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ORIGINAL ARTICLE

Evidence for factor IX-independent roles for factor XIa in blood coagulation

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Summary. *Background:* Factor XIa is traditionally assigned a role in FIX activation during coagulation. However, recent evidence suggests this protease may have additional plasma substrates. *Objective:* To determine whether FXIa promotes thrombin generation and coagulation in plasma in the absence of FIX, and to determine whether FXI-deficiency produces an antithrombotic effect in mice independently of FIX. *Methods:* FXIa, FXIa variants and anti-FXIa antibodies were tested for their effects on plasma coagulation and thrombin generation in the absence of FIX, and for their effects on the activation of purified coagulation factors. Mice with combined FIX and FXI deficiency were compared with mice lacking either FIX or FXI in an arterial thrombosis model. *Results:* In FIX-deficient plasma, FXIa induced thrombin generation, and anti-FXIa antibodies prolonged clotting times. This process involved FXIa-mediated conversion of FX and FV to their active forms. Activation of FV by FXIa required the A3 domain on the FXIa heavy chain, whereas activation of FX did not. FX activation by FXIa, unlike FIX activation, was not a calcium-dependent process. Mice lacking both FIX and FXI were more resistant to ferric chloride-induced carotid artery occlusion than FXI-deficient or FIX-deficient mice. *Conclusion:* In addition to its predominant role as an activator of FIX, FXIa may contribute to coagulation by activating FX and FV. As the latter reactions do not require calcium, they may make important contributions to *in vitro* clotting triggered by contact activation. The reactions may be relevant to FXIa's roles in hemostasis and in promoting thrombosis.

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Introduction

In the cascade/waterfall hypotheses of plasma coagulation [1–3], thrombin generation is the result of a series of proteolytic reactions initiated by conversion of factor XII to the protease FXIIa. In the activated partial thromboplastin time (aPTT) assay used in clinical practice, FXIIa forms when blood comes into contact with a charged surface through a process called contact activation [4,5]. FXIIa activates FXI to FXIa, which in turn converts FIX to FIXaβ when plasma is recalcified. This set of reactions, referred to as the intrinsic pathway, although required for clot formation in the aPTT assay, probably plays a minor role in hemostasis. FXII deficiency is not associated with abnormal bleeding [4,5], and FXI deficiency causes relatively mild bleeding as compared with FIX deficiency [4–6]. However, there is mounting evidence that FXIa and FXIIa contribute to thrombosis [7–9], suggesting that reactions similar to those in the intrinsic pathway are involved in pathologic coagulation.

FXIa is usually assigned a single function in coagulation: activation of FIX [3,4]. Recently, Whelihan *et al.* observed that citrate-anticoagulated plasma supplemented with FIXaβ clots more slowly after recalcification than the same plasma after recalcification in the aPTT assay [10]. They concluded that FXIa-mediated FIX activation alone cannot account for the rate of clot formation in the assay, and showed that FXIa cleaves FVIII and FV to active forms. Here, we show that FXIa induces thrombin generation in FIX-deficient plasma by activating FV and FX. In a mouse arterial thrombosis model, combined

FIX and FXI deficiency produced a greater antithrombotic effect than deficiency of either protein alone, consistent with the hypothesis that FXIa interacts with substrates other than FIX.

Materials and methods

Reagents

FV was prepared as previously described [11]. Normal human plasma was obtained from Precision Biologics (Dallas, TX, USA). FIX-deficient and FV-deficient plasma were obtained from George King Bio-Medical (Overland Park, KS, USA). FIX-depleted plasma and PTT-A reagent were obtained from Diagnostica Stago (Asniéres sur Seine, France). FXIIa, fibrinogen and corn trypsin inhibitor (CTI) were obtained from Enzyme Research (South Bend, IN, USA). FVa, FIXaβ, FX, FXa, FXIa and prothrombin were obtained from Haematologic Technologies (Essex Junction, VT, USA). Aprotinin and hirudin were obtained from Sigma (St. Louis, MO, USA). Apixaban was obtained from Chemscene (Monmouth Junction, NJ, USA). Phosphatidylcholine (PC)/phosphatidylserine (PS) vesicles were obtained from Avanti Polar Lipids (Alabaster, AL, USA). S-2366 (L-pyro-Glu-L-Pro-L-Arg-*p*-nitroanilide), S-2765 (Z-D-Arg-Gly-Arg-*p*-nitroanilide) and S-2238 (H-D-Phe-L-Pip-L-Arg-*p*-nitroanilide) were obtained from DiaPharma (Westchester, OH, USA). Z-Gly-Gly-Arg-AMC was obtained from Bachem (Torrance, CA, USA).

Recombinant proteins

Wild-type FXI (FXI^{WT}), FXI with the prekallikrein (PK) A3 domain (FXI/PKA3) and the FXIa catalytic domain ($FXIa^{CD}$) were prepared as previously described [12]. FXI^{WT} and FXI/PKA3 were activated by incubation in 50 mM Tris-HCl (pH 7.4) and 100 mM NaCl (Tris-buffered saline [TBS]) with FXIIa.

mAbs

Anti-FIX IgG SB249417 (from J. Toomey, GlaxoSmithKline, King of Prussia, PA, USA) [13] is referred to as Anti-FIX^{Gla} in this article. Anti-FXI IgG aXIMab (O1A6) [14] is referred to as Anti-FXI^{A3}. IgG Anti-FXI^{AS} was raised by immunizing FXI-deficient Balb-C mice with $FXIa^{CD}$.

Clotting assays

FIX-deficient plasma was incubated for 30 min at 4 °C with vehicle or 300 nM Anti-FIX^{Gla}, Anti-FXI^{A3}, or Anti-FXI^{AS}. Plasma (35 μL) and PTT-A reagent (35 μL) were mixed and incubated for 5 min at 37 °C. $CaCl_2$ (35 μL, 25 mM) was added, and time to clot formation was measured on an ST-4 Analyzer (Diagnostica Stago). For FXIa-initiated clotting, plasma was supplemented with 5 μM PC/PS vesicles. FXIa in TBS with 0.1% bovine serum albumin (BSA) was incubated for 30 min at 4 °C with vehicle or 300 nM IgG (Anti-FIX^{Gla}, Anti-FXI^{A3}, or Anti-FXI^{AS}). FIX-deficient human plasma, or plasma from mice with combined deficiencies of FIX, FXI, and FXII (35 μL), were incubated with FXIa (35 μL) for 5 min at 37 °C. Clotting was initiated with 25 mM $CaCl_2$ (35 μL).

Cleavage of FV, FIX, FX, and prothrombin

FV (1 μM), FX (500 nM) or prothrombin (500 nM) was incubated in TBS/0.1% poly(ethylene glycol) (PEG) 8000 ± 1.2 mM $CaCl_2$ and FXIa (50 nM for FV and prothrombin, 125 nM for FX) at 37 °C. Aliquots were removed at various times and placed in non-reducing sample buffer. Prothrombin, FV, FIX and FX (500 nM) were incubated with 50 nM FXIIa in TBS/0.1% PEG 8000 for 60 min at 37 °C. Reactions were size-fractionated on 4–20% gradient polyacrylamide SDS gels, stained with GelCode Blue, and imaged on an Odyssey infrared imager (LI-COR Biosciences, Lincoln, NE, USA) [12].

FVa activity

FV (30 nM) was incubated with FXIa (15 nM) in TBS with 0.1% PEG 8000 and 1.2 mM $CaCl_2$ at 37 °C. Aliquots of 35 μL were supplemented with aprotinin, and mixed with 35 μL of FIX-deficient plasma containing PC/PS vesicles (5 μM). FXa (1.25 nM) and $CaCl_2$ (6.7 mM) were added, and time to clot formation was determined. Values were converted to nanomolar FVa activity by comparison with a standard curve prepared with FVa.

FXa activity

FX (150 nM) was incubated with 15 nM FXIa at 37 °C in the absence or presence of 1.2 mM Ca^{2+}, 1.0 mM Mg^{2+}, or 10 μM Zn^{2+}. Reactions were stopped with aprotinin (10 μM), and FXa cleavage of S-2765 was measured by following the change in $OD_{405\ nm}$ on a microplate reader. Values were converted to pM FXa by comparison with a standard curve. In separate assays, FX (150 nM) was incubated with FXIa (15 nM) in TBS with 0.1% PEG 8000 at 37 °C with 300 nM Anti-FIX^{Gla} or Anti-FXI^{A3}. Aliquots were supplemented with aprotinin (10 μM), and mixed with prothrombin (100 nM), FVa (1 nM), PC/PS vesicles (5 μM), and $CaCl_2$ (1.2 mM). Thrombin cleavage of S-2388 was measured by following the change in $OD_{405\ nm}$. Values were converted to pM FXa generated by use of a standard curve.

FXa activity in plasma

Normal or FIX-deficient plasma (100 μL) with or without apixaban (10 μM) was incubated with PTT-A reagent

(50 μL) at 37 °C. At various time points, reactions were stopped with CTI (4 μM), aprotinin (10 μM), and hirudin (10 μM). FXa cleavage of S-2765 was determined by following the change in $OD_{405\ nm}$. Values were converted to pM FXa by comparison with a FXa standard curve.

Thrombin activity

Prothrombin (500 nM) was incubated with FXIa (125 nM) in TBS with 1.2 mM $CaCl_2$ and 0.1% PEG 8000 at 37 °C. Reactions were stopped with aprotinin (10 μM). Cleaved prothrombin (60 nM) was tested for its ability to hydrolyze S-2238 (500 μM), and to convert fibrinogen (5 μM) to fibrin as measured with the ST-4 Coagulation Analyzer.

Thrombin generation assay

Plasma was supplemented with 415 μM Z-Gly-Gly-Arg-AMC, 5 μM PC/PS vesicles, 4 μM CTI or vehicle, and 300 nM Anti-FXI^{A3}, 900 nM Anti-FIX^{Gla} or vehicle. Supplemented plasma (40 μL) was mixed with 10 μL of Tyrode buffer (pH 7.4) containing FXIa (final concentration 2.5–15 nM) or FIXaβ (11–900 pM). Ten microliters of 20 mM Hepes (pH 7.4), 100 mM $CaCl_2$ and 6% BSA was added, and fluorescence (excitation, 390 nm; emission, 460 nm) was monitored at 37 °C on a Thrombinoscope (Maastricht, The Netherlands) [14]. Each condition was tested three times in duplicate. Peak thrombin generation and endogenous thrombin potential (ETP) were determined (Thrombinoscope Analysis software, 3.0).

Arterial thrombosis

C57Bl/6 mice were used in the study. FIX-deficient ($FIX^{-/-}$) mice were supplied by D. Stafford (University of North Carolina – Chapel Hill) [15]. FXI-deficient ($FXI^{-/-}$) mice have been described previously [16]. $FIX^{-/-}$ and $FXI^{-/-}$ mice were crossed to generate mice lacking both proteins ($FIX^{-/-}/FXI^{-/-}$). Mice (20–25 g) were anesthetized with intraperitoneal pentobarbital (50 mg kg^{-1}). The right common carotid artery was exposed and fitted with a flow probe (Model 0.5 VB; Transonic Systems, Ithaca, NY, USA). Thrombus formation was induced by applying two 1 × 1.5-mm filter papers saturated with $FeCl_3$ (2.5–15%) to opposite sides of the artery for 3 min; this was followed by rinsing with normal saline [16,17]. Flow was monitored for 30 min. Results were compared by use of the χ^2-test.

Results

Anti-FIX and anti-FXI antibodies in clotting assays

Three antibodies were used to study FXIa activity in plasma. Anti-FIX^{Gla} is a potent inhibitor of FIX activation and FIXaβ activity [13]. Anti-FXI^{A3} blocks an exosite required for FIX activation on the FXIa A3 domain [12], and also inhibits FXI activation by FXIIa. Anti-FXI^{AS} was raised against $FXIa^{CD}$. It appropriately recognizes FXI and $FXIa^{CD}$ on western blots (Fig. 1A), and inhibits FXIa cleavage of S-2366 (Fig. 1B), indicating that it binds at the active site or alters its conformation. The aPTT of normal plasma (45 ± 4 s) was prolonged by Anti-FXI^{A3} (180 ± 24 s). Anti-FXI^{AS} had a smaller effect (67 ± 4 s), indicating that it is not as potent as Anti-FXI^{A3} at inhibiting FIX activation. Neither Anti-FXI^{A3} (232 ± 16 s) nor Anti-FXI^{AS} (274 ± 21 s) affected clotting in FXI-deficient plasma (259 ± 29 s; Fig. 1C), consistent with specificity for FXI/FXIa.

The aPTT of plasma from an FIX-deficient patient (Fig. 2A; 106 ± 2 s, antigen <0.1% of normal), as expected, changed little with addition of Anti-FIX^{Gla} (114 ± 12 s), but was prolonged with Anti-FXI^{A3} (154 ± 3 s) and Anti-FXI^{AS} (199 ± 2 s) (Fig. 2A). In contrast to its effect on normal plasma, Anti-FXI^{AS} had

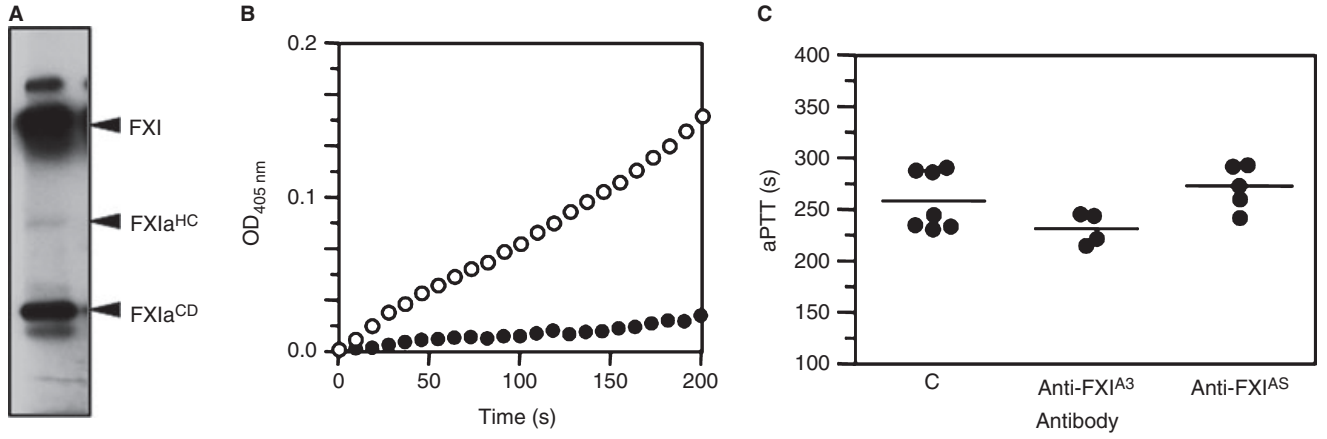

Fig. 1. Anti-FXI IgGs. (A) Western blot of a mixture of reduced FXI and FXIa with Anti-FXI^{AS} for detection. The positions of FXI, the FXIa heavy chain ($FXIa^{HC}$) and the FXIa catalytic domain ($FXIa^{CD}$) are indicated. (B) Cleavage of S-2366 by FXIa in the absence (○) or presence (●) of Anti-FXI^{AS}. (C) Effects of Anti-FXI^{A3} or Anti-FIX^{AS} on the activated partial thromboplastin time (aPTT) of FXI-deficient plasma.

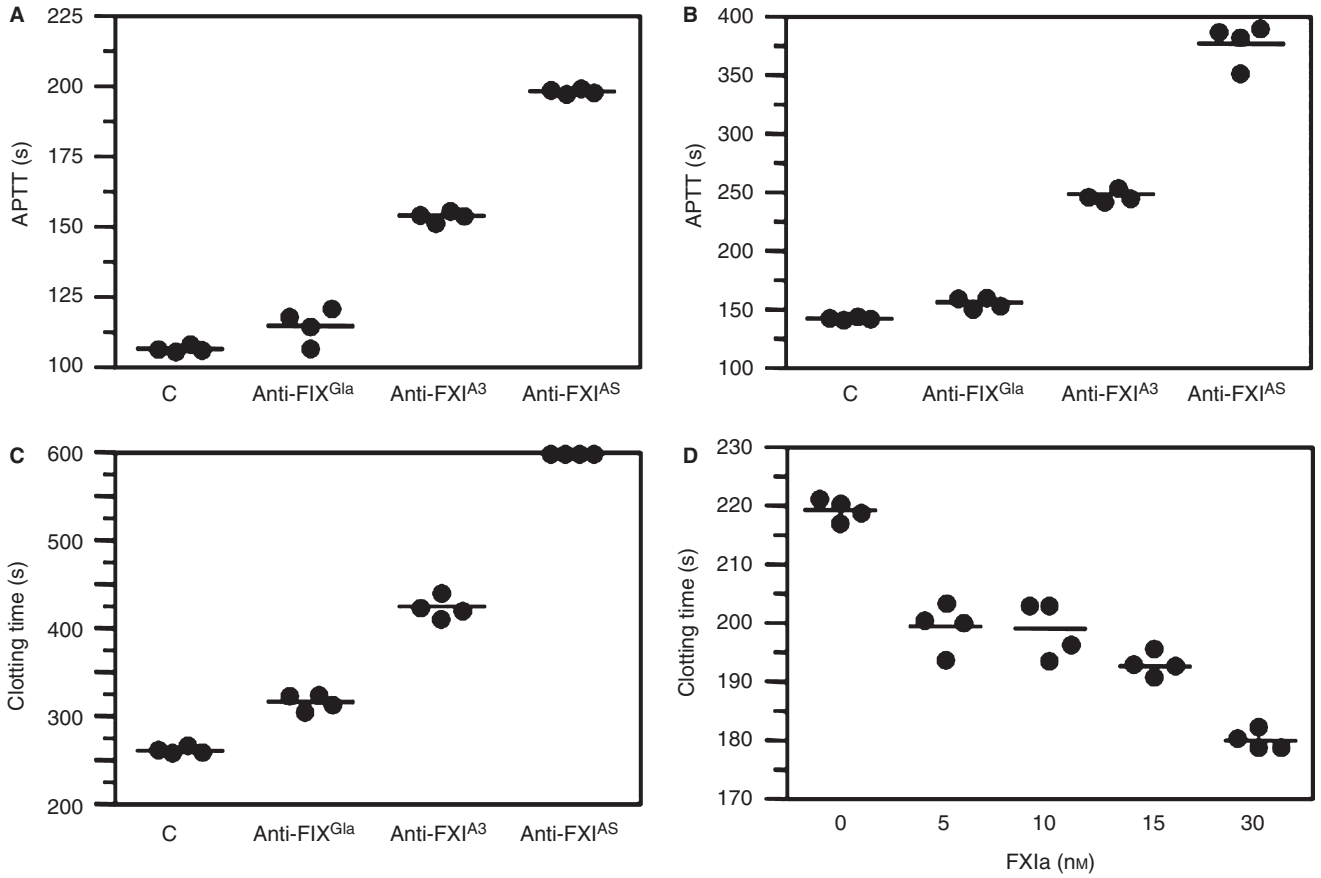

Fig. 2. Clotting assays. aPTTs of FIX-deficient (A) or FIX-depleted (B) plasma with control (C), Anti-FIX^{Gla}, Anti-FXI^{A3} or Anti-FIX^{AS}. (C) Clotting time of FIX-deficient plasma supplemented with 3 nM FXIa and antibodies. (D) Clotting time of plasma from mice lacking FIX, FXI and FXII supplemented with human FXIa. Each circle indicates one clotting time. Bars indicate mean clotting time.

a larger effect than Anti-XFI^{A3} in FIX-deficient plasma. Similar results were obtained with FIX-depleted plasma (vehicle, 142 ± 2 s; Anti-FIX^{Gla}, 156 ± 9 s; Anti-FXI^{A3}, 248 ± 10 s; Anti-FXI^{AS}, 379 ± 36 s; Fig. 2B).

Addition of FXIa (3 nM) directly to FIX-depleted plasma induced clotting in 261 ± 8 s. Anti-FXI^{A3} (432 ± 26 s) and Anti-FXI^{AS} (> 600; Fig. 2C) again prolonged time to clot formation (Fig. 2A,B), indicating they influence clotting by inhibiting FXIa, despite the absence of FIX. Clotting times in FXIa-initiated assays are longer than in aPTT assays, because the aPTT reagent induces the generation of higher FXIa concentrations.

Although a trace of FIX in FIX-deficient plasma could facilitate FXIa-initiated coagulation, it would not account for the large effects of the anti-FXI antibodies. We tested the ability of human FXIa to shorten the clotting time of plasma from mice deficient in all three intrinsic pathway proteases (FIX, FXI, and FXII; Fig. 2D). The gene disruptions in these animals prevent FIX, FXI and FXII synthesis [15,16]. This plasma was used because clotting times of plasma from mice lacking only one intrinsic protease are relatively short as compared with those of comparable human plasmas, making them unsuitable for the assay. The results (vehicle, 219 ± 3 s; 30 nM FXIa, 179 ± 3 s) support the premise that FXIa can promote clotting in an FIX-independent manner, although the effect is modest, perhaps because of the relatively short baseline clotting time of the plasma.

FXIa cleavage of FV

FV and FX are involved in the step downstream of FIX in the coagulation cascade. Whelihan *et al.* showed that FXIa cleaves FV, generating FVa activity [10]. The cleavage pattern is more complex than for FVa generated by thrombin, but the light and heavy chains and the B domain of FVa are evident (Fig. 3A). Interestingly, the reaction proceeds faster without Ca^{2+}, suggesting that FXIa activation of FVa in the aPTT assay occurs primarily during the contact phase before recalcification. FXIa with the A3 domain replaced with the PK A3 domain (FXIa/PKA3) cleaves the tripeptide S-2366 similarly to wild-type FXIa (FXIa^{WT}), but has a significant defect in FIX activation [12]. In reactions with FXIa/PKA3, the FV band disappeared at half the rate as in reactions with

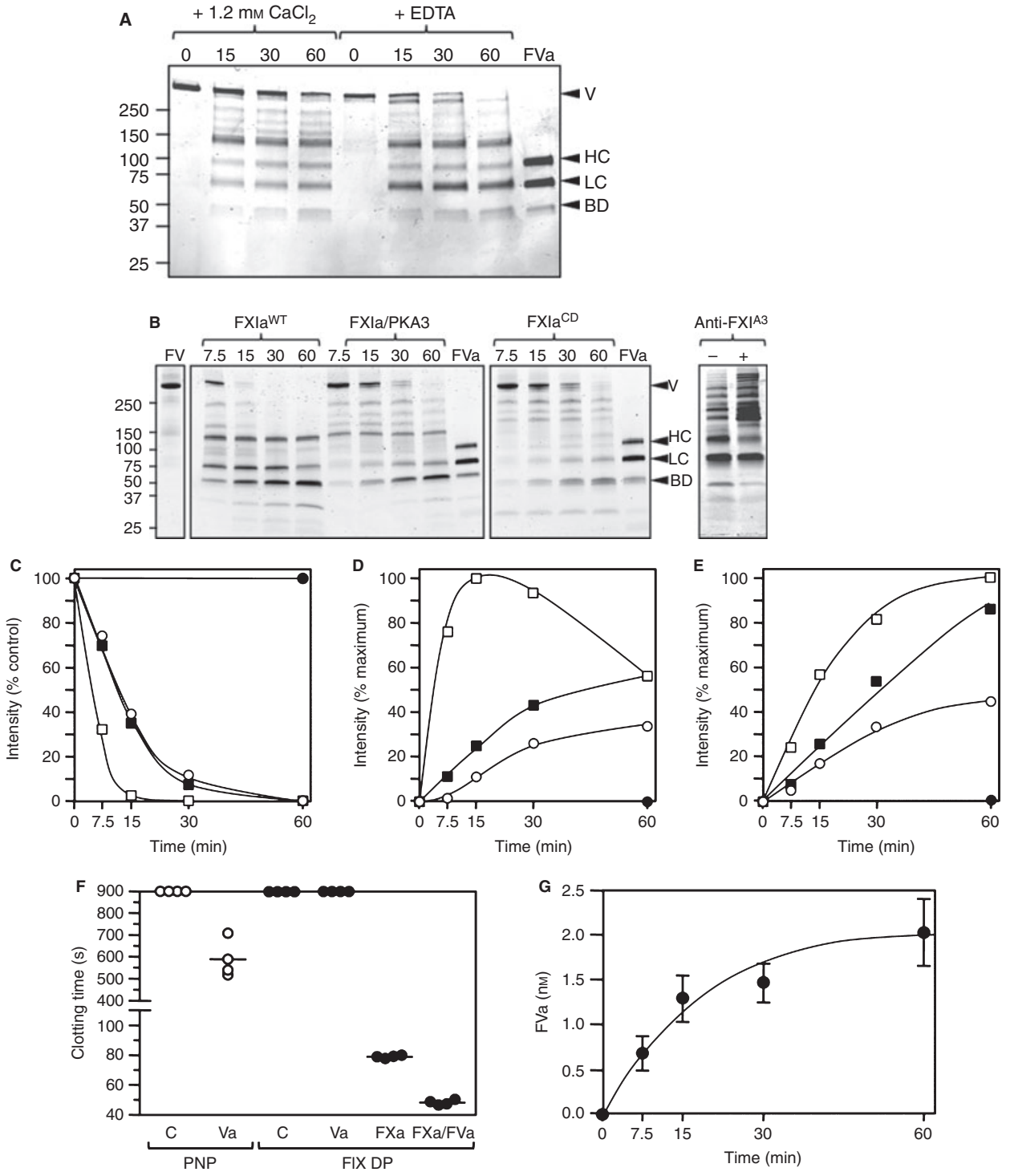

Fig. 3. FV cleavage by FXIa. (A) Non-reducing SDS-PAGE of FV (150 nM) incubated with plasma FXIa (15 nM) in the presence or absence of 1.2 mM Ca^{2+}. (B) SDS-PAGE of FV (500 nM) incubated with vehicle (left panel), or 50 nM wild-type FXIa ($FXIa^{WT}$), FXIa with the A3 domain replaced with the prekallikrein A3 domain (FXIa/PKA3), or the FXIa catalytic domain ($FXIa^{CD}$). Right panel: FV incubated with $FXIa^{WT}$ for 60 min with (+) or without (–) Anti-FXI^{A3}. For (A) and (B), minutes of incubation are indicated at the top, the positions of mass standards (kDa) on the left, and the positions of FV, FVa heavy chain (HC), FVa light chain (LC) and B domain (BD) standards on the right. (C–E) Disappearance of FV (C), and appearance of FVa light chain (D) and B domain (E), determined by densitometry of the gels in (B). Symbols: (●), vehicle (V); (○), $FXIa^{WT}$; (■), FXIa/PKA3; (□), $FXIa^{CD}$. (F) Clotting times for normal (PNP) or FIX-deficient (FIX DP) plasma supplemented with 30 nM FVa, 1.25 nM FXa, or both. Each symbol indicates one clotting time. Bars indicate mean clotting time. (G) FV (30 nM) incubated with FXIa (15 nM). At various times, FVa activity was determined as described in Materials and methods.

$FXIa^{WT}$ (Fig. 3B,C), with slower FVa light chain (Fig. 3D) and B domain (Fig. 3E) accumulation. Similar results were obtained with $FXIa^{CD}$, which also lacks the A3 domain (Fig. 3B–E). Anti-FXI^{A3}, which binds to the A3 domain, reduced FVa light chain generation by FXIa by ~ 50% (Fig. 3B), consistent with a role for the A3 domain in FV activation.

Addition of 30 nM standard FVa (generated with thrombin) induced clotting in recalcified normal plasma (586 ± 173 s) but not in FIX-deficient plasma (> 900 s) (Fig. 3F). FVa requires FXa to produce thrombin, and the result could be explained by a higher FXa concentration in normal plasma than in FIX-deficient plasma. Addition of FXa to FIX-deficient plasma gave clotting times of 78 ± 3 s, and addition of FXa and FVa shortened the clotting time further (45 ± 3 s; Fig. 3F). On the basis of these observations, we developed a FVa activity assay with FIX-deficient plasma supplemented with FXa. FV cleaved by FXIa had ~ 6% of the activity of an equivalent amount of standard FVa (Fig. 3G), consistent with data from Whelihan *et al*. [10] showing a lower specific activity than for standard FVa. The results also show that FV activation alone is insufficient to explain the capacity of FXIa to induce clotting in FIX-deficient plasma.

FX activation by FXIa

Activation of the homologs FIX and FX involves the release of an activation peptide. FXIa cleaved FX to a product that migrated similarly to FXa (Fig. 4A) on SDS-PAGE, although high FXIa concentrations were required to make this evident. At more physiologic concentrations, FX cleaved by FXIa shortened the clotting time of FIX-deficient plasma (216 ± 2 s), whereas FX incubated without FXIa did not (Fig. 4B). This effect was blocked by the FXIa inhibitor aprotinin (> 450 s), but not appreciably by Anti-FXI^{A3} (265 ± 3 s; Fig. 4B), indicating a minor role for the FXIa A3 domain in the reaction. Consistent with this, FX was activated similarly by $FXIa^{WT}$, FXIa/PKA3 or $FXIa^{CD}$ (Fig. 4C). Addition of divalent cations had little effect on FX activation by FXIa (Fig. 4D), again suggesting that FX, unlike FIX, does not bind the FXIa A3 domain. FX cleaved by FXIa converted prothrombin to

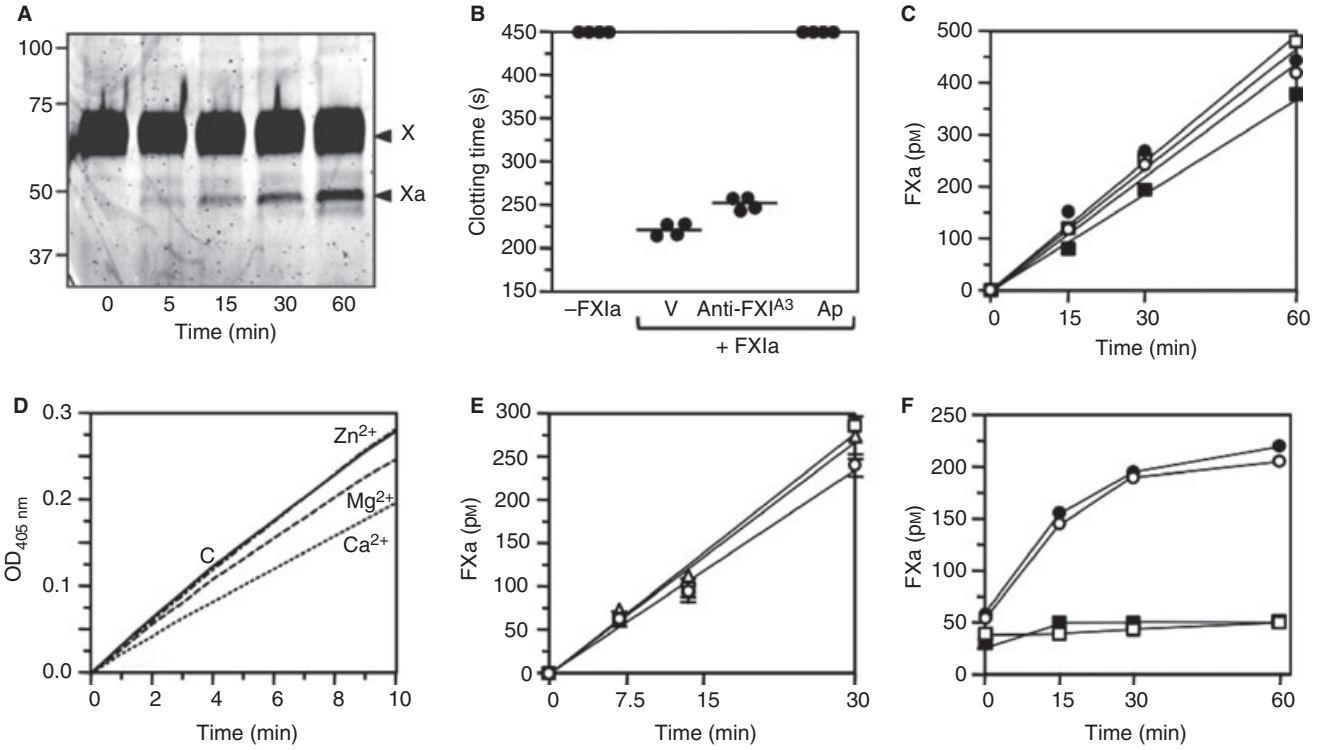

Fig. 4. FX cleavage by FXIa. (A) Non-reducing SDS-PAGE of FX (500 nM) incubated with FXIa (125 nM). The positions of FX and FXa are indicated on the right. Mass standards (kDa) are shown on the left. (B) FX (150 nM) was incubated for 1 h with (+ FXIa) or without (− FXIa) 15 nM FXIa, in the presence of vehicle (V), Anti-FXI^{A3}, or aprotinin (Ap). Aliquots were added to FIX-deficient plasma (18.75 nM final concentration), and clotting was initiated with $CaCl_2$. Each circle indicates one clotting time. Bars indicate mean clotting time. (C) FX (150 nM) was incubated with 15 nM plasma FXIa (○), wild-type FXIa (●), FXIa with the A3 domain replaced with the prekallikrein A3 domain (□), or the FXIa catalytic domain (■). FXa was measured with a chromogenic assay. (D) Cleavage of S-2765 (250 μM) by FX (150 nM) incubated for 1 h with 15 nM FXIa in the absence (C) or presence of divalent cations (1.2 mM Ca^{2+}, 1 mM Mg^{2+}, or 10 μM Zn^{2+}). (E) FX (150 nM) was incubated with FXIa (15 nM) and vehicle (○), Anti-FIX^{Gla} (Δ), or anti-FXI^{A3} (□). FXa was measured with a prothrombinase assay. (F) Normal (○, □) or FIX-deficient (●, ■) plasma was incubated with PTT-A reagent. Aliquots were mixed with corn trypsin inhibitor, aprotinin, and hirudin, with (□, ■) or without (○, ●) apixaban. FXa was measured with a chromogenic assay.

α-thrombin in the presence of FVa and Ca^{2+} (Fig. 4E). Purified plasma FX may contain FIX. Using a FIX-specific ELISA, we determined that 10 μg of FX contained ~ 0.3 ng of FIX (0.003%). Anti-FIX^{Gla} prevented FX activation by FIXaβ in the prothrombin activation assay (data not shown), but did not affect FX activation by FXIa (Fig. 4E), supporting the premise that FXIa, and not FIXaβ, activates FX.

These observations suggest that FXIa can convert FX to FXa during the aPTT contact phase. This was demonstrated by incubating normal and FIX-deficient plasmas with PTT-A reagent without recalcification. After incubation, FXIIa, FXIa and α-kallikrein were inhibited with CTI and aprotinin, and thrombin was inhibited with hirudin. Amidolytic activity towards the tripeptide S-2765 was noted in both plasmas that could be neutralized by the FXa-specific inhibitor apixaban (Fig. 4F), consistent with FXa generation during contact activation.

Thrombin generation in the absence of FIX

Subpicomolar FXIa induces thrombin generation in normal plasma [14]. Nanomolar FXIa induced thrombin generation in FIX-deficient plasma (Fig. 5A), an effect that did not occur in the presence of apixaban or the absence of FV (Fig. 5B). Thrombin was generated by as little as 10 pM FIXaβ (Fig. 5C), and this was blocked by Anti-FIX^{Gla} (Fig. 5D). Anti-FIX^{Gla} was included in the studies in Fig. 5A to account for possible FIX contamination. Cumulatively, the data support the hypothesis that FXIa activates FV and FX in the absence of FIX.

ETP (1970 ± 10 vs. 1170 ± 15 nM min^{-1}) and peak thrombin generation (150 nM vs. 65 nM) were greater in FIX-deficient plasma supplemented with $FXIa^{WT}$ than in FIX-deficient plasma supplemented with FXIa/PKA3 (Fig. 5E). Recall that these proteases are equally effective FX activators, but that $FXIa^{WT}$ cleaves FV more effectively than FXIa/PKA3. The results in Fig. 5E are consistent with a scenario in which both proteases activate FX comparably, with FXIa/PKA3 having a reduced capacity to activate FV. In support of this, Anti-FXI^{A3}, which interferes with FXIa activation of FV, but not FXIa activation of FX, reduced thrombin generation induced by $FXIa^{WT}$ (1470 ± 10 vs. 650 ± 40 nM min^{-1}, respectively; Fig. 5F).

FIX-independent FXIa activity in vivo

Removing the tail tip of $FIX^{-/-}$ mice with a scalpel typically leads to exsanguination [15], whereas bleeding in $FXI^{-/-}$ mice is mild and comparable to that in wild-type mice [16]. $FIX^{-/-}/FXI^{-/-}$ mice are viable, with a bleeding propensity similar to that of $FIX^{-/-}$ mice. The double deficiency did not affect reproduction. Measurements of plasma FVIII, FIX, FXI and FXII with one-stage clotting assays confirmed a lack of activity resulting from specific gene disruptions, without significant differences in other factors (data not shown), indicating that loss of an intrinsic pathway factor does not cause large compensatory changes in other factors.

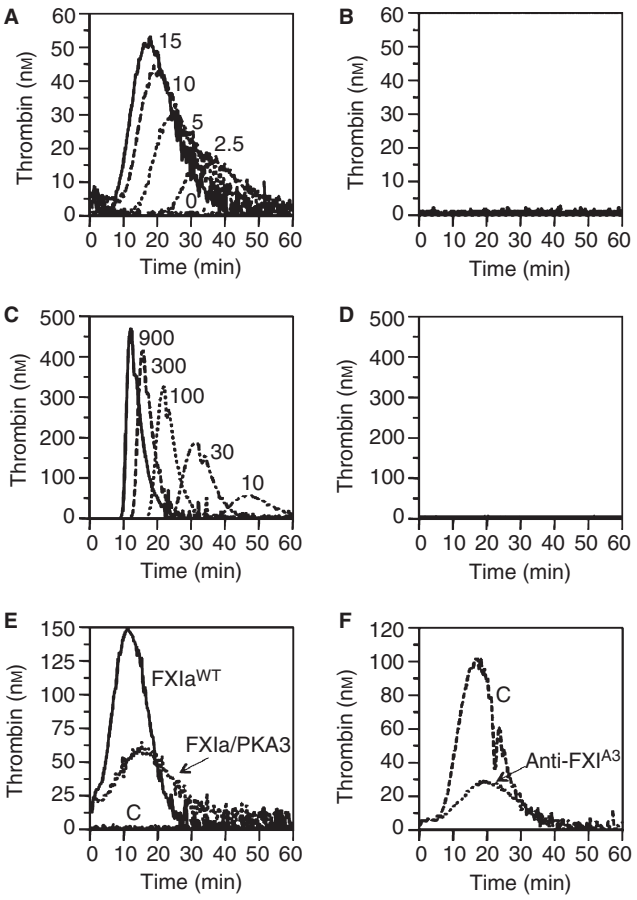

Fig. 5. Thrombin generation. (A–C) Thrombin generation in: (A) FIX-deficient plasma supplemented with FXIa (0–15 nM) and 900 nM Anti-FIX^{Gla}; (B) FIX-deficient plasma containing apixaban or FV-deficient plasma initiated by 15 nM FXIa; and (C) FIX-deficient plasma initiated by FIXaβ (10–900 pM). (D) Same as (C), with 900 nM IgG Anti-FIX^{Gla} included. (E) FIX-deficient plasma supplemented with 900 nM IgG Anti-FIX^{Gla} and 15 nM wild-type FXI ($FXIa^{WT}$), FXIa with the A3 domain replaced with the prekallikrein A3 domain (FXIa/PKA3), or control (C). (F) FIX-deficient plasma supplemented with 900 nM IgG Anti-FIX^{Gla} and 15 nM $FXIa^{WT}$ in the presence of control (C) or Anti-FXI^{A3}. All plasmas were supplemented with 4 μM corn trypsin inhibitor to inhibit FXIIa.

Despite the differences in bleeding propensity, $FIX^{-/-}$ and $FXI^{-/-}$ mice were comparably resistant to $FeCl_3$-induced carotid artery thrombosis (Fig. 6) [17]. Wild-type mice developed artery occlusion with ≥ 3.5% $FeCl_3$, whereas $FIX^{-/-}$ and $FXI^{-/-}$ mice were resistant to occlusion with 5% $FeCl_3$, and partially resistant at 7.5% ($P = 0.001$ and $P = 0.025$, respectively, as compared with wild-type mice). If the thrombotic effect of FXIa is mediated exclusively through FIX activation, $FIX^{-/-}/FXI^{-/-}$ and $FIX^{-/-}$ mice should behave similarly in this assay. However, whereas $FIX^{-/-}$ and $FXI^{-/-}$ mice consistently showed occlusion with 10% $FeCl_3$, some $FIX^{-/-}/FXI^{-/-}$ mice did not show occlusion even at 12.5% $FeCl_3$ ($P = 0.025$ and $P = 0.06$ as compared

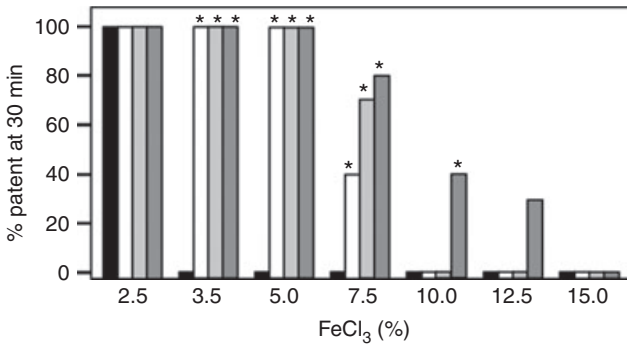

Fig. 6. $FeCl_3$-induced carotid artery thrombosis. Occlusion of the carotid artery in C57Bl/6 mice was induced by applying $FeCl_3$ (2.5–15%) to the vessel. Groups of 10 wild-type (black bars), $FXI^{-/-}$ (white bars), $FIX^{-/-}$ (light gray bars) and $FIX^{-/-}/FXI^{-/-}$ (dark gray bars) mice were tested at each $FeCl_3$ concentration. Bars represent percentages of mice with patent arteries 30 min after $FeCl_3$ exposure. For each $FeCl_3$ concentration, results marked with asterisks (*) were significantly different from the wild type for that concentration ($P < 0.05$).

with other genotypes for 10% and 12.5% $FeCl_3$, respectively). This is consistent with FXIa activating substrates in addition to FIX.

Discussion

The catalytic domains of α-thrombin and the enzymes that contribute to its generation are homologs of the pancreatic protease trypsin [18]. Although coagulation proteases show varying propensities to behave like trypsin (cleaving proteins after basic amino acids) when tested with pure substrates, they show more restricted specificity in plasma. For these proteases, substrate affinity and specificity are governed by exosite interactions [19,20]. Exosites are substrate-binding sites that are distinct from the protease active site. FXIa contributes to thrombin generation primarily through Ca^{2+}-dependent activation of FIX. The gene for FXI is the result of a duplication of the PK gene [21]. For FXI, adaptation to a role as an FIX activator included changes to the parent PK sequence, producing a high-affinity FIX-binding exosite on the A3 domain [12,22]. It is clear that FIX is the major plasma substrate for FXIa, but there is evidence that it can act on a wider range of targets.

In the aPTT assay, a charged substance added to plasma containing the Ca^{2+} chelator sodium citrate results in Ca^{2+}-independent contact activation of FXII, FXI, and PK [4,5]. With recalcification, FXIa converts FIX to FIXaβ, leading to clot formation. Welihan *et al.*, noting the limited capacity of FIXaβ to activate FX without FVIIIa, and of FXa to activate prothrombin without FVa, proposed that FVIIIa and FVa must form before recalcification (prior to FIX activation by FXIa) to explain the rate of clot formation in the aPTT assay [10]. In support of this hypothesis, we showed that FXIa-mediated thrombin generation in FIX-deficient plasma depends on its capacity to activate FV in a reaction involving the FXIa A3 domain. This is consistent with work from Maas *et al.* showing that FV and FVa bind FXI through the A3 domain [23].

In our study, FX activation by FXIa was required for thrombin generation in the absence of FIX, a result supported by recent data from Puy *et al.* [24]. The FIX gene arose from a duplication of the FX gene [25], and FIX and FX are structurally very similar. In the aPTT assay, Ca^{2+} is required for proper folding of the FIX Gla domain, which binds to the FXIa A3 domain [3,12,22]. FX activation by FXIa does not involve the A3 exosite, explaining why the reaction is not Ca^{2+}-dependent, and probably why it is much less efficient than FIX activation. The observation that FX activation and FV activation by FXIa are not Ca^{2+}-dependent (indeed, FV activation is faster in the absence of Ca^{2+}) suggests that FXa and FVa are produced during contact activation. This has implications for preparing plasma for assays that are sensitive to the activated forms of these factors. Failure to collect blood in a manner that limits contact activation (such as phlebotomy directly into a CTI-containing solution) may result in sufficient activation of FX and FV (and FVIII [10]) to affect the results.

We did not find evidence that FXIa activated substrates further down the coagulation cascade from FV/FX. Although FXIa readily cleaves prothrombin, generating a species that runs in a similar position to α-thrombin on SDS-PAGE, the species lacks activity in a chromogenic assay, and fails to convert fibrinogen to fibrin (Fig. S1). FXIa does not convert fibrinogen to fibrin (not shown). Furthermore, FXIIa does not convert fibrinogen to fibrin (not shown) or cleave FII, FIX, or FX (Fig. S2A), making it unlikely that this contact protease activates proteins downstream of FXI. FXIIa does cleave FV; however, it does not appear that FVa heavy or light chain are formed.

The observation that FXIa is a more promiscuous protease than originally suspected may be relevant to its role in hemostasis and thrombosis. Despite the difference in bleeding phenotype, mice lacking either FXI or FIX are comparably resistant to arterial thrombosis induced by $FeCl_3$, consistent with the premise that FXIa activates FIX in this model [16,17]. However, mice lacking both FIX and FXI are more resistant than mice lacking only one of the factors. Although this observation has only been made in one thrombosis model, the results do support the hypothesis that FXIa can act on targets other than FIX in some situations. Despite its modest role in hemostasis, there is mounting evidence for the thrombogenic potential of FXIa in humans. Plasma FXI levels correlate with the risk of myocardial infarction [26], stroke [27], and venous thrombosis [28]. Initial attempts to use FXI concentrate to treat FXI deficiency were associated with a significant incidence of thrombosis [29,30], probably because of trace contamination with FXIa. FXI is a common

contaminant in gammaglobulin concentrates, because IgG and FXI are difficult to separate chromatographically [31,32]. FXIa concentrations as low as 100 pM in gammaglobulin are associated with thrombotic events [32]. Our data raise the possibility that the capacity of FXIa to activate plasma proteins in addition to FIX may contribute to its thrombogenic potential.

Addendum

A. Matafonov: conducted studies of plasma coagulation, FXIa cleavage of clotting factors, and thrombin generation, and wrote the original draft of the manuscript. Q. Cheng: performed mouse thrombosis studies. Y. Geng: prepared and tested recombinant FXIa variants. I. M. Verhamme: designed experiments on FX and FV activation and interpreted the results. O. Umunakwe: conducted studies on FX activation by FXIa. E. I. Tucker: generated and characterized antibodies against the FXIa A3 and catalytic domains. M.-F. Sun: prepared recombinant proteins and identified binding epitopes of monoclonal IgGs. V. Serebrov and A. Gruber: contributed to the overall study design and interpretation of data. D. Gailani: responsible for oversight of the project and preparation of the final manuscript.

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Disclosure of Conflict of Interests

E. I. Tucker and A. Gruber have a significant financial interest in Aronora, Inc., a company that may have a commercial interest in the results of this research. This potential conflict of interest has been reviewed and managed by the Oregon Health & Science University OHSU Conflict of Interest in Research Committee. D. Gai-lani is a consultant for several pharmaceutical companies.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Fibrinogen and prothrombin.
Figure S2. Factor XIIa cleavage of coagulation proteins.

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