Characterization of the H-kinogen-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 recombiant 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 IC50 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 XIa, 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 XIa. 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 polypeptide chains 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 XIa (FXIa) (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 D6H (21), to which FXI binds with high affinity (apparent KD = 1.8 × 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 Phe56 and Ser86 within the first apple domain (P1). The second, third, and fourth FXI apple domains (P2, 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
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 P followed by the domain number. Murine monoclonal antibodies are indicated by the symbol "a" 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, aP2 is an antibody raised against PPK that recognizes the P2 domain. The names of some antibodies used in previous studies are maintained to comply with the new nomenclature system. Specifically, anti-PPK antibodies aP1, aP2, and aP4 were formerly designated PKH19, PKH6, and PKH1, respectively (25), and anti-FXI antibodies aF1, aF2, and aF4 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 P2 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-e-aminoacaproyl-N-hydroxysuccinimide (Pierce) in 0.1 x NaHCO₃ for 4 h at 4 °C. Unbound biotin-e-aminoacaproyl-N-hydroxysuccinimide was separated from biot-HK by centrifuging three times at 2,000 x 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 NaH₂PO₄, 10 mM Na₂HPO₄, pH 7.4. Human factor XII was purchased from Enzyme Research Laboratories (South Bend, IN) and activated by incubation with glass beads for 7.4 °C. Human factor XII was purchased from Enzyme Research Laboratories (South Bend, IN) and activated by incubation with glass beads for 4 h at 4 °C. Human factor XII was purchased from Enzyme Research Laboratories (South Bend, IN) and activated by incubation with glass beads for 4 h at 4 °C. Human factor XII was purchased from Enzyme Research Laboratories (South Bend, IN) and activated by incubation with glass beads for 4 h at 4 °C.

**EXPERIMENTAL PROCEDURES**

**Expression and Purification of FXI Truncation Protein FXI/F2N—**

The cDNA for the FXI splice variant FXI/F2N (28) was a generous gift from Dr. Peter Walsh (Temple University School of Medicine, Philadelphia, PA). The cDNA was subcloned into the EcoRI site of the pcDNA3 (+) vector, and the HEK293 cells were transiently transfected 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 Translation Protein FXI/F2N—**

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**Expression and Purification of PPK Translation Protein PPK/F2N—**

The cDNA for the PPK splice variant PPK/F2N (28) was a generous gift from Dr. Peter Walsh (Temple University School of Medicine, Philadelphia, PA). The cDNA was subcloned into the EcoRI site of the pcDNA3 (+) vector, and the HEK293 cells were transiently transfected 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).

**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). After extensive washing with PBS, bound protein/FXI/F2N was eluted with 1% NaSCN in PBS, dialyzed against PBS followed by gel filtration high pressure liquid chromatography on a Sephadex 200 column (Amersham, Buckinghamshire, England) to equilibrate the protein. Equilibrated protein-containing fractions were concentrated using an Amicon concentrator, and protein concentration was determined by ELISA (see below).

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**Immunoprecipitation of Recombinant Proteins—**

HK-binding Site on Factor XI

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.

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HK-binding Site on Factor XI

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, followed by affinity purification. A, 100 ng (1.8 pmol) of domain 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. The recombinant fusion proteins were soluble and did not show any apparent dissociation constants for HK binding to FXI and between HK and PPK. 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 blot analysis by using monoclonal antibody αP2 or αF4.

HK Binding Assays—Maxisorp™ microtiter plates (used throughout; from Nunc) were coated overnight with 25 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 with tPA starting at 500 nM (25 μg/ml) with serial 1:2 dilutions of individual FXI or PPK apple domains fused with tPA starting at 500 nM (25 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, fragment and the substrate solution, i.e., 0.15% (w/v) 2,2'-azido-bis-(3-ethyl-2,3-dihydrobenzthiazoline-6-sulfonate), 0.010% (v/v) H2O2 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 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 ng (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 μM (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 chimera, 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 (Kd) and maximum binding (Bmax) 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 analyzed using the Prism 2.0a software (GraphPad Software, San Diego, CA). Relative Bmax values for the recombinant constructs PPK, PPK/F2, FXI/F2, and FXI/F2N differed by ~15% from the Bmax 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/F2N (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 Fc, 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.

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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 HK binding to FXI in a competitive ELISA. αF2 specifically blocked HK binding to FXI with an apparent IC₅₀ of 8 nM (Fig. 3A). This anti-PPK antibody was even more effective than its anti-FXI counterpart αF2 (apparent IC₅₀ = 35 nM). Antibodies αF4 and αF1 inhibited binding with IC₅₀ values of 1 μM and 2 μM, respectively, whereas αP4 and αP1 failed to interfere with FXI-HK complex formation (IC₅₀ > 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 IC₅₀ values of 35 and 50 nM, respectively, whereas domains F4 and F1 (IC₅₀ = 1.3 and 4 μM, respectively) and P4 and P1 (IC₅₀ > 10 μM) were much less effective in blocking biot-HK binding to FXI (Fig. 3B). 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 αF2 epitopes of FXI and PPK and establish αF2 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-PKα) that binds to HK and antibody αF2 in a manner similar to zymogen PPK (25). With continued incubation, a second proteolytic cleavage occurs in the P2 domain creating β-kallikrein (β-PKα) (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.

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.

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) αF1 ( ), αF2 ( ), αF4 ( ), 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. Picturegrams 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/F2N is a FXI variant whose cDNA was cloned from a megakaryocytic cell line. FXI/F2N lacks Ala⁹¹–Arg¹⁴⁴ in the N terminus of P2 because exon 5 of the FXI cDNA has been removed by alternative splicing. Black triangles, FXIα activation cleavage sites. White triangle, cleavage site to produce β-kallikrein.
HK-binding Site on Factor XI

Characterization of FXI Lacking the N-terminal Portion of F2 (FXI/F2N)—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 Ala3-Ala144) represents a nonsecreted platelet specific form of factor XI (FXI/F2N; Fig. 4) (29). FXI/F2N 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/F2N, wild-type FXI, or FXI/P2. After 72 h of incubation in medium containing [35S]Cys/Met, factor XI proteins were immunoprecipitated from culture supernatants (Fig. 6A); however, FXI/F2N was not detected in the supernatant. A faint band representing FXI/F2N (63 kDa) was detected in immunoprecipitates from whole cells (Fig. 6A). Cellular FXI/F2N was not detected by αF4, an antibody that also recognizes FXI and FXI/P2 (Fig. 6B). These results indicate that the deletion in F2, while destroying the epitope recognized by αF2, does not alter the conformation of the F4 domain sufficiently to prevent αF4 binding.

FXI/F2N Binding to HK—Because failure to bind the αF2 antibody is associated with poor HK binding to other recombinant proteins, we examined HK binding to FXI/F2N. HEK293 cells were transiently transfected with expression vectors for wild-type FXI or PPK, PPK/F2, FXI/P2, FXI/F2N, 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 αF2 and αF4 to demonstrate expression of proteins of proper molecular size (Fig. 7A). Direct binding of HK to the various proteins was examined in a microtiter plate assay (Fig. 7B). HK bound to immobilized PPK and the FXI construct where the F2 domain had been replaced by P2, with highest affinities (ap-
FIG. 7. HK binding to recombinant proteins. HEK293 cells were transfected with pcDNA3(+) vector constructs containing wild-type FXI, FXI/F2N, unrelated control (cont), wild-type PPK, PPK/F2, 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 aF2 for FXI and FXI/F2, control (cont), PPK/F2, or FXI/F2N. 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.

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-PKα) 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

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-PKα) 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

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/F2 chimera, or splice variant FXI/ΔF2N. A, cells were metabolically labeled with [35S]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 aP2 (upper panel) or aF4 (lower panel) as primary antibodies.

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/F2 holding apple domain P2 competed with biot-HK binding to immobilized FXI with apparent IC₅₀ values of 9 and 10 nM, respectively. Replacement of P2 by F2 in constructs PPK/F2 and FXI lowered the apparent IC₅₀ values to 27 and 29 nM, respectively, whereas truncation of F2 in FXI/ΔF2N increases the apparent IC₅₀ value to 312 nM (Fig. 7C). 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

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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/F2, 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 aF2 for FXI and FXI/F2, control (cont), PPK/F2, or FXI/F2N. 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.

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HK-binding Site on Factor XI

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Localized to amino acids Phe56 important for the interaction between HK and FXI have been conformationally constrained peptides representing various with HK are quite different. A series of experiments using peptides localized the binding site to F2 (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 Phe56–Ser56 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-PKα. Prolonged incubation with FXIIa results in a second cleavage in the P2 domain, producing the three-chain form β-PKα (31). Although the enzymatic activities of β-PKα and α-PKα are similar (31), the HK binding capacity of β-PKα is decreased >20-fold compared with α-PKα 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-PKα 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 P4 may well reflect different requirements for continuous cell-associated activity between α-PKα 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 Ala101–Arg144 in P2) 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 F3/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 polyvalent 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 F3 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.

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2 T. Tarumi, M. Zhao, S. Williams, and D. Guilani, unpublished observations.
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REFERENCES

1. Mann, K., Jenny, R., and Krishnaswamy, S. (1988) Annu. Rev. Biochem. 57, 915–956
2. Furie, B., and Furie, B. (1988) Cell 53, 505–518
3. Davie, E., Fujikawa, K., and Kisiel, W. (1991) Biochemistry 30, 10363–10370
4. Gailani, D., and Broze, G. (2001) in Biochemistry (Scriver, C., Beaudet, A., Sly, W., Valle, D., Childs, B., Kinzler, K., and Vogelstein, B., eds) Vol. 3, 8th Ed., pp. 4433–4453, McGraw-Hill, New York
5. Fujikawa, K., Chung, D., Hendrickson, L., and Davie, E. (1986) Biochemistry 25, 2417–2424
6. McMullen, B., Fujikawa, K., and Davie, E. (1991) Biochemistry 30, 2066–2080
7. Zhou, B. N., Voswijk, R. A., and Griffin, J. H. (1985) Blood 62, 1123–1131
8. Martincic, D., Kravtsov, V., and Gailani, D. (1999) Blood 94, 3397–3404
9. Mandle, R. J., Colman, R. W., and Kaplan, A. P. (1976) Proc. Natl. Acad. Sci. U. S. A. 73, 4179–4183
10. Henderson, L. M., Figueroa, C. D., Mu, R. W., and Mu, R. A., Scott, C. F., de Agostini, A., Burger, D., and Schapira, M. (1985) J. Biol. Chem. 260, 3109–3115
11. Mandle, R. J., Colman, R. W., and Kaplan, A. P. (1976) Proc. Natl. Acad. Sci. U. S. A. 73, 4179–4183
12. Chung, D. W., Fujikawa, K., McMullen, B. A., and Davie, E. W. (1986) Biochemistry 25, 2410–2417
13. Henderson, L. M., Figueroa, C. D., Muller-Esterl, W., and Bhoola, K. D. (1994) Biochemistry 33, 8446–8452
14. Renné, T., Dedio, J., David, G., and Muller-Esterl, W. (2000) J. Biol. Chem. 275, 33688–33696
15. Renne, T., and Muller-Esterl, W. (2001) FEBS Lett. 500, 36–41
16. Herwald, H., Dedio, J., Kellner, R., Loos, M., and Muller-Esterl, W. (1996) J. Biol. Chem. 271, 13040–13047
17. Greengard, J. S., Heeb, M. J., Erstad, E., Walsh, P. N., and Griffin, J. H. (1986) Biochemistry 25, 3884–3890
18. Ho, D., Badellino, K., Baglia, F., Sun, M.-F., Zhao, M., Gailani, D., and Walsh, P. (1998) J. Biol. Chem. 273, 25139–25145
19. Baglia, F., and Walsh, P. (1998) Biochemistry 37, 2271–2281
20. Baglia, F., and Walsh, P. (2000) J. Biol. Chem. 275, 20514–20519
21. Tait, J. F., and Fujikawa, K. (1967) J. Biol. Chem. 242, 11561–11566
22. Tait, J. F., and Fujikawa, K. (1968) J. Biol. Chem. 261, 15396–15401
23. Herwald, H., Jahnne-Dechent, W., Alla, S. A., Hock, J., Bouma, B. N., and Muller-Esterl, W. (1995) J. Biol. Chem. 268, 14527–14535
24. Herwald, H., Renne, T., Meijers, J. C. M., Chung, D. W., Page, J. D., Colman, R. W., and Muller-Esterl, W. (1996) J. Biol. Chem. 271, 13061–13067
25. Renne, T., Dedio, J., Meijers, J. C., Chung, D., and Muller-Esterl, W. (1999) J. Biol. Chem. 274, 25777–25784
26. Baglia, F. A., Jameson, B. A., and Walsh, P. N. (1990) J. Biol. Chem. 265, 4149–4154
27. Baglia, F. A., Jameson, B. A., and Walsh, P. N. (1992) J. Biol. Chem. 267, 4247–4252
28. Hsu, T. C., Shore, S. K., Seshamma, T., Bagasra, O., and Walsh, P. N. (1998) J. Biol. Chem. 273, 13787–13793
29. Hsu, C. J., Baglia, F. A., Mills, D. C., Konkle, B. A., and Walsh, P. N. (1998) Blood 91, 3800–3807
30. Wullimim, W., Minnema, M., Meijers, J. J., Roem, D., Eerenberg, A., Nuijens, J., ten Cate, H., and Hack, C. (1995) Blood 85, 1517–1526
31. Colman, R. W., Wachtalg, Y. T., Kuzich, U., Weinbaum, G., Hahn, S., Fixler, R. A., Scott, C. F., de Agostini, A., Burger, D., and Schapira, M. (1985) Blood 63, 311–318
32. Hock, J., Vogel, R., Linke, R. P., and Muller-Esterl, W. (1996) J. Biol. Chem. 265, 12005–12011
33. Meijers, J. C., Mulvihill, E. E., Davie, E. W., and Chung, D. W. (1992) Biochemistry 31, 4680–4684
34. Johannesen, M., Dines, V., Pingle, K., Petersen, L. C., Rao, D., Linsbin, P., O’Harra, P., and Mulvihill, E. (1990) Thromb. Haemostasis 63, 54–59
35. Horton, R. M., Hunt, H. D., Ho, S. N., Pullen, J. K., and Pease, L. R. (1989) Gene (Amst.) 77, 61–68
36. Lipsomb, M., and Walsh, R. (1979) J. Clin. Invest. 63, 1066–1014
37. Cochrane, C., and Revak, S. (1986) J. Exp. Med. 152, 608–619
38. Remans, K., and Seidah, N. G. (1991) Biochemistry 30, 3884–3890
39. Asakai, R., Davie, E., and Chung, D. (1987) J. Biol. Chem. 262, 1516–1522
40. Page, J. D., You, J. L., Harris, R. B., and Colman, R. W. (1994) Arch. Biochem. Biophys. 314, 159–164
41. Baglia, F. A., Jameson, B. A., and Walsh, P. N. (1991) J. Biol. Chem. 266, 24190–24197