing evidence that the fXII gene was lost along the lineage leading from reptiles to birds.\textsuperscript{1} FXI and its predecessor, therefore, persist in the absence of fXII, supporting the conclusion that other proteases can activate these proteins to allow them to fulfill their functions.

**ACKNOWLEDGEMENT**

The authors thank Dr. Earl Davie (U. of Washington) for his thoughtful comments on an early version of the manuscript. This work was supported by grants HL58837 and HL81326 to D.G., and HL46213 to P.N.W, from the National Heart, Lung, and Blood Institute. **Contributions of authors:** D.V. Kravtsov and A Matafonov performed the thrombin generation experiments and analyzed all data. E.I. Tucker developed and characterized monoclonal antibodies. M. Sun generated and characterized the recombinant proteins. P.N. Walsh analyzed the thrombin activation mutant of factor XI and contributed to manuscript writing. A. Gruber developed and characterized monoclonal antibodies, analyzed data, and assisted in manuscript writing. D. Gailani designed experiments and wrote the manuscript. None of these authors has a conflict of interest, financial or otherwise, to declare.
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FIGURE LEGENDS

Figure 1. Effect of factor XI on thrombin generation in factor XI deficient plasma.

Thrombin generation in plasma is shown as Areas Under the Curve for A and B, or thrombin generation over time for C-E. Coagulation was initiated with (A) TF and Ca\(^{2+}\) in the presence (white) or absence (black) of fXI; (B) TF (0.23 pM) and Ca\(^{2+}\) (white) or Ca\(^{2+}\) alone (black) at various fXI concentrations; (C) Ca\(^{2+}\) and 1.6 pM (1 and 2) or 0.23 pM (3 and 4) TF in the presence (1 and 3) or absence (2 and 4) of fXI; (D) Ca\(^{2+}\) and 30 pM (1 and 2) or 6 pM (3 and 4) fXa, in the presence (1 and 3) or absence (2 and 4) of fXI; and (E) 5 nM \(\alpha\)-thrombin in the presence (1) or absence (2) of fXI. Error bars for A and B are one S.D.

Figure 2. Effect of factor XIa on thrombin generation in factor XI deficient plasma.

Thrombin generation in plasma supplemented with (A) vehicle or (B) 30 nM fXI. Coagulation was initiated with (1) 300, (2) 30, (3) 3.0, (4) 0.3, or (5) 0.0 pM fXIa. Note that curves 4 and 5 in panel A, and curve 5 in panel B, are essentially flat lines (no thrombin generated).

Figure 3. Effect of recombinant factor XI on thrombin generation in factor XI deficient plasma. Thrombin generation with (A and B) recombinant (1) fXI\(^{WT}\), (2) vehicle, (3) fXI-Ala\(^{557}\), or (4) fXI-Ala\(^{195-197}\); or (D) (1) fXI\(^{WT}\), (2) vehicle, or (3) fXI-Ala\(^{83-84}\). Assays contain phospholipid (A and D) or gel filtered platelets (B). (C) Activation of 25 nM FXI\(^{WT}\) (□,○) or fXI-Ala\(^{83-84}\) (■,●) by 5 nM fXIIa (□,■) or 15 nM \(\alpha\)-thrombin (○,●). FXIa generation was followed by cleavage of S2366, as described under methods.
Figure 4. Anti-factor XI monoclonal antibodies. Western blots of fXI and PK using (A) anti-human fXI IgG O1A6 or (B) anti-murine fXI IgG 14E11 as primary antibody. Abbreviations at the top indicate human fXI (H); murine fXI (M); human fXI with the human PK A1, A2, A3, or A4 domains; and human PK (PK). Positions of molecular mass standards are shown on the left. The uppercase D and M to the right of each panel indicate positions of fXI dimer and monomer (no inter-chain disulfide bond), respectively. Note that fXI/PKA4 is half the molecular mass of other fXI species because the fXI A4 domain mediates dimer formation. (C) Activation of 25 nM fXI with 5 nM fXIIa (○,●) or 15 nM α-thrombin (□,△) in the presence (●,△) or absence (○,□) of 100 nM IgG 14E11.

Figure 5. Thrombin generation in factor XII deficient plasma. Thrombin generation was initiated by addition of Ca²⁺ and (A) 0.23 pM TF or (B) 5 nM (1 and 2) or 50 nM (3 and 4) α-thrombin. Reactions were run in the absence (1 and 3) or presence (2 and 4) of 50 nM O1A6. (C) Thrombin generation initiated with Ca²⁺ and 1 nM fXIIa in the presence of (1) vehicle, (2) 50 μg/ml CTI, or (3) 50 nM 14E11. (D) Thrombin generation initiated with Ca²⁺ and 5 nM α-thrombin (1-3) or no initiator (4). Reactions were run with (1) vehicle, (2) 50 nM O1A6 or (3) 50 nM 14E11.
Figure 2

A

Thrombin (nM)

Time (min)

B

Thrombin (nM)

Time (min)
Figure 4

A

B

C

Time (min)

V (mOD/min)
Factor XI contributes to thrombin generation in the absence of factor XII

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