High-molecular-weight kininogen interactions with the homologs prekallikrein and factor XI: importance to surface-induced coagulation

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Essentials

- High molecular weight kininogen (HK) circulates in plasma free or in non-covalent complexes with factor XI (FXI) or prekallikrein (PK) through its D6 domain.
- FXI is needed for hemostasis, but HK is not.
- FXI/HK interaction is more important for surface driven clotting and thrombosis than PK/HK interaction.
- HK knockout mice resistance to thrombosis can be reversed with murine HK and not human HK.

Abstract

Background: In plasma, high-molecular-weight kininogen (HK) is either free or bound to prekallikrein (PK) or factor XI (FXI). During contact activation, HK is thought to anchor PK and FXI to surfaces, facilitating their conversion to the proteases plasma kallikrein and FXIa. Mice lacking HK have normal hemostasis but are resistant to injury-induced arterial thrombosis.

Aims: Identify amino acids on the HK-D6 domain involved in PK and FXI binding and study the importance of the HK-PK and HK-FXI interactions to coagulation.

Methods: Twenty-four HK variants with alanine replacements spanning residues 542-613 were tested in PK/FXI binding and aPTT clotting assays. Surface-induced FXI and PK activation in plasma was studied in the presence or absence of HK. *Kng1*$^{-/-}$ mice lacking HK were supplemented with human or murine HK and tested in an arterial thrombosis model.

Results: Overlapping binding sites for PK and FXI were identified in the HK-D6 domain. HK variants with defects only in FXI binding corrected the aPTT of HK-deficient plasma poorly compared to a variant defective only in PK-binding. In plasma, HK deficiency appeared to have a greater deleterious effect on FXI activation than PK activation. Human HK corrected the defect in arterial thrombus formation in HK-deficient mice poorly due to a specific defect in binding to mouse FXI.

Conclusion: Clinical observations indicate FXI is required for hemostasis, while HK is not. Yet, **t**he HK-FXI interaction is required for contact activation-induced clotting *in vitro* and *in vivo* suggesting an important role in thrombosis and perhaps other FXI-related activities.

Keywords: High-molecular-weight Kininogen; Factor XI; Prekallikrein; Factor XII; thrombosis

Introduction

High-molecular-weight kininogen (HK) is a multi-domain glycoprotein [1-4] that circulates in plasma either in free form or in non-covalent complexes with the zymogens prekallikrein (PK) or factor XI (FXI) [5,6]. HK and PK, together with factor XII (FXII), comprise the kallikrein-kinin system (KKS) [3,4,7]. In plasma [8,9] or on vascular endothelial cells [10,11] PK and FXII reciprocally convert each other to the proteases plasma kallikrein (PKa) and FXIIa. PKa cleaves HK at two sites, liberating the peptide bradykinin, which contributes to regulation of vascular permeability and tone by binding to specific receptors [1,3,4]. When KKS proteins assemble on macro-molecules or surfaces, FXII undergoes autocatalytic activation and reciprocal activation with PK is enhanced in a process called contact activation [7,9,12]. During contact activation, HK facilitates PK binding to the surface, in addition to serving as a PKa substrate [4,7,12]. Contact activation-induced bradykinin production at injury sites likely contributes to swelling and pain sensation [13,14]. Accelerated PKa and FXIIa generation in the disorder hereditary angioedema triggers episodes of bradykinin-induced soft tissue swelling [15-19]. In these pathophysiologic scenarios, the importance of the PK-HK interaction for bradykinin production seems clear.

The coagulation zymogen FXI is a homolog of PK [20]. During contact activation, FXI, like PK, binds to surfaces and is converted to the protease FXIa by FXIIa [7,20]. FXIa then promotes plasma coagulation by activating factor IX. The importance of FXIIa-initiated coagulation through FXI in the normal host-response to trauma is uncertain. Indeed, of the proteins involved in contact activation (FXII, PK, HK and FXI), only FXI is required to stop bleeding at an injury site [21]. This suggests that FXIIa activation of FXI [22], and the FXI-HK interaction, are not required for hemostasis. However, these reactions may be highly relevant for other situations, such as when contact activation is induced in blood when it is exposed to non-biological surfaces of medical devices or the cell walls of microorganisms. In this study we investigated HK binding to PK and FXI, and the importance of the FXI-HK interaction to clot/thrombus formation *in vitro* and *in vivo*.

Methods

Materials FXII, FXI, PK, and HK purified from human plasma were from Enzyme Research Laboratory. Low-molecular-weight kininogen (LK) was from Sigma-Aldrich. Human pooled normal plasma (PNP), FXI-deficient plasma and kininogen-deficient plasma from an individual lacking both HK and LK were from George King Bio-Medical. Human plasma immunodepleted of HK (HK-DP) was from Diapharma. Anhydrous iron (III) chloride ($FeCl_3$) was from Sigma-Aldrich. Streptavidin conjugated to horseradish peroxidase (HRP) was from Pierce. PTT-A silica-based activated partial thromboplastin time (aPTT) reagent was from Diagnostic Stago.

Antibodies. Affinity-purified rabbit anti-mouse kininogen IgG was raised against a polypeptide containing residues 607-638 in the murine HK D6 domain (comparable to Pro^{589} to Asp^{620} in human HK) [23]. Monoclonal anti-human PK IgG 5C8, anti-human FXI IgG 1G5, and anti-mouse FXI IgG 15B4 recognize the protease domains of their respective proteins [24,25]. Biotinylated mouse anti-mouse FXI IgG 14E11 recognizes the A2 domain of human and mouse FXI [26]. HRP-conjugated IgG against a hemagglutinin tag was from Invitrogen. HRP-conjugated polyclonal goat anti-human FXII, anti-human PK, and anti-human FXI IgGs were from Affinity Biologicals. Goat-anti-human-HK polyclonal IgG was from Nordic-MUbio, Sustren, The Netherlands.

Recombinant proteins. cDNAs encoding human HK (NCBI Ref Seq NM_001102416.3), and the mouse HK isoforms mHK1 (NCBI NM_001102411.1) and mHK3 (NM_001102412.1) in expression vector pJCMV were modified by addition of a nine amino acid (Tyr-Pro-Tyr-Asp-Val-Pro-Asp-Tyr-Ala) hemagglutinin tag to the C-termini of the coding sequences, as described [27]. Alanine substitutions were introduced into the human HK cDNA using a QuickChange mutagenesis kit (Agilent). Groups of three consecutive residues between Phe^{542} and Lys^{613}, except for Cys^{596}, were replaced with three alanine residues (Supplemental Figure 1A) [28]. HEK293 cells were stably transfected with expression constructs as described [27], and proteins were purified from conditioned media on an anti-HA IgG agarose column (ThermoFisher, Supplemental Figures 1B and 1C). Human FXI (NCBI NM_000128.3) and mouse FXI (mFXI,

NCBI 028066.2) were prepared as described (Supplemental Figure 1D) [29]. Proteins were stored in 25mM Tris-HCl pH7.4, 100mM NaCl at −80°C.

HK binding assays (Supplemental Figure 2)***.*** Immulon 4HB microtiter plates were coated overnight with 100μL 50mM carbonate buffer pH 9.6 containing 20μg/mL IgG 5C8 or 1G5. Plates were blocked with phosphate buffer saline (PBS) containing 2%BSA at RT for 90 min. After washing 3× with PBS/0.1%Tween-20, wells were filled with 100μL diluent buffer (90mM HEPES pH7.2, 100 mM NaCl 1%BSA, 0.1%Tween-20) containing PK (5μg/mL, 60nM) for 5C8-coated plates, or FXI (10μg/mL, 60nM) for 1G5-coated plates, and incubated at RT for 90 min. After washing 3×, 100μL diluent buffer containing recombinant HK (various concentrations) was added and incubated at RT for 90 min. After washing 3×, HRP-linked anti-HA antibody (1:5000) in 100μL diluent buffer was added, and incubated for 90 min. After washing 3×, 100μL of Substrate Solution (5mg *o*-phenylenediamine tablet [Sigma-Aldrich #P-691] in 12ml citrate-phosphate pH5.0 with 12μl 30% H_2O_2) was added. Reactions were stopped after 5 min with 50μl 2.5M H_2SO_4, and OD 490 nm measured. Results were compared to control curves made with known concentrations of wild-type HK (Supplemental Figure 3). For experiments examining mHK1 binding to FXI, platelets were coated with 15B4 IgG, which recognizes human and murine FXI [25].

Plasma clotting assays. HK-DP has a PK concentration ~30% of normal and undetectable FXI due to the depletion process (Supplemental Figure 4A). HK-DP was supplemented with human plasma-derived PK and FXI to concentrations of 500nM and 30nM, respectively (Supplemental Figure 4A). The repleted plasma is designated HK-RP. aPTT assays were performed on a STart4 analyzer (Diagnostica Stago). In brief, 25μl recombinant HK (400nM, final concentration 100 nM) in 25mM Tris-HCl, 100mM NaCl, 0.1% BSA pH7.4 (TBSA) was mixed with 25μl HK-RP. PTT-A reagent (35μl) was added. After 5 min incubation at 37°C, 25μl $CaCl_2$ (25mM) was added, and time to clot formation determined. aPTTs for recombinant HKs were compared to a control curve established by testing different plasma-derived HK concentrations in the aPTT assay with HK-RP

(Supplemental Figure 4B). To quantify FXI in kininogen-deficient (*Kng1*$^{-/-}$) mouse plasma, FXI-deficient human plasma was mixed with serial dilutions of wild-type or *Kng1*$^{-/-}$ mouse plasma anticoagulated with sodium citrate, and aPTTs were determined (Supplemental Figure 4C).

Zymogen activation on titanium. Ten microliters of PNP or kininogen-deficient plasma supplemented with HK (640nM) or LK (2.4μM), were mixed with titanium nanospheres (avg. diameter 70nm, 20m^2/g, surface area used in reactions 20cm^2, US Research Nanomaterials, Inc., Houston, TX, USA) in PEG-20,000-coated polypropylene tubes as described [30]. Mixtures were incubated at 37°C for 0-60 min, then titanium was pelleted at 14,000g for 1 min. Supernatants were mixed with non-reducing SDS-sample buffer. Pellets were washed 3× with PBS, then mixed with non-reducing SDS-sample buffer. Samples of supernatant and pellet eluate were size fractionated on non-reducing 6.5% polyacrylamide-SDS gels and transferred to nitrocellulose. Blots were probed with HRP-conjugated polyclonal IgG to human FXII or PK. For FXI, biotinylated monoclonal IgG 14E11 was used with streptavidin-HRP. Detection was by chemiluminescence.

Western blots of mouse plasma. Mouse plasma (1μL) prepared from blood anticoagulated with sodium citrate was size-fractionated under non-reducing conditions on 7.5% acrylamide-SDS gels and transferred to nitrocellulose membranes. FXI was detected with biotinylated IgG 14E11 [26], and HRP-conjugated streptavidin. FXII and PK were detected with HRP-conjugated polyclonal IgGs to FXII or PK, respectively. HK in mouse plasma was detected with anti-mouse HK IgG [23], and HRP-conjugated goat anti-rabbit IgG. Detection in all cases was by chemiluminescence.

Carotid artery thrombosis model. All procedures were approved by the Vanderbilt University Medical Center Institutional Animal Care and Use Committee. C57Bl/6 mice lacking PK (*Klkb1*$^{-/-}$) [31], FXI (*F11*$^{-/-}$) [32], HK (*Kng1*$^{-/-}$) [33], or FXII (*F12*$^{-/-}$) [34] have been described. Mice were anesthetized with 50mg/kg intraperitoneal pentobarbital. The right common carotid artery and jugular vein were exposed, and the artery was fitted with a Doppler probe (model 0.5 VB; Transonic System) connected to an ML866 PowerLab 4/30 data acquisition system (AD Instruments). Five minutes before arterial injury, mice received infusions through the jugular vein

of (1) plasma-derived human FXII (25μg), PK (50μg), FXI (10μg) or HK (50μg) in 150 μL PBS or (2) 300 μL of mouse plasma prepared from blood anticoagulated with sodium citrate obtained from WT, *Kng1*$^{-/-}$, *Klkb*$^{-/-}$, or *F11*$^{-/-}$ mice. Blood vessel injury was induced with two 1 × 1.5 mm filter papers (GB003, Schleicher & Schuell, Keene, NH) saturated with 3.5% $FeCl_3$ solution applied to opposite sides of the artery for three min. Blood flow was monitored for 30 min.

Expression of HK variants in mice by hydrodynamic tail-vein injection (HTI). cDNAs for human HK, full-length mouse HK (mHK1), and a splice variant of mouse HK lacking the D5 domain and part of D6 (mHK3, Supplemental Figure 5) were introduced into an EEV600A expression vector (System Biosciences) [33]. *Kng1*$^{-/-}$ mice (5 mice per construct, for each time point) were anesthetized with pentobarbital (50mg/kg intraperitoneally) and 3.5μg HK/EEV600A construct in 2mL lactated Ringer's solution was infused into a tail vein over 30 seconds. Seven days after HTI, mice were tested in the carotid artery thrombosis model as described above.

Statistical analysis. Means of duplicate runs and standard deviations were determined for clotting assays. For the thrombosis model, data were presented as percentage occlusion and average time to occlusion. Animals in which the carotid artery did not occlude were assigned the maximum experimental observation period of 30 min. Times to occlusion was reported as means ± 1 standard deviation and compared using parametric one-way ANOVA, while the binary variable "occluded" vs "not occluded" was presented as a percentages. For all analyses a *p*-value of <0.05 was considered significant.

Results

Binding sites for PK and FXI on HK. PK and FXI polypeptides contain four apple domains (A1 to A4 from the N-terminus) that form a planar structure on which the protease domain rests (Figure 1A) [20,35,36]. HK binds to the face of the planar structure opposite the protease domain through its D6 domain (amino acids 503 to 626, Figure 1B and Supplemental Figure 6) [37-40]. Using peptide inhibition techniques, Tait and Fujikawa showed that PK binds within the 31 amino acids bounded by HK Ser^{565} and Lys^{595} (Figure 1C, *top*) [38], while a larger area is involved in FXI

binding (58 residues, Pro^{556}-Met^{613}, Figure 1C, *bottom*) [39]. We prepared 24 recombinant HKs (HK-1 to HK-24, Figure 1D and Supplemental Figures 1A and 1B). In each, three consecutive amino acids were replaced with three alanines. The strategy covered all residues between Phe^{542} and Met^{613}, except Cys^{596}, which was not changed to leave the disulfide bond between the HK N- and C-termini intact (Figure 1B).

HK binding to PK and FXI was assessed with a microtiter plate assay in which PK or FXI is immobilized with an IgG to the catalytic domain, leaving the apple domains free to bind HK (Supplemental Figure 2). Compared to wild-type HK, five variants (HK-7, HK-9 through HK-11 and HK-16, Figures 1D and 2A) displayed reduced PK binding, and ten reduced FXI binding (HK-5 to HK-8, HK-10 to HK-13, HK-16, and HK-19, Figures 1D and 2B). The apparent PK and FXI binding sites are displaced slightly toward the HK N-terminus relative to sites predicted with peptide mapping (compare Figure 1C and 1D) [38,39]. FXI binding required all HK residues involved in PK binding except for the three aspartic acids (Asp^{566}-Asp^{558}) replaced in HK-9.

PK and FXI binding to HK in the aPTT assay. HK variants were used to reconstitute HK-RP in silica-initiated aPTT assays (Figure 2C). Clotting times for plasmas containing HK with a defect in FXI binding were prolonged to the same extent as the HK-RP control. Only HK-9 was defective in PK binding while binding normally to FXI. The aPTT in plasma containing HK-9 was slightly prolonged compared to HK-RP containing wild-type HK. aPTTs for HK-RP supplemented with HKs that were defective in binding to both FXI and PK were slightly prolonged compared to results for plasmas with HKs defective only in FXI binding. These results suggest that HK binding to FXI may be more important for clotting in the aPTT assay than is HK binding to PK.

Protease activation on titanium. To investigate the importance of HK in surface-dependent PK and FXI activation further, we used a system in which contact activation is induced in plasma by exposure to titanium nanospheres [30]. The metal is a potent inducer of contact activation and HK binds avidly to it [30,41]. After incubation, the nanospheres can be easily separated from plasma by brief centrifugation, allowing assessment of surface bound and non-bound proteins.

There are no appreciable changes in FXII, PK or FXI in kininogen-deficient plasma supplemented with plasma-derived HK over sixty-minutes of incubation in the absence of titanium (Figure 3A). When titanium nanospheres are added to the same plasma there is rapid transfer of FXII, PK and FXI to the metal, followed by evidence of zymogen activation (Figure 3B). For FXII and PK, high molecular weight species representing FXIIa and PKa in complex with plasma protease inhibitors accumulate (Figure 3B, *left and center columns*) [30]. FXI is a homodimeric molecule, while PK is a monomer [20]. Conversion of FXI to FXIa is detectable on non-reducing western blots because FXIa with one or two activated subunits migrates slower than FXI on non-reducing SDS-PAGE (Figure 3B, *right column*) [30,42]. Results for kininogen-deficient plasma supplemented with HK are comparable to those obtained with normal plasma (Supplemental Figure 7) [30].

When titanium is added to plasma lacking HK (Figure 3C), FXII still associates rapidly with the metal and is activated. Most PK remains in plasma, consistent with an important role for HK in PK surface binding. However, despite the reduced binding, PKa-inhibitor complex is detectable indicating PK activation occurs. When FXII binds to a surface it assumes a conformation that renders it highly susceptible to activation by PKa [9]. FXIIa then reciprocally activates PK, either on the surface or in solution. The process is less efficient than in the presence of HK (compare Figure 3C to 3B), but activation of both proteases appears to occur. This may explain why HK-9 with an isolated defect in PK binding only displays a slight defect in the aPTT assay (Figure 2C).

Unlike PK, most FXI binds to titanium in the absence of HK (Figure 3C). Yet FXI activation appears to be reduced when HK is not present (compare Figure 3C to 3B). While some reduction may be secondary to reduced FXIIa formation when HK is missing, the results suggest FXI activation on titanium requires the HK-FXI interaction to proceed optimally. This is consistent with the prolonged clotting times of plasmas containing HK variants with an isolated FXI binding defect in the aPTT assay (Figure 2C), where FXII activation should not be impaired. Unlike HK, LK lacks a D6 domain and does not bind FXI or PK. Supplementing kininogen-deficient plasma with LK gives results like those for plasma lacking kininogen (Supplemental Figure 8).

***Reconstituting Kng1$^{-/-}$* mice with human HK**. Based on results for the *in vitro* assays, we planned to express human wild-type HK and some of the HK binding variants in kininogen-deficient (*Kng1*$^{-/-}$) mice by hydrodynamic tail vein injection to test their effects on a $FeCl_3$-induced arterial thrombosis. Wild-type mice develop carotid artery occlusion consistently after vessel exposure to 3.5% $FeCl_3$, while mice lacking FXI (*F11*$^{-/-}$), FXII (*F12*$^{-/-}$), PK (*Klkb1*$^{-/-}$) or kininogen (*Kng1*$^{-/-}$) are resistant to occlusion at this $FeCl_3$ concentration (Figures 4A and 4B, compare red bars to black bar) [43,44]. This suggests that thrombosis in this model requires contact activation. Intravenous infusion of plasma-derived human FXI, FXII or PK into mice with the corresponding factor deficiency restored thrombus formation. (Figures 4A and 4B, compare blue bars to black bar). However, infusing human HK into *Kng1*$^{-/-}$ mice did not completely restore the wild-type pattern (Figures 4A and 4B, compare yellow bar to black bar). Human HK infusion led to thrombus formation in 60% of *Kng1*$^{-/-}$ mice with average time to occlusion of 19.1 min $\pm$ 9.6 min (Figures 4B). In *Kng1*$^{-/-}$ mice with occluded arteries (6 out of 10 mice), the average time to occlusion (11.7 $\pm$ 4.8 min) was longer than for wild-type mice (7.4 $\pm$ 0.8 min). Because of this, we could not use *Kng1*$^{-/-}$ mice to assess the function of human recombinant kininogens. Instead, we conducted experiments to determine the cause of the incomplete response to human HK in *Kng1*$^{-/-}$ mice.

Human HK in *Kng1*$^{-/-}$ mice. Several possibilities could account for the failure of human HK to completely restore thrombus formation in *Kng1*$^{-/-}$ mice. HK-deficient humans often have low plasma PK concentrations, possibly because HK stabilizes PK in plasma [45,46]. This is also the case in *Kng1*$^{-/-}$ mice (Figure 4C). Reduced plasma FXI is not typically a feature of HK-deficiency in humans, and the FXI signal on Western blots appear comparable for plasmas from wild-type and *Kng1*$^{-/-}$ mice (Figure 4C)**.** In clotting assays, FXI activity in *Kng1*$^{-/-}$ plasma was modestly reduced relative to wild-type plasma (~65% of normal, Supplemental Figure 4C). As PK-deficient (*Klkb1*$^{-/-}$) mice and *Kng1*$^{-/-}$ mice are comparably resistant to $FeCl_3$-induced arterial thrombosis [43], the defect attributed to HK deficiency may be due, in part or in whole, to PK deficiency. However, infusing mixtures of human HK and PK into *Kng1*$^{-/-}$ mice gave similar results to infusing

HK alone (Figure 5A). Furthermore, infusions of PK-deficient mouse plasma restored thrombus formation (Figure 5B), arguing against PK deficiency, or an incompatibility between human HK and murine PK, as causes for the defect in *Kng1*$^{-/-}$ mice.

Results in Figures 4A and 4B could be explained by human HK instability in mouse plasma. However, plasma taken after each run of the thrombosis model identified intact human HK (Supplemental Figure 9). We conducted additional reconstitution experiments and noted that the combination of human HK and FXI (with or without PK) restored normal thrombus formation in *Kng1*$^{-/-}$ mice, while human FXI alone or combined with PK did not (Figures 5A). Infusing mouse plasma lacking FXI also restored thrombus formation (Figures 5B). Taken as a whole, the data support the conclusion that mouse HK restores thrombus formation in *Kng1*$^{-/-}$ mice, but human HK does not, probably because the latter does not interact normally with mouse FXI.

HK binding to human and mouse FXI. There is substantial sequence variation between the putative FXI binding sites in the D6 domains of human and mouse HK (Supplemental Figure 6). We tested human HK and full-length mouse HK (mHK1) binding to human or murine FXI using the microtiter plate assay described above. Binding of human HK to mouse FXI is approximately an order of magnitude weaker than binding to human FXI (Figure 6A), while mouse HK binds comparably to mouse and human FXI (Figure 6B). The data support those from the reconstitution studies in *Kng1*$^{-/-}$ mice and reinforce the importance of the FXI-HK interaction in clot formation in the *in vitro* binding and plasma studies.

Mouse HK isoforms and thrombosis. Mouse plasma contains two HK isoforms. mHK1 has similar domain structure to human HK, while mHK3 lacks the D5 domain that is thought to mediate surface binding [27,33]. It also lacks the N-terminal portion of the D6 domain, but retains the area implicated in FXI and PK binding (Supplemental Figure 5). mHK1 and mHK3 were expressed in *Kng1*$^{-/-}$ mice by HTI. The isoforms were detectable in plasma by Western blot three days after DNA injection, with expression persisting for at least seven days (Supplemental Figure 10). All

mice expressing mHK1 or mHK3 had normal thrombus formation in the $FeCl_3$ model (Figure 6C), indicating both HK isoforms support PK and FXI activation in this model.

Discussion

In humans, the *Kng1* gene encodes two types of kininogen through alternatively spliced mRNAs [3,47,48]. The more abundant LK lacks sequences in the D5 domain that bind surfaces and the D6 domain that binds PK or FXI. As a result, LK does not support contact activation. HK has a different and larger D5 domain than LK with putative binding sites for anionic surfaces [49-52], while its unique D6 domain has binding sites for PK and FXI [38-40]. Most PK and nearly all FXI in human plasma appears to circulate bound to HK [5,6,37,45]. In 1987, Tait and Fujikawa mapped overlapping binding sites for PK and FXI on HK D6 using a peptide inhibition approach [39]. More recently Sing et al. and Chen et al. showed that monoclonal IgGs against these areas of D6 displace PK and FXI from HK and reduce thrombus formation in rodents [53,54].

We used alanine scanning mutagenesis to map the PK and FXI binding sites on human HK. Our data support those of Tait and Fujikawa [38,39], and provide detail on specific residues involved in binding. HK residues 569-574 (Trp-Ile-Pro-Asp-Ile-Gln) and 587-589 (Asp-Phe-Pro) are required for binding to PK and FXI. Three consecutive aspartic acid residues (566-568) are required specifically for PK binding, while the N-terminus of the FXI binding site (residues 554 to 565) is larger than in the PK binding site. Recently, Li and colleagues reported structures for the PK and FXI apple domain disks co-crystalized with the 31-amino acid HK D6 peptide described by Tait and Fujikawa [40]. In these structures, the Asp-Phe-Pro motif (HK residues 587-589) inserts into a pocket on the A2 domains of PK and FXI, consistent with earlier work from Renné *et al.* showing that the isolated A2 domains of PK and FXI bind HK with higher affinity than do other apple domains [55,56]. Interestingly, in the PK structure the 31-mer adopts a bent configuration with the Trp-Ile-Pro-Asp-Ile-Gln sequence binding to the A1 domain [40], while in the FXI structure the peptide runs across the A2 domain, adopting a linear configuration with the

N-terminus binding to the A3 domain (Figure 7) [40]. The differences in the putative HK binding sites on PK and FXI may reflect accommodations for the dimeric conformation of FXI.

The ability to facilitate PK and FXI surface binding is thought to be key to the HK cofactor effect during contact activation. PK and FXI bind to surfaces in the absence of HK in assays using purified proteins, but the interactions may be weaker in a plasma environment. In our study the capacity of HK variants to bind FXI was an important determinant of their abilities to restore clotting in the aPTT assay. This was supported by the fortuitous observation that human HK, which binds poorly to mouse FXI, did not restore thrombus formation to normal in *Kng1*$^{-/-}$ mice. Isolated defects in FXI binding were associated with aPTTs comparable to the HK-deficient control plasma, and supplementing the plasma with variants defective in binding to both FXI and PK resulted in aPTTs that were only slightly longer than those with isolated FXI-binding defects, The one variant with an isolated defect in PK binding (HK-9) displayed a relatively mild defect in the aPTT assay.

Recently, we described a system for studying plasma contact activation using titanium nanospheres [30]. Titanium-based alloys are widely used in biomedical devices because of their strength and durability. When this system was tested with normal plasma, PK and FXI bound rapidly to the metal surface, and became activated [30]. Interestingly, in the current study, in the absence of HK, most PK remains in the fluid phase while FXI is still largely surface-bound. FXI has two anion-binding sites that are not present on PK that may contribute to surface-binding independently of HK [57]. But despite being bound to the surface, we did not observe FXI activation in the absence of HK. PK and FXII can reciprocally activate each other without a surface [9,23,58]. One of the major contributions that surfaces make to contact activation is to change the structure of FXII so that it can be rapidly activated by PKa [9,58]. FXIIa then drives surface-dependent and surface-independent PK activation to accelerate reciprocal activation. Because FXII does not required HK to bind to surfaces, PK-FXII reciprocal activation will be enhanced to some extent by a surface, even in the absence of HK [9]. Our data indicate that FXI, despite its

ability to bind surfaces independently of HK, requires HK for normal surface-dependent activation by FXIIa, a premise supported by our observation that loss of the normal HK-FXI interaction disrupted thrombus formation in mice.

There was an unexpected finding in our study related to the way HK is thought to bind to surfaces that has interesting implications for our conclusions. In mice, there are two HK isotypes. As discussed, the D5 and D6 domains of mHK1 are structurally analogous to those of human HK, while the shorter mHK3 isotype is missing the D5 domain and a portion of D6 [27,33]. A body of work indicates that D5 mediates human HK surface-binding [49-52]. If this is the case with the mouse protein, we might not expect mHK3 to support contact activation. However, when expressed in *Kng1*$^{-/-}$ mice, mHK3 had the same capacity to correct thrombus formation as mHK1. Several possibilities could explain this. Human and mouse HK, despite relatively similar structures, may not interact with surfaces in the same manner. In mice, components of HK other than D5 could facilitate surface binding. Or perhaps the process involved in thrombus formation in the $FeCl_3$ model does not involve classic contact activation, even though it does require the plasma contact factors. A third possibility is that during contact activation, FXI binds directly to surfaces through its anion-binding sites, while HK performs a cofactor function that facilitates FXI activation, but that is not directly related to FXI surface binding. Additional work is clearly required to fully characterized the nature of HKs cofactor activity as it relates to FXI.

Available data indicate that FXII and PK are not major contributors to common thrombotic disorders in humans such as venous thromboembolism (VTE), myocardial infarction or stroke [59-66]. In contrast, several studies link FXI to thrombosis, and particularly to VTE and ischemic stroke [43,67-69]. This suggests that mechanisms for FXI activation distinct from contact activation may operate in some human thrombotic disorders. FXI is activated by thrombin [2,20], and it is conceivable other proteases are also FXI activators. The importance of HK to FXI activation and activity *in vivo* is unclear, but it is interesting that HK persists in some mammals that lack a full contact activation system. Cetaceans (whales, porpoises, and dolphins) lack functional genes for

FXII and PK, while retaining those for FXI and HK [2]. The HK from one cetacean, the false killer whale (*Pseudorca crassidens*), can reconstitute human HK-deficient plasma in an aPTT mixing study, indicating it has similar pro-coagulant cofactor function to human HK [2]. Thus, while clinical observations suggest that the FXI-HK interaction is not necessary for hemostasis, HK cofactor activity may contribute to other FXI functions and have a role in thrombogenicity.

AUTHORSHIP

B.M.M performed animal studies, recombinant protein production, and western blot protein detection assays and contributed to writing the manuscript. M.S carried out recombinant protein production and purification, plasma clotting assays, and developed the HK binding assay. Q.C performed animal studies and western blot protein detection assays. M.L. carried titanium binding studies. K.R.M developed kininogen deficient mice and antibodies to murine HK and contributed insight to kininogen biology. J.E. contributed structural and modeling insights. O.J.T.M contributed to study design and data interpretation. D.G. oversaw the project and preparation of the manuscript.

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POTENTIAL CONFLICTS OF INTEREST

D.G. is a consultant for pharmaceutical companies (Anthos Therapeutics; Aronora, Inc.; Bayer Pharma; Bristol-Myers Squibb; Ionis Pharmaceuticals; Janssen, Pharmaceuticals) with interests in targeting factor XI, factor XII, and prekallikrein for therapeutic purposes. The other authors have no conflicts to report.

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Figure Legends

Figure 1. Binding sites for PK and FXI on HK. (A) Model for prekallikrein and a subunit of FXI homodimer. In both, four apple domains (A1-A4 from the N-terminus) form a planar structure on which the catalytic domain (CD) rests. The activation cleavage site is located between A4 and the CD (black line). HK binds to the apple domain planar structure on the side opposite the CD. **(B)** Schematic diagram showing the 6 domains of HK (D1-D6) and the disulphide link between D1 and D6. **(C)** Amino acid sequence of HK D6 residues Phe542 through Lys614 (numbering does not include the signal peptide). Highlighted in red is the 31-mer peptide covering the binding site for PK and the 58-mer peptide covering the binding site for FXI on HK D6 as identified by Tait and Fujikawa (references 38 and 39). **(D)** Summary of the results of binding studies for HK variants covering HK residues Phe542 through Lys614. Twenty-four recombinant HK variants were produced in cell culture. In each, three consecutive amino acid are replaced with three alanines. Highlighted in blue and green are the residues identified by binding studies to be involved in PK binding and FXI binding, respectively.

Figure 2. HK binding of FXI and PK and HK activity in an aPTT assay. (A) Binding of HK variants to immobilized PK. Shown are percent HK binding for each variant to PK as a percent of wild-type (WT) recombinant HK binding. Shown along the bottom of the graph are triplet letters indicating the amino acids replaced with alanine in each variant. Data for WT HK are on the right. **(B)** Binding of HK variants to immobilized FXI. As in panel A except that the immobilized protein was FXI. **(C)** Effects of HK and HK variants on surface-initiated plasma coagulation. HK-depleted plasma (HK-DP) in these studies has significantly reduced levels of PK and FXI. Plasma-derived human PK and FXI were added to replete the plasma (HK-RP). Recombinant HK was added to HK-RP to a final concentration of 400nM, and the aPTT clotting time determined as described under methods. Controls: WT HK and Plasma HK are HK-RP supplemented with wild-type recombinant HK and human plasma-derived HK, respectively; No HK is HK-DP repleted only with plasma-derived PK and FXI; No FXI is HK-DP only repleted with plasma-derived PK and

recombinant HK, but not FXI. HKs with normal binding to PK and FXI in panels A and B are indicated by black diamonds. Variants defective in binding only to FXI are indicated in blue and those defective in binding to both FXI and PK are indicated in green. The single variant with an isolated defect in PK binding is indicated in red.

Figure 3. Binding and activation of plasma FXII, PK and FXI to titanium nanospheres. Time courses were conducted with plasma from a kininogen-deficient human with or without supplemental HK. (**A**) kininogen-deficient plasma supplemented with human plasma-derived HK (640 nM) not exposed to titanium. (**B**) kininogen-deficient plasma supplemented with human plasma-derived HK (640 nM) exposed to titanium nanospheres (20cm^2 surface area). (**C**) kininogen-deficient plasma lacking HK exposed to titanium nanospheres. Plasma samples (10μl) were incubated at 37ºC for 0, 1, 2, 3, 5, 15 or 60 minutes. Control plasmas (C) were not incubated and were not exposed to titanium. After incubation samples underwent centrifugation for 1 min (14,000g, 4ºC). Supernatant plasma was mixed with non-reducing SDS sample buffer. Pellets were washed 3X with ice-cold PBS, then mixed with SDS sample buffer. Samples were size-fractionated by non-reducing SDS-PAGE (6.5% acrylamide) and transferred to nitrocellulose membranes. For each protein and condition the top blot shows results for plasma supernatants and the lower blot for nanosphere eluates (Pellet). Blots were probed with horseradish peroxidase–conjugated polyclonal IgG to human FXII (left column) or PK (center column) or biotinylated monoclonal IgG 14E11/streptavidin-HRPFXI (right column) and detected with chemiluminescence. Positions of molecular mass standards are shown to the left of each figure. Positions of migration for FXII and FXIIa [FXII(a)], PK and PKa [PK(a)], FXI and FXIa and active proteases in complex with plasma inhibitors (INH) are indicated at the right of each panel.

Figure 4. Murine arterial thrombosis model. Effects of contact factor deficiencies on $FeCl_3$-induced carotid artery thrombosis in mice. **(A)** Percent mice with occluded carotid arteries over 30 min and **(B)** Average time to occlusion in minutes. WT mice (black bars) all developed occlusive thrombus formation within 30 minutes after vessel exposure to 3.5% $FeCl_3$, while all

mice lacking a contact factor (red bars) were resistant to occlusive thrombus formation. The wild-type phenotype was restored in *F12*$^{-/-}$, *F11*$^{-/-}$, and *Klkb*$^{-/-}$ mice (blue bars) by intravenous bolus infusion of the human version of the missing contact factor given five minutes before exposure to $FeCl_3$. Infusion of human HK (yellow bars) did not fully restore thrombus formation in *Kng1*$^{-/-}$ mice (60% of mice occluded) and the time to occlusion was prolonged compared to mice of other genotypes. Each experimental group contain 10 animals. **(C)** Western blots for FXII, FXI, PK and HK in plasmas of wild-type (WT), *F12*$^{-/-}$, *F11*$^{-/-}$, *Klkb*$^{-/-}$ and *Kng1*$^{-/-}$ mice. Note that in kininogen-deficient *Kng1*$^{-/-}$ mice the plasma PK level is also reduced. While not evident on the western blot, the FXI level is moderately reduced in *Kng1*$^{-/-}$ mice (**supplemental Figure 4C**).

Figure 5. Murine HK but not human HK reconstitutes *Kng1*$^{-/-}$ mice in the FeCl3 carotid artery thrombosis model. (A) Time to carotid artery occlusion after exposure to 3.5% $FeCl_3$ in *Kng1*$^{-/-}$ mice after infusion of human plasma-derived HK, PK, or FXI, alone or in combination. Red lines indicate average time to occlusion for each group. Only infusions containing human FXI and HK completely restored thrombus formation in *Kng1*$^{-/-}$ mice. **(B)** *Kng1*$^{-/-}$ mice were infused with citrated plasma prepared from the blood of *Kng1*$^{-/-}$, WT, *Klkb*$^{-/-}$, and *F11*$^{-/-}$ mice. The plasmas containing mouse HK (all except *Kng1*$^{-/-}$ plasma) restored thrombus formation in *Kng1*$^{-/-}$ mice.

Figure 6. Mouse FXI binding to HK. (A-B) HK binding to murine FXI and human FXI. Murine FXI or human FXI were immobilized to microtiter plates using capture IgG 15B4 which recognizes FXI from both species (**Supplemental figure 2**). Human HK and the full-length form of murine HK (mHK1) were tested for their abilities to bind to FXI. **(A)** mHK1 binds relatively weak to human FXI, when compared with human FXI, while **(B)** human and mouse FXI bind comparably to mHK1. **(C)** *Kng1*$^{-/-}$ mice underwent hydrodynamic tail injection (HTI) to induce hepatic expression of either full-length murine HK (mHK1) or an alternatively spliced variant (mHK3) lacking the D5 domain and part of the D6 domain. Seven days after DNA infusion, mice were tested in the $FeCl_3$ carotid artery thrombosis model. Both mHK1 and mHK3 expression (**Supplemental figure 9**) restored thrombus formation in *Kng1*$^{-/-}$ mice.

Figure 7. Crystal structures of PK and FXI apple domains in complex with HK D6 31-mer peptide. Surface representations of the PK31-mer (left) and FXI-31-mer (right) complexes with the apple domains colored red (A1), green (A2), blue (A3) and purple (A4). The 31-mer peptide (HK residues 565-595) is shown in white and rendered transparent revealing the peptide backbone (Black). At areas corresponding to peptide residues Trp-Ile-Pro-Asp-Ile-Gln (HK residues 569-574) and Asp-Phe-Pro (HK residues 587-589), stick representations (yellow) indicate specific residue structures. Images were generated in UCSF Chimera v1.17 using PDB ID 7QOX for PK and PDB ID 7QOT for FXI.