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Characterization of the H-kininogen-binding Site on Factor XI

A COMPARISON OF FACTOR XI AND PLASMA PREKALLIKREIN*

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Factor XI (FXI), the zymogen of the blood coagulation protease FXIa, and the structurally homologous protein plasma prekallikrein circulate in plasma in noncovalent complexes with H-kininogen (HK). HK binds to the heavy chains of FXI and of prekallikrein. Each chain contains four apple domains (F1–F4 for FXI and P1–P4 for prekallikrein). Previous studies indicated that the HK-binding site on FXI is located in F1, whereas the major HK-binding site on prekallikrein is in P2. To determine the contribution of each FXI apple domain to HK-FXI complex formation, we examined binding of recombinant single apple domain-tissue plasminogen activator fusion proteins to HK. The order of affinity from highest to lowest is F2 ≫ F4 > F1 ≫ F3. Monoclonal antibodies against F2 are superior to F4 or F1 antibodies as inhibitors of HK binding to FXI. Antibody αP2, raised against prekallikrein, cross-reacts with FXI F2 and inhibits FXI-HK binding with an IC_{50} of 8 nM. HK binding to a platelet-specific FXI variant lacking the N-terminal half of F2 is reduced > 5-fold compared with full-length FXI. A chimeric FXI molecule in which F2 is replaced by P2 is cleaved within P2 during activation by factor XIIa, resulting in greatly reduced HK binding capacity. In contrast, wild-type FXI is not cleaved within F2, and its binding capacity for HK is unaffected by factor XIIa. Our data show that HK binding to FXI involves multiple apple domains, with F2 being most important. The findings demonstrate a similarity in mechanism for FXI and prekallikrein binding to HK.

Coagulation factor XI (FXI)[1] is the zymogen of a plasma serine protease (FXIa) that contributes to blood coagulation by proteolytically activating factor IX (1–4). Deficiency of FXI results in a mild to moderate bleeding disorder, whereas elevated FXI levels have been associated with increased risk for deep venous thrombosis (5, 6). Human FXI is a 160-kDa dimeric glycoprotein consisting of two identical 80-kDa polypeptides connected by a single disulfide bond. The N-terminal "heavy chain" portion of each polypeptide is comprised of four 90–91-amino acid repeats called apple domains, whereas the C-terminal "light chain" region is a typical trypsin-like serine protease domain (7, 8). FXI circulates in plasma as a noncovalent complex with H-kininogen (HK), the high molecular mass precursor of kinin hormones (7, 9). Plasma prekallikrein (PPK), another protease zymogen that circulates as a complex with HK (11), has a similar structural organization to the 80-kDa FXI polypeptide, with which it shares 58% amino acid identity (12). The interaction of HK with FXI or PPK facilitates binding of the latter two proteins to surfaces such as cell membranes (13). HK docks to cell surface heparan and chondroitin sulfate-type proteoglycans, indirectly anchoring FXI and PPK to the cells (14, 15). In the case of PPK, this may be a critical step in localizing kinin production to the surface of vascular endothelial cells (14, 16). Along similar lines, it has been shown that HK facilitates the binding of FXI to activated platelets (17, 18). Once bound to the platelet surface, FXI is efficiently activated to FXIa by coagulation proteases such as thrombin and factor XIIa (FXIIa) (4, 19, 20).

The FXI-binding site of HK has been mapped to a contiguous sequence of 56 amino acids in the extreme C-terminal domain $D6_H$ (21), to which FXI binds with high affinity (apparent $K_D = 1.8 \times 10^{-8}$ M). The FXI-binding site on HK overlaps with the PPK-binding site, and the two zymogens compete for binding to HK (21, 22). Therefore, it appears that there are similarities in the mechanism by which HK binds to FXI and to PPK. A series of studies using recombinant whole molecules and individual apple domains, as well as monoclonal antibodies, have determined that PPK binding to HK requires the first, second, and fourth apple domains (P1, P2, an P4, respectively) but not the third apple domain (P3) (23–25). The P2 domain appears to be most important for this process (25). In contrast, studies of FXI binding to HK using conformationally constrained peptides suggest that HK is bound through a relatively small area between amino acids Phe^{56} and Ser^{86} within the first apple domain (F1). The second, third, and fourth FXI apple domains (F2, F3, and F4, respectively) appear to contribute little to the interaction with HK (26, 27). The significant differences in the interactions of FXI and PPK with HK suggested by published studies are surprising considering the high degree of structural similarity between the proteins. To address this issue, we have conducted studies of FXI binding to HK using recombinant FXI

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[1] The abbreviations used are: FXI, factor XI; HK, H-kininogen; biot-HK, biotinylated HK; ELISA, enzyme-linked immunosorbent asysay; PBS, phosphate-buffered saline; PPK, plasma prekallikrein; tPA, tissue plasminogen activator; BSA, bovine serum albumin.

apple domains, recombinant FXI/PPK chimeras, and monoclonal antibodies directed against specific FXI apple domains. The results indicate that the binding interaction between FXI and HK is similar to the interaction between PPK and HK, involving multiple apple domains, with the second apple domain being most important. We tested these findings with additional experiments on a recently identified FXI splice variant lacking the N-terminal half of F2 (28) that is present in platelets (29). This protein has greatly reduced HK binding compared with full-length FXI with potential (patho)physiological consequences.

EXPERIMENTAL PROCEDURES

Nomenclature—For clarity the following nomenclature was followed throughout the manuscript. FXI apple domains are designated by a capital F followed by the number of the domain, whereas PPK apple domains are designated by capital P followed by the domain number. Murine monoclonal antibodies are indicated by the symbol α followed by F for antibodies raised against FXI and P for antibodies raised against PPK and then the number of the apple domain recognized by the antibody. For example, αP2 is an antibody raised against PPK that recognizes the P2 domain. The names of some antibodies used in previous studies were changed to comply with the new nomenclature system. Specifically, anti-PPK antibodies αP1, αP2, and αP4 were formerly designated PKH19, PHK6, and PKH1, respectively (25), and anti-FXI antibodies αF1, αF2, and αF4 were previously named XI-5, XI-3, and XI-1, respectively (30). For chimeric FXI/PPK proteins, the molecule that makes up the majority of the chimera is listed first, while the abbreviation for the apple domain substituted into the protein is listed second. For example, FXI in which the F2 domain has been replaced with the P2 domain from PPK is designated FXI/P2.

Sources of Plasma Proteins and Antibodies—HK was isolated from human plasma according to established methods (14). To prepare biotinylated HK (biot-HK), 100 μg of HK was incubated with 10 μg of biotin-ϵ-aminocaproyl-*N*-hydroxysuccinimide (Pierce) in 0.1 M $NaHCO_3$ for 4 h at 4 °C. Unbound biotin-ϵ-aminocaproyl-*N*-hydroxysuccinimide was separated from biot-HK by centrifuging three times at 2,000 $\times$ *g* at 4 °C using a Microcon-10 column (Amicon, Beverly, MA) with a 10,000-Da molecular mass cut-off. The buffer used for the repeat centrifugations was 150 mM NaCl, 100 mM NaH_2PO_4, 10 mM Na_2HPO_4, pH 7.4. Human factor XII was purchased from Enzyme Research Laboratories (South Bend, IN) and activated by incubation with glass beads for 30 min at 37 °C. To activate FXI, PPK, and chimeric proteins (see below), protein was incubated with FXIIa in a 250:1 molar ratio in 6.5 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 2.7 mM KCl, 150 mM NaCl, pH 7.4 (PBS) for 72 h at 37 °C as described (25, 31). The generation and characterization of murine monoclonal antibodies against FXI (αF1, αF2, and αF4), PPK (αP1, αP2, and αP4), and tPA have been described (30, 32, 33). The properties of monoclonal anti-HK antibody HKH14 have been published (25). Polyclonal antisera AS199 and AS176 against human FXI and PPK, respectively, were raised in New Zealand White rabbits.

Cell Culture—Human embryonic kidney cells (HEK293-ATCC: CRL-1573) were grown in Dulbecco's modified Eagle's medium (Invitrogen) containing 4.5 g/liter glucose, 10% (v/v) fetal bovine serum, penicillin (50 mg/ml), and streptomycin (50 mg/ml). Baby hamster kidney cells (BHK-ATCC: CRL-10314) were grown in Dulbecco's modified Eagle's medium supplemented with fetal bovine serum (5% v/v), and 50 mg/ml penicillin/streptomycin. All cultures were kept in a humidified 5% CO_2 atmosphere at 37 °C.

SDS-PAGE and Western Blotting—Proteins were resolved by electrophoresis on 12.5% polyacrylamide gels containing 0.1% (w/v) SDS at 30 mA for 90 min. The proteins were visualized either by silver staining or staining with Coomassie Brilliant Blue. Alternatively, proteins were transferred to nitrocellulose at 100 mA for 30 min using a semi-dry technique for Western blotting. The membranes were blocked with PBS containing 5% (w/v) dry milk powder and 0.05% (v/v) Tween 20. Typically the primary detection antibody was diluted 1:1,000 in PBS/milk, and bound antibody was detected by a horseradish peroxidase-coupled secondary antibody against mouse immunoglobulin (DAKO, Hamburg, Germany), followed by chemiluminescence detection according to the manufacturer's instructions (Amersham Biosciences, Inc.).

Expression of FXI Apple Domain-tPA Fusion Proteins—Complementary DNAs for the four apple domains of FXI, F1 (Glu^1–Ser^{90}), F2 (Ala^{91}–Leu^{180}), F3 (Ala^{181}–Val^{271}), and F4 (Phe^{272}–Glu^{361}) were amplified by PCR with *Taq* polymerase using primers that introduced a *Bgl*II site and a *Xho*I site at the 5′- and 3′-ends of the PCR products, respectively (33). The primers also introduced an additional six amino acids (Pro-Arg-Ile-Lys-Gly-Gly) between the apple domains and the tPA molecule (33) to allow for maximum flexibility. The cDNAs encoding single apple domains were excised by *Bgl*II and *Xho*I digestion and ligated into the corresponding sites in the modified tPA expression vector ZpL7(S478A) as described previously (33, 34). The sequences of the PCR products were verified by dideoxy chain termination sequencing. The constructs encode fusion proteins that contain the signal peptide sequence of tPA, followed by a single FXI apple domain (F1, F2, F3, or F4), a spacer sequence of 6 amino acids, the kringle 1 and kringle 2 domains of tPA, and the tPA catalytic chain containing an alanine substitution for the active site serine (S478A). Expression plasmids were stably transfected into baby hamster kidney cells grown in the presence of 1 μM methotrexate, and fusion proteins were purified from serum-free medium (Opti-MEM; Invitrogen) by affinity chromatography using a monoclonal antibody to tPA as described previously (24). Protein concentrations were determined with an ELISA system for tPA (33).

Expression of Full-length FXI, PPK, and FXI/PPK Chimeric Constructs—Two different methods were used to prepare recombinant proteins. The preparation of mammalian tissue culture expression constructs in vector pJVCMV for wild-type FXI, wild-type PPK, and chimera FXI/P2 has been described previously (35). The proteins were expressed in stably transfected HEK293 cells in serum-free medium (Cellgro Complete; Mediatech, Herndon, VA), and purified on an anti-human FXI monoclonal antibody affinity column (35). Alternatively, chimeric constructs were prepared using overlap extension with PCR in vector pZEM (36). The sequence of all constructs was verified by dideoxy sequencing. Chimeric cDNAs were excised from pZEM with *Eco*RI and cloned into the *Eco*RI site of mammalian expression vector pcDNA3(+) (Invitrogen). Constructs were transiently transfected into HEK293 cells using LipofectAMINE (Invitrogen) according to the manufacturer's instructions. Transfection efficiency was ≥40% as monitored by co-transfection with a vector encoding green fluorescent protein. Recombinant proteins were collected in serum-free medium (Dulbecco's modified Eagle's medium).

Expression and Purification of FXI Truncation Protein FXI/ΔF2N—The cDNA for the FXI splice variant FXI/ΔF2N (28) was a generous gift from Dr. Peter Walsh (Temple University School of Medicine, Philadelphia, PA). The cDNA was subcloned into the *Eco*RI site of the pcDNA3(+) vector, and the HEK293 cells were transiently transfected using LipofectAMINE. The transfected cells were resuspended in 2× PBS supplemented with 10 μg/ml each of soybean trypsin inhibitor, benzamidine, leupeptin (Sigma), and 0.1 mM Pefabloc SC (Roth, Karlsruhe, Germany), placed on ice, and lysed by repetitive application of weak ultrasonic pulses for 3 min. Following centrifugation at 20,000 $\times$ *g* for 10 min at 4 °C, the cell lysates were applied to an affinity chromatography column containing polyclonal anti-FXI antibodies (affinity-purified from AS 199 antiserum) immobilized on AffiGel-10 matrix (Bio-Rad). After extensive washing with PBS, bound FXI/ΔF2N was eluted with 1 M NaSCN in PBS, dialyzed against PBS followed by gel filtration high pressure liquid chromatography on a Sephadex 200 column (Amersham Biosciences, Inc.) equilibrated in PBS. Protein-containing fractions were concentrated using an Amicon concentrator, and protein concentration was determined by ELISA (see below).

Determination of Protein Concentration—Protein concentrations were determined by ELISA. Polyclonal anti-FXI antiserum (AS199) at a 1:1,000 dilution was used to coat the wells of microtiter plates (Nunc, Wiesbaden, Germany). After blocking with 1% BSA in PBS, culture supernatants or purified proteins were added in serial 1:2 dilutions. Bound protein was detected using monoclonal antibodies directed against the PPK or FXI light chain (25, 32). Purified FXI and PPK or supernatants from mock-transfected cells supplemented with known concentrations of FXI or PPK were used as standards. Protein concentration was determined by the Bradford assay (Bio-Rad) or by biospecific interaction analysis using surface plasmon resonance spectroscopy (BIAcore, Freiburg, Germany). CM5 sensor chips were coated with α-FXI (AS199) or α-PPK (AS176) antibodies using the amine coupling kit provided by the manufacturer. Serial 1:2 dilutions of the supernatants were applied at a continuous flow rate of 20 μl/min, and association was followed for 90 s. Dissociation of the complex induced by applying PBS alone was monitored for 3 min. For calibration, supernatants from cells transfected with control vector supplemented with purified FXI or PPK were used. The chip was reconstituted by briefly washing with 30 mM HCl. The relative concentrations of proteins were determined from the measured response units with the BIAevaluation 2.1 program (BIAcore). The concentrations calculated by ELISA or biospecific interaction analysis differed by <10%.

Immunoprecipitation of Recombinant Proteins—HEK293 cells were

transiently transfected with pcDNA3 vectors containing wild-type FXI, FXI/P2, or FXI/ΔF2N using the LipofectAMINE method. After 60 h the cells were washed with Cys/Met-free Dulbecco's modified Eagle's medium and incubated with the same medium for 45 min at 37 °C. The cells were labeled with 100 μCi/ml [^{35}S]Cys/Met (Trans-label, ICN, Eschwege, Germany) for 12 h at 37 °C. The supernatants were collected, centrifuged at 2,500 × *g* for 10 min, and used directly for immunoprecipitation. Adherent cells were washed four times with PBS and lysed in 150 mM NaCl, 50 mM Tris-HCl, pH 8.0, 0.1% SDS, 0.5% deoxycholic acid, 1% Nonidet P-40, 10 μg/ml phenylmethylsulfonyl fluoride, 10 μg/ml benzamidine-HCl (RIPA buffer) under rotation for 1 h at 4 °C. The cell lysates were centrifuged at 14,000 × *g* for 15 min at 4 °C to separate insoluble components. The cell lysates and supernatants were incubated for 60 min at 4 °C with 20 μl of polyclonal anti-FXI antibodies (AS199) bound to staphylococcus A (Pansorbin; Calbiochem, La Jolla, CA) cells. The mixtures were pelleted at 8,000 × *g* for 2 min at 4 °C and washed four times with RIPA buffer. Immunoprecipitates were dissolved in SDS sample buffer and size-fractionated by SDS-PAGE. The gels were fixed with sodium acetate, incubated with 15% (m/v) sodium salicylate for 30 min at room temperature, dried for 2 h at 55 °C, and exposed to Fuji x-ray film for 6 or 24 h at −80 °C (14, 25). Alternatively, immunoprecipitated proteins from unlabeled cells were transferred to nitrocellulose and analyzed by Western blot analysis using monoclonal antibody αP2 or αF4.

HK Binding Assays—Maxisorp™ microtiter plates (used throughout; from Nunc) were coated overnight with 25 nM (3 μg/ml) HK in 100 mM NaCl, pH 5.5 ("coating solution"). Coated plates were washed six times with PBS and blocked with 1% (w/v) BSA in PBS and incubated with serial 1:2 dilutions of individual FXI or PPK apple domains fused to tPA starting at 500 nM (25 μg/ml PBS/BSA). After washing six times with PBS, bound apple domains were probed by 13.3 nM (2 μg/ml) of α-tPA (from rabbit) specific for the tPA portion shared by all constructs, followed by a horseradish peroxidase-coupled secondary antibody to rabbit immunoglobulin F_t fragment and the substrate solution, *i.e.* 0.15% (w/v) diammonium 2,2′-azido-bis-(3-ethyl-2,3-dihydrobenzthiazoline-6-sulfonate), 0.012% (v/v) H_2O_2 in 100 mM citric acid, pH 4.5. After 30 min the absorbance at 405 nm was monitored by an ELISA plate reader (Dynatech, Deppendorf, Germany). Alternatively, a sandwich ELISA was employed. Microtiter plates were coated with 20 nM (3 μg/ml) α-tPA in coating buffer, washed, blocked with 1% BSA, and incubated with serial 1:2 dilutions of apple-tPA fusion proteins (starting concentration, 50 nM in PBS/BSA). Plates were washed, and 8.3 nM (1 μg/ml) biot-HK in PBS/BSA was applied. Bound biot-HK was probed by streptavidin-peroxidase (1 μg/ml; Roche Molecular Biochemicals) and the substrate solution, as above.

For competitive ELISAs, the microtiter plates were coated with 45.4 nM (8 μg/ml) of FXI. Serial 1:2 dilutions of monoclonal antibodies (starting concentration, 1.2 μM = 180 μg/ml), FXI apple-tPA constructs (starting concentration, 4 μM = 240 μg/ml), or full-length recombinant proteins FXI or PPK (starting concentration, 2 μM) were prepared and made 8.3 nM in biot-HK (final concentration, 1 μg/ml), and the resultant mixtures were applied to the coated plates. Bound biot-HK was probed by streptavidin-peroxidase (1 μg/ml) and the substrate solution. To follow binding of HK to immobilized target proteins, 5 nM of full-length FXI and PPK, FXI/PPK chimeras, or proteolytic cleavage products thereof were coated on microtiter plates, followed by incubation with serial 1:2 dilutions (starting at 2 μM = 240 μg/ml) of biot-HK in PBS/BSA. Bound biot-HK was measured as above.

To determine apparent dissociation constants (K_D) and maximum binding (B_{max}) for HK binding to the individual constructs, nonspecific binding of biot-HK to recombinant full-length constructs was determined in the presence of 100-fold molar excess of unlabeled HK. Binding data were calculated using the Prism 2.0a software (GraphPad Software, San Diego, CA). Relative B_{max} values for the recombinant constructs PPK, PPK/F2, FXI/P2, and FXI/Δ2N differed by <15% from the B_{max} value of HK binding to FXI, which was arbitrary set to 100%.

To measure binding of FXI constructs to immobilized HK, 4 nM (4.8 μg/ml) HK was coated on microtiter plates, and serial 1:2 dilutions of FXI or FXI/Δ2N (starting concentration 300 nM) were applied, followed by 6.7 nM (1 μg/ml) of α-FXI (AS 199), an horseradish peroxidase-coupled secondary antibody to rabbit F_c, and the substrate solution. If not otherwise stated, incubation steps were at 37 °C for 45 min, except for the coating step, which was done at 4 °C overnight.

RESULTS

Binding of HK to Single FXI Apple Domains—Initially, we investigated the capacity of HK to bind to individual FXI apple domains. FXI apple domains were expressed as fusion proteins with tPA in baby hamster kidney cells and purified using antibodies against the tPA portion of the fusion protein (Fig. 1*A*). The assay measures binding of the fusion proteins to HK immobilized on a microtiter plate. A rank order of F2 ≫ F4 > F1 ≫ F3 was found for the binding of individual apple domains to HK (Fig. 1*B*). The binding of F3 to HK was not significantly different from that of the control (tPA). An identical rank order was found when fusion proteins were bound to microtiter plates via antibodies to their common tPA portion and probed by increasing concentrations of biot-HK (data not shown). Interestingly, these results are very similar to previously published data examining binding of HK to individual PPK apple domains, using identical techniques: P2 ≫ P4 > P1 ≫ P3 ≈ control (25). These results indicate that the F2 domain is crucial for FXI binding to HK and emphasize a similarity in the interactions between HK and FXI and between HK and PPK.

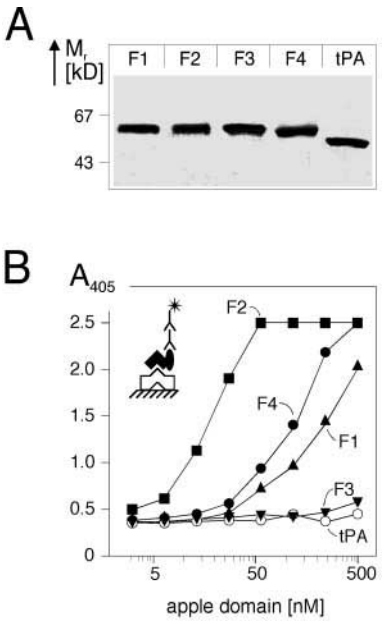

FIG. 1. **Individual FXI apple domains bind differentially to HK.** Single FXI apple domains were expressed in baby hamster kidney cells as fusion proteins with tPA, followed by affinity purification. *A*, 100 ng (1.8 pmol) of domains F1, F2, F3, F4 fused to tPA, and control protein (tPA alone) were separated by SDS-PAGE under reducing conditions and visualized by silver staining. *B*, microtiter plates were coated with 25 nM of HK (*open polygon*), followed by incubation with recombinant fusion proteins in serial 1:2 dilutions starting at 500 nM (*filled polygon*). Complex formation between HK and fusion proteins was detected by α-tPA (*inverted Y*) and a horseradish peroxidase-conjugated secondary antibody (*inverted Y* with *asterisk*), followed by the chromogenic substrate diammonium 2,2′-azido-bis-(3-ethyl-2,3-dihydrobenzthiazoline-6-sulfonate). The figure is representative for a series of five independent experiments. A *schematic diagram* of the setup is given on the *upper left*.

Anti-FXI and PPK Monoclonal Antibodies Interfere with FXI-HK Complex Formation—Previously, we developed a panel of monoclonal antibodies against epitopes on the FXI heavy chain (30). Using the recombinant apple domain-tPA fusion proteins, we identified several antibodies from this panel that interact with individual apple domains on Western blots (Fig. 2*A*). Because of the structural similarity of PPK to FXI, we tested the monoclonal anti-FXI antibodies and a similar panel of monoclonal anti-PPK antibodies for cross-reactivity using full-length FXI and PPK. The anti-PPK antibody αP2 (25, 32) readily detected FXI, whereas all other antibodies tested selectively detected their target protein only (Fig. 2*B*). αP2 cross-reacted selectively with recombinant F2 domain (Fig. 2*C*), suggesting that the epitope for αP2 is conserved in the apple 2 domains of FXI and PPK.

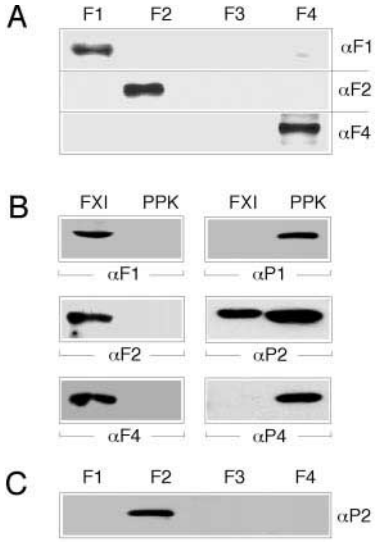

FIG. 2. **Characterization of monoclonal anti-FXI and anti-PPK antibodies.** 20 ng (0.36 pmol) each of FXI apple domains F1–F4 fused to tPA or full size FXI and PPK were separated by SDS-PAGE under reducing conditions and analyzed by Western blotting. *A*, monoclonal antibodies raised against FXI were tested with FXI apple-tPA fusion proteins. The antibodies are named according to the domain recognized. *B*, monoclonal anti-PPK antibodies αP1, αP2, and αP4 and antibodies αF1, αF2, and αF4 raised against native FXI were used to detect for full-length FXI and PPK. *C*, FXI apple domains F1–F4 fused to tPA were probed by αP2.

To investigate the role of individual apple domains in HK binding in the context of a complete FXI molecule, we tested the capacities of monoclonal anti-FXI and anti-PPK antibodies and individual apple domains to interfere with FXI binding to HK in a competitive ELISA. αP2 specifically blocked HK binding to FXI with an apparent IC_{50} of 8 nM (Fig. 3*A*). This anti-PPK antibody was even more effective than its anti-FXI counterpart αF2 (apparent IC_{50} = 35 nM). Antibodies αF4 and αF1 inhibited binding with IC_{50} values of 1 μM and 2 μM, respectively, whereas αP4 and αP1 failed to interfere with HK-FXI complex formation ($IC_{50} > 5$ μM). We obtained similar results using individual apple domains of FXI and PPK fused to tPA. Apple domains F2 and P2 most efficiently inhibited HK-FXI complex formation with apparent IC_{50} values of 35 and 50 nM, respectively, whereas domains F4 and F1 (IC_{50} = 1.3 and 4 μM, respectively) and P4 and P1 ($IC_{50} > 10$ μM) were much less effective in blocking biot-HK binding to FXI (Fig. 3*B*). These results again point to the F2 domain as playing a key role in formation of the FXI-HK complex formation. Furthermore the cross-reactivity studies highlight the similarity in the αP2 epitopes of FXI and PPK and establish αP2 as a generic probe for studying HK binding to apple 2 domains.

Cleavage within the Apple 2 Domain Impairs HK Binding—Activation of PPK by FXIIa initially produces a two-chain protease called α-kallikrein (α-PKa) that binds to HK and antibody αP2 in a manner similar to zymogen PPK (25). With continued incubation, a second proteolytic cleavage occurs in the P2 domain creating β-kallikrein (β-PKa) (31). We have previously demonstrated that this cleavage in P2 greatly reduces binding of both HK and αP2 (25). To further characterize the importance of the apple 2 domain for HK binding to FXI and PPK, we constructed chimeric molecules containing homologous exchanges of the apple 2 domain between PPK and FXI. HEK293 cells were stably transfected with expression constructs for FXI/P2, PPK/F2, wild-type FXI, or PPK (schematics shown in Fig. 4), and recombinant proteins were affinity-purified from culture supernatants. The proteins were activated by incubation with FXIIa, and the reaction mixtures were analyzed under reducing conditions by SDS-PAGE (Fig. 5*A*). To determine the identity of the bands, identical gels underwent Western blot analysis using domain specific antibodies. Zymogen PPK (migrating at 88 kDa) was cleaved into the three fragments of β-kallikrein: the light chain (37 kDa), fragment N (25 kDa), and fragment C (31 kDa). In contrast, activated FXI (FXIa) has two fragments: the light chain migrating at 33 kDa and the heavy chain (49 kDa). FXI/P2 activation results in a

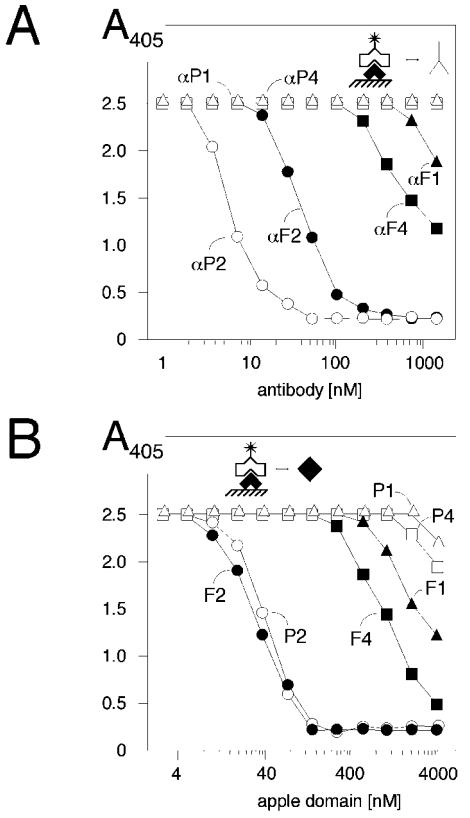

FIG. 3. **Antibodies and individual apple domains interfere with FXI-HK complex formation.** Microtiter plates coated with 45.4 nM FXI were incubated with 8.3 nM biotinylated HK and serial 1:2 dilutions of competitors. *A*, antibodies (starting concentration, 1.2 μM) αP1 (△), αP2 (○), αP4 (□), αF1 (▲), αF2 (●), and αF4 (■). *B*, tPA fusion proteins with individual apple domains (starting concentration, 4 μM) P1 (△), P2 (○), P4 (□), F1 (▲), F2 (●), and F4 (■) were applied. Bound HK was detected by the streptavidin-peroxidase method. A representative result of three independent experiments is shown. A *schematic diagram* of the assay is shown on the *top right*; the *symbols* described in the legend of Fig. 1 are used. The polygon with the *asterisk* is biot-HK.

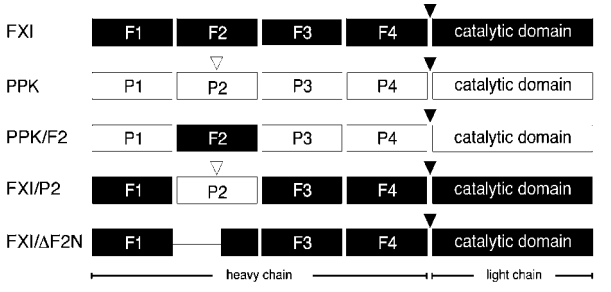

FIG. 4. **Schematic representation of FXI-PPK constructs.** Pictograms show the domain structures of mature PPK, FXI, and chimeric molecules. Domains derived from PPK are shown in *white*, and PPK apple domains are designated with a *P* followed by the domain number. FXI domains are shown in *black* and with the *letter F*. FXI/ΔF2N is a FXI variant whose cDNA was cloned from a megakaryocytic cell line. FXI/ΔF2N lacks Ala^{91}–Arg^{144} in the N terminus of F2 because exon 5 of the FXI cDNA has been removed by alternative splicing. *Black triangles*, FXIIa activation cleavage sites. *White triangle*, cleavage site to produce β-kallikrein.

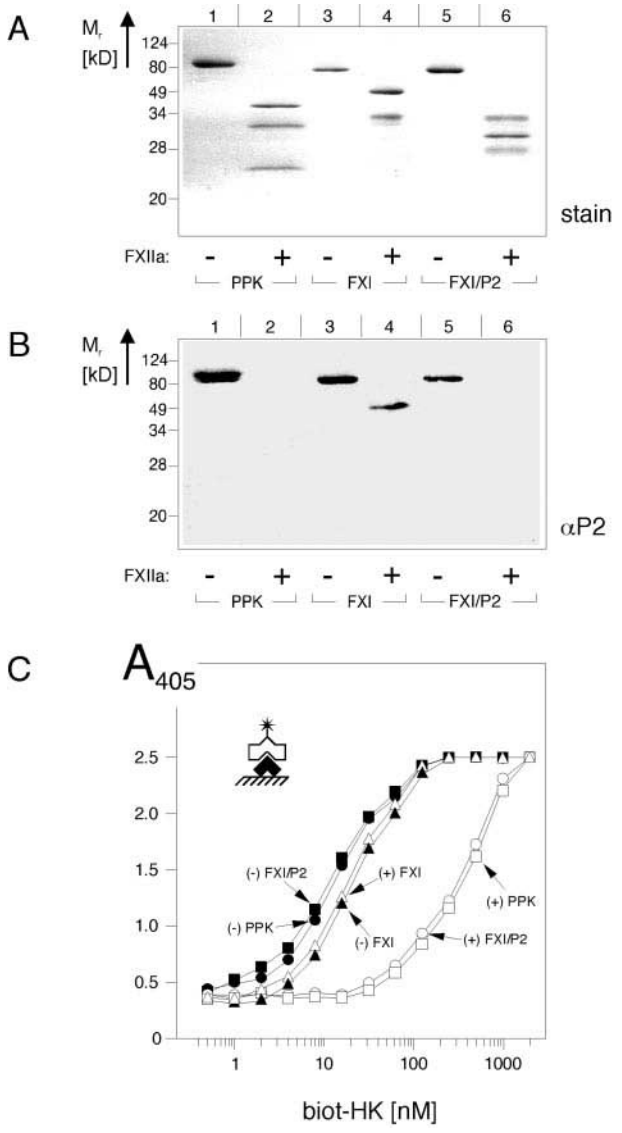

FIG. 5. **Cleavage in the apple 2 domain impairs αP2 and HK binding.** Noncleaved (−) zymogen forms of PPK, FXI, and FXI/P2 were activated with FXIIa for 72 h at 37 °C. Zymogens (−) and activated products (+) were subjected to electrophoresis on a 10% polyacrylamide-SDS gel under reducing conditions. *A*, Coomassie Brilliant Blue-stained gel. The positions of the molecular mass standards in kDa are indicated along the *left edge* of the figure. *B*, Western blot of a gel identical to the one in *A* with 1 pmol of protein/*lane* transferred to nitrocellulose using the anti-PPK antibody αP2 (1 μg/ml) as primary antibody. *C*, HK binding assay. Microtiter plates were coated with 5 nM (0.9 μg/ml) of zymogen (−) or activated (+) recombinant proteins. Following incubation with biot-HK (serial 1:2 serial dilution; starting concentration, 2 μM), bound HK was detected with the biotin-avidin-peroxidase method. A *schematic diagram* of the assay is shown at the *top* of *C*; the *symbols* are identical to those in Fig. 3. A representative result of a series of five independent experiments is shown.

three-chain form (light chain, 33 kDa; fragment N, 28 kDa; and fragment C, 31 kDa), demonstrating that introduction of the P2 domain into FXI also introduces the β-kallikrein cleavage site. PPK/F2, in contrast, is activated to a two-chain form (light and heavy chains; data not shown) consistent with a loss of the β-kallikrein cleavage site.

Next we investigated the consequence of cleavage within the apple 2 domain for binding of αP2, the monoclonal antibody most effective in blocking HK binding to FXI (Fig. 5*B*). αP2 detected the zymogen forms of PPK, FXI, and FXI/P2 (*lanes 1*, *3*, and *5* of Fig. 5*B*) and the heavy chain of FXIa (*lane 4* of Fig. 5*B*). However, αP2 failed to detect any fragment of the cleaved heavy chains of PPK or FXI/P2 (*lanes 2* and *6* of Fig. 5*B*). This clearly indicates that the epitope for αP2 is critically dependent on the conformation of the apple 2 domain and that proper conformation is lost upon proteolytic cleavage of the domain. Based on the premise that the epitope of αP2 overlaps with the HK-binding site, we tested HK binding to the zymogen and activated proteins in a direct binding assay (Fig. 5*C*). Biotinylated HK bound to the zymogens of recombinant PPK (apparent K_D = 10 ± 3 nM) and the FXI/P2 chimera (apparent K_D = 11 ± 3 nM) with almost identical affinities. Cleavage of these zymogens within the apple 2 domain decreases the apparent HK binding affinity more than 25-fold to K_D = 270 ± 37 nM for cleaved PPK and K_D = 284 ± 41 nM for cleaved FXI/P2. In contrast the binding of HK to FXI was not affected by FXI activation (K_D = 19 ± 5 nM for FXI and K_D = 20 ± 4 nM for FXIa), indicating that HK binding is not changed by a cleavage separating the light and heavy chain portions of FXI. Together these data indicate that loss of HK binding during activation is caused by a cleavage in the apple 2 domain of PPK and FXI/P2 and support the hypothesis that the integrity of the apple 2 domain is important for HK binding to both FXI and PPK.

Characterization of FXI Lacking the N-terminal Portion of F2 (FXI/ΔF2N)—Recently a splice variant of FXI mRNA lacking exon 5 was identified in a megakaryocytic leukemia cell line (28). It has been proposed that this message, coding for a factor XI protein lacking the N-terminal half of the F2 domain (amino acids Ala^{91}–Arg^{144}) represents a nonsecreted platelet specific form of factor XI (FXI/ΔF2N; Fig. 4) (29). FXI/ΔF2N offers an opportunity to test the hypothesis that an intact F2 domain is critical for HK binding in a system of possible physiologic relevance. HEK293 cells were transiently transfected with cDNAs coding for FXI/ΔF2N, wild-type FXI, or FXI/P2. After 72 h of incubation in medium containing [^{35}S]Cys/Met, factor XI proteins were immunoprecipitated from culture supernatant and whole cell lysates using polyclonal anti-FXI antibody (AS199) and size fractionated on SDS-polyacrylamide gels. Bands for wild-type FXI and FXI/P2 migrating at 80 kDa were identified in precipitates of culture supernatants (Fig. 6*A*); however, FXI/ΔF2N was not detected in the supernatant. A band representing FXI/ΔF2N (~73 kDa) was identified in immunoprecipitates from whole cells (Fig. 6*A*). A faint band representing intracellular wild-type FXI can also be seen (Fig. 6*A*). Similar results were obtained when COS-7 cells were transfected with the cDNAs (data not shown). On Western blots of nonlabeled immunoprecipitates, antibody αP2 detected FXI and FXI/P2 but failed to recognize FXI/ΔF2N (Fig. 6*B*). However, cellular FXI/ΔF2N was detected by αF4, an antibody that also recognizes FXI and FXI/P2 (Fig. 6*B*). These results indicate that the deletion in F2, while destroying the epitope recognized by αP2, does not alter the conformation of the F4 domain sufficiently to prevent αF4 binding.

FXI/ΔF2N Binding to HK—Because failure to bind the αP2 antibody is associated with poor HK binding to other recombinant proteins, we examined HK binding to FXI/ΔF2N. HEK293 cells were transiently transfected with expression vectors for wild-type FXI or PPK, PPK/F2, FXI/P2, FXI/ΔF2N, or mock vector (control), and proteins were affinity-purified using columns with immobilized polyclonal anti-PPK or anti-FXI antibodies. Protein concentrations were determined by ELISA and by biospecific plasmon-resonance spectroscopy (data not shown). We employed Western blots using antibodies αP2 and αF4 to demonstrate expression of proteins of proper molecular size (Fig. 7*A*). Direct binding of HK binding to the various proteins was examined in a microtiter plate assay (Fig. 7*B*). HK bound to immobilized PPK and the FXI construct where the F2 domain had been replaced by P2, with highest affinities (ap-

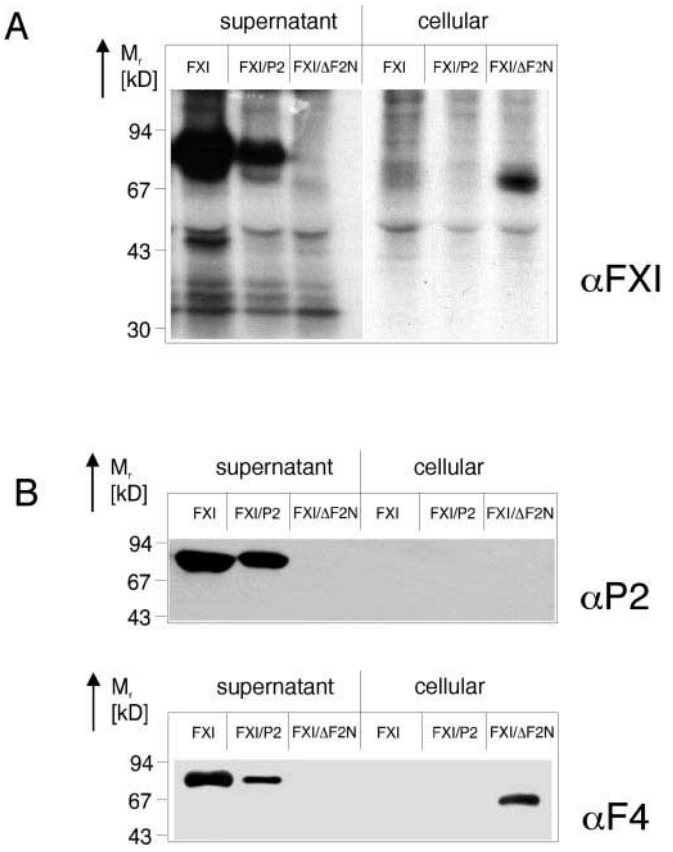

FIG. 6. **Expression and characterization of the FXI splice variant FXI/ΔF2N.** HEK293 cells were transiently transfected with pcDNA3(+) vector constructs containing wild-type FXI, FXI/P2 chimera, or splice variant FXI/△F2N. *A*, cells were metabolically labeled with [^{35}S]Cys/Met, and the culture supernatants (*left*) and lysed cells (*right*) were subjected to immunoprecipitation using anti-FXI antibodies. Immunoprecipitates were resolved on SDS-PAGE under reduced conditions followed by autoradiography. *B*, Western blots of immunoprecipitates from nonlabeled transfected cell supernatants or lysates using αP2 (*upper panel*) or αF4 (*lower panel*) as primary antibodies.

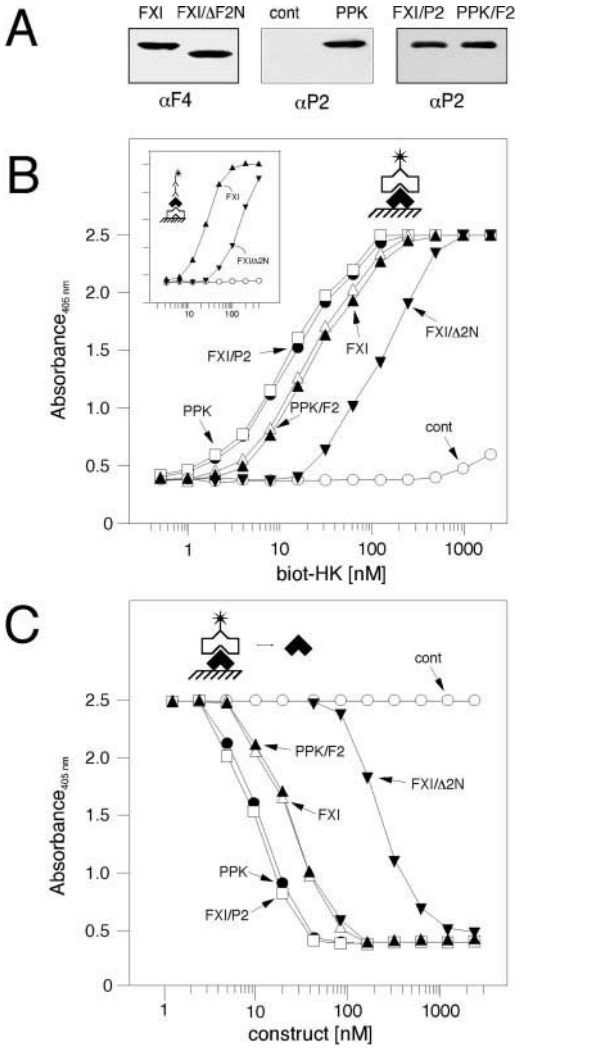

FIG. 7. **HK binding to recombinant proteins.** HEK293 cells were transfected with pcDNA3(+) vector constructs containing wild-type FXI, FXI/ΔF2N, unrelated control (*cont*), wild-type PPK, PPK/P2, or FXI/F2, and recombinant proteins were immunopurified. *A*, 1 pmol of each protein was subjected to SDS-PAGE under reducing conditions, followed by Western blotting with antibody αP2 for PPK, control, and chimeras or F4 antibody for FXI and FXI/ΔF2N. *B*, microtiter plates were coated with 5 nM of PPK (□), FXI (▲), control (*cont*, ○), FXI/P2 (●), PPK/F2 (△), or FXI/Δ2N (▼). Following incubation with a serial 1:2 dilutions of biot-HK (starting from 2 μM), bound HK was measured by the biotin-avidin-peroxidase method. Alternatively, HK-coated microtiter plates (4 nM) were incubated with serial 1:2 dilutions of FXI/ΔF2N starting from 300 nM (*B*, *inset*). *C*, microtiter plates coated with 45.5 nM FXI were incubated with solutions containing a constant concentration of biot-HK (8.3 nM) and decreasing concentrations (serial 1:2 dilutions down from 2 μM) of PPK, FXI, control (*cont*), FXI/P2, PPK/F2, or FXI/Δ2N. A representative of three independent experiments is shown. *Schemes* of the assay setups are shown at the *tops* of *B* and *C*; the *symbols* are identical to those in Fig. 3.

parent K_D = 10 ± 3 and 11 ± 3 nM, respectively). Replacement of P2 of PPK by F2 of FXI lowered the affinity for the PPK/F2 construct (K_D = 18 ± 5 nM) almost to that of native FXI (K_D = 19 ± 5 nM). Deletion of the N-terminal portion of F2 (FXI/ΔF2N) further reduced HK binding affinity to an apparent K_D of 107 ± 19 nM. However, FXI/ΔF2N still bound HK significantly over background (Fig. 7*B*), indicating that other portions of the FXI molecule contribute to HK binding, although to a minor extent. This conclusion was confirmed by an assay system using a "reverse" set-up, where microtiter plate-bound HK was probed by FXI and FXI/ΔF2N (Fig. 7*B*, *inset*).

To rule out the possibility that immobilization of the various constructs to microtiter plates may induce subtle conformational changes and thus contribute to differential binding affinities, we employed a competitive ELISA. Constructs such as PPK and FXI/P2 holding apple domain P2 competed with biot-HK binding to immobilized FXI with apparent IC_{50} values of 9 and 10 nM, respectively. Replacement of P2 by F2 in constructs PPK/F2 and FXI lowered the apparent IC_{50} values to 27 and 29 nM, respectively, whereas truncation of F2 in FXI/ΔF2N increases the apparent IC_{50} value to 312 nM (Fig. 7*C*). Taken together, these results underline the importance of the apple 2 domain in HK binding to FXI and PPK. Furthermore, the data indicate that FXI/ΔF2N binds poorly to HK.

DISCUSSION

HK circulates in plasma in binary complexes with FXI or PPK (9, 11). The protease zymogens remain bound to HK upon attachment of the kinin precursor to its acceptor structures on cell membranes such as heparan and chondroitin sulfate proteoglycans (13, 14). In this way HK works as an adapter that links the proenzymes indirectly to the cell. For PPK the physiological consequence of the association between the proenzyme and prohormone (HK) on the cell surface is obvious. In the course of contact phase reactions, PPK is converted to the active enzyme α-kallikrein (α-PKa) that proteolytically cleaves HK, liberating the vasoactive peptide hormone bradykinin (38, 39). Because bradykinin has a half-life of <15 s in plasma, the generation of the hormone from its precursor in proximity to its cellular receptors is a prerequisite for an effective hormone

response (14, 16, 40). In contrast the physiological consequences of the interaction between FXI and HK are less clear. HK is required for proper FXI activation during contact activation-initiated coagulation *in vitro*. However, although congenital FXI deficiency is associated with a bleeding disorder, deficiency of HK is not (39, 41, 42). Several recent studies have shed light on this conundrum. It has been shown that HK facilitates FXI binding to the surface of activated platelets, where it is rapidly activated to FXIa (18, 20, 43). Prothrombin may serve as a substitute for HK in this role, providing a plausible explanation as to why HK-deficient humans do not bleed (19).

Despite their different biological functions, PPK and FXI share 58% amino acid identity and identical domain organizations (7, 12, 46). This homology is reflected in the organization of the PPK and FXI genes, both containing 15 exons and identical intron-exon boundaries (47, 48). In fact, the two genes are separated by <10 kilobases on the distal end of the long arm of chromosome 4, indicating they are the products of a duplication event involving a common ancestral gene.[2] Despite the many similarities between FXI and PPK, previously published data suggest that the mechanisms by which these proteins interact with HK are quite different. A series of experiments using conformationally constrained peptides representing various portions of the FXI apple domains have assigned binding sites for several macromolecules on FXI (49–51). Sequence segments important for the interaction between HK and FXI have been localized to amino acids Phe^{56}–Ser^{86} of the F1 domain, based on the capacity of peptides from this area to competitively inhibit HK binding to FXI. Peptides representing F2, F3, and F4 were poor inhibitors of HK binding (26, 27). In contrast, an approach using recombinant individual apple domains, monoclonal antibody interference, PPK deletion mutants, and PPK/FXI chimeras indicates that the HK-binding site on PPK is discontinuous, involving three apple domains (P1, P2, and P4) with P2 being most important (25). Using a similar strategy to analyze the HK interaction with FXI we have found that the apple 2 domain of FXI is most important for HK binding, with minor contributions coming from the F1 and F4 domains. The results are strikingly similar to our previous results with HK binding to PPK and suggest that the interactions between HK and FXI and between HK and PPK are similar.

The reasons for the disparity between our results and those of the previously published peptide inhibition studies are not clear. Both approaches determined the F1 domain to be important for HK binding. It is possible that the peptides for F2 and F4 used in the previous study (26) were not in the proper conformation to inhibit FXI-HK complex formation. Indeed, early studies examining HK binding to PPK, using peptides based on the PPK sequence, identified P1 and P4 as important for HK binding but failed to identify P2 as a critical element of the binding site (23, 52). This may be indicative of a general problem with using peptides to model complex protein structures. Each apple domain in FXI and PPK contains three or four disulfide bonds, and the resulting structures may be difficult to reproduce with a short linear peptide. Supporting this notion, mapping studies of the factor IX-binding site on FXI using peptides localized the binding site to F2 (53), whereas studies using FXI/PPK chimeras and alanine scanning mutagenesis make a strong case for F3 (35). Our approach using fusion proteins of tPA and individual apple domains may have some limitations as well. For example, the bulky tPA portion of ~50 kDa could sterically hinder the access of HK to binding site(s) exposed by the smaller apple domain of ~10 kDa. To minimize this possibility, we have inserted spacer sequences of 6 amino acids, each separating the apple domain from the flanking regions of tPA. Although we cannot entirely exclude an underestimation of the HK binding capacity of individual apple domains by the strategy employing fusion proteins, our alternative approaches using FXI deletion mutants or domain-directed antibodies are consistent with the conclusions drawn from individual apple domain experiments.

Factor XIIa cleaves PPK to form the active two-chain enzyme, α-PKa. Prolonged incubation with FXIIa results in a second cleavage in the P2 domain, producing the three-chain form β-PKa (31). Although the enzymatic activities of β-PKa and α-PKa are similar (31), the HK binding capacity of β-PKa is decreased >20-fold compared with α-PKa or PPK (24, 25). Because kallikrein docking to cell surfaces is mediated through HK, the dramatic loss in affinity for HK will promote dissociation of β-PKa from cells. It is possible, therefore, that FXIIa proteolysis of PPK serves two purposes: the initiation and the termination of kinin release on endothelial cells. The present study clearly demonstrates that human FXI is not susceptible to secondary cleavage in its heavy chain by FXIIa. Thus the FXIa-HK complex may persist on cell surfaces such as platelet membranes for extended periods of time. The differences in the sequences of P2 and F2 may well reflect different requirements for continuous cell-associated activity between α-PKa and FXIa, respectively.

Recently, a splice variant of FXI mRNA lacking the sequence encoded by exon 5 of the FXI gene (coding for amino acids Ala^{91}–Arg^{144} in F2) has been described (28). Flow cytometry studies indicate that this variant is expressed on platelet membranes independent of plasma FXI expression and thus may compensate partially for plasma FXI deficiency (29). Although the (patho)physiological relevance of this variant is debated (10), the protein offers an opportunity to test our model for HK binding with a molecule of possible physiologic importance. Initial studies predicted that FXI/ΔF2N is a tetramer comprised of identical 50–55-kDa subunits that migrates at 220 kDa (37, 44). In contrast, the protein we precipitated from transfected cells has an apparent molecular mass of 73 kDa. This is consistent with the loss of the 53 amino acids encoded by exon 5 from the 80-kDa FXI polypeptide. We note that FXI/ΔF2N is expressed poorly compared with FXI but was recoverable using affinity purification with polyclonal anti-FXI antibodies. Furthermore, a monoclonal antibody (αF4) raised against full-length FXI recognizes FXI/ΔF2N on Western blot. This indicates that FXI/ΔF2N may share a similar conformation with FXI outside of the F2 domain. Most importantly for the present study, FXI/ΔF2N bound poorly to HK compared with FXI, supporting an important role for F2 in HK binding.

We note limitations in our experiment analyzing antibody interference with FXI-HK complex formation. We did not have monoclonal antibodies directed against F3 (or P3) to make a complete study. However, there are several lines of evidence to indicate that F3, as in the case of P3, is not involved in HK binding. We demonstrated that the recombinant F3 domain bound HK poorly. Furthermore, a previous study involving saturation mutagenesis of the F3 domain failed to identify abnormalities in HK binding using surface plasmon resonance techniques (18). These findings, in conjunction with the observation that P3 does not contribute to HK binding to PPK (25), support the premise that F3 is not involved in HK binding to FXI. Because F3 appears to be important for binding to other components of the coagulation mechanism, such as factor IX (35, 45), platelet membranes (51), and heparin (52), it makes sense that it would not be involved in the interaction with HK

[2] T. Tarumi, M. Zhao, S. Williams, and D. Gailani, unpublished observations.

(43). Taken together, our data are consistent with a model of a discontinuous HK-binding site jointly formed by F2, F1, and F4 of FXI, where domain F2 harbors the core binding sequence and F1 and F4 contribute directly and/or indirectly to this high affinity binding site. The generation of constructs comprising doublets or triplets of distinct apple domains and of recombinant proteins holding duplicated or multiple individual apple domains should allow us to address the issue of cooperativity among the apple domains in more detail.

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