Mapping of the Discontinuous H-kininogen Binding Site of Plasma Prekallikrein

EVIDENCE FOR A CRITICAL ROLE OF APPLE DOMAIN-2

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 Plasma prekallikrein, a zymogen of the contact phase system, circulates in plasma as heterodimeric complex with H-kininogen. The binding is mediated by the prekallikrein heavy chain consisting of four apple domains, A1 to A4, to which H-kininogen binds with high specificity and affinity (KD = 1.2 × 10⁻⁸ M). Previous work had demonstrated that a discontinuous kininogen-binding site is formed by a proximal part located in A1, a distal part exposed by A4, and other yet unidentified portion(s) of the kallikrein heavy chain. To detect relevant binding segment(s) we recombinantly expressed single apple domains and found a rank order of binding affinity for kininogen of A2 > A4 ∼ A1 > A3. Removal of single apple domains in prekallikrein deletion mutants reduced kininogen binding by 21 (A1), 64 (A2), and 24% (A4), respectively, whereas deletion of A3 was without effect. Transposition of homologous A2 domain from prekallikrein to factor XI conferred high-affinity kininogen binding from the former to the latter. The principal role of A2 for H-kininogen docking to the prekallikrein heavy chain was further substantiated by the finding that cleavage of a single peptide bond in A2 drastically diminished the H-kininogen binding affinity. Furthermore, the epitope of monoclonal antibody PKH6 which blocks kallikrein-kininogen complex formation with an IC₅₀ of 8 nM mapped to the center portion of domain A2. Our data indicate that domain A2 and two flanking sequence segments of A1 and A4 form a discontinuous binding platform for H-kininogen on the prekallikrein heavy chain. Domain-specific antibodies directed to these critical sites efficiently interfered with contact phase-induced bradykinin release from H-kininogen.

Human plasma prekallikrein (PPK), the zymogen of the plasma serine proteinase α-kallikrein is involved in the intrinsic pathway of blood coagulation (1, 2) in pro-urokinase-dependent fibrinolysis (3, 4), and in local inflammation (5). The zymogen is converted into its active form by surface-bound activated factor XII (FXIIa) (6, 7) via cleavage of a single peptide bond at position 371. The active enzyme, α-kallikrein, is composed of a catalytically active light chain of 35 kDa and a heavy chain of 50 kDa, linked together by a single disulfide bridge (8). Autocatalytic cleavage at Lys140-Ala141 of its heavy chain further converts α-kallikrein into a three-chain form, β-kallikrein (9). Analyses revealed that the 371 residues of the PPK heavy chain is composed of four tandem repeats of 90–91 amino acid residues each (10) with a unique disulfide bridge pattern where the first and sixth, second and fifth, and third and forth cysteine residues are linked (11). These repetitive modules, aptly dubbed “apple” domains A1 to A4, mediate the high-affinity binding of PPK to its major substrate, high molecular mass kininogen (H-kininogen, HK), with an apparent KD of 1.2 × 10⁻⁶ M (12). The bimolecular complex docks to the plasma membranes of many cells via specific and affine cell-binding sites exposed on HK domains D3 and D6H. Local accumulation of the prohormone and its cognate processing enzyme on the surface of target cells such as neutrophils, platelets, and endothelial cells allows the extremely short-lived effector of the system, bradykinin (BK, t₁/₂ < 15 s), to act on cellular receptors next to the site of release (13).

To meet the requirements of a locally operating effector system, an elaborate network of complementary structures ensures that HK and PPK interact both in solution and on surfaces. HK exposes a continuous segment of 27 amino acids in the carboxyl-terminal portion of domain D6H of its light chain to which PPK binds (12, 14) whereas the corresponding HK-binding site on the prekallikrein heavy chain is highly discontinuous. Affinity cross-linking studies indicated that one interacting segment is localized in the amino-terminal portion of A1 (15). An antibody-based strategy as well as peptide competition studies identified a second binding segment in the center part of A4 (16–18) and indicated that other, yet unknown portion(s) of the PPK heavy chain contribute to H-kininogen binding (16). On the amino acid level PPK exhibits 58% sequence identity to FXI, another serine proteinase of the contact activation system (1, 19, 20). Like PPK, FXI complexes with HK via its heavy chain though with lower affinity (KD = 1.8 × 10⁻⁵ M) than PPK (21). Since the FXI-binding site on HK overlaps with the PPK-binding site, the two zymogens mutually displace each other from the HK light chain (14, 21).

The aim of the present study was the identification of crucial structures and domains that make up the discontinuous HK-binding site on PPK. This paper is available online at http://www.jbc.org
binding site in PPK. By direct binding studies with recombinantly expressed single apple domains, by analysis of deletion mutants and chimeras of PPK and FXI where apple domains had been removed and exchanged, and by antibody competition experiments we provide convincing evidence that apple domain A2 in PPK is crucial to HK binding. We demonstrate that blockage of the relevant subsites in domains A1, A2, and A4 efficiently attenuates contact phase-dependent bradykinin release from the HK-PPK complex.

EXPERIMENTAL PROCEDURES

Proteins and Antibodies—HK and PPK were isolated from human plasma according to established protocols (22, 23). Human FXII was from Enzyme Research Laboratories (South Bend, IN) and activated by incubation with glass beads for 30 min at 37 °C. The generation and characterization of the mouse monoclonal antibodies to human PPK, i.e. PKH1, PKH4, PKH6, and PKL16, have been previously detailed (22). Monoclonal antibody PKH19 was raised against the synthetic peptide PK31 of the human PPK sequence (15). Antisera AS176 and AS199 were raised in rabbits against purified human PPK and FXI, respectively. Recombinantly expressed human PPK apple domain A3 fused to human tissue-type plasminogen activator (tPA; see below) was used to generate antibodies (“anti-rA3”) in mice following standard immunization protocols. Monoclonal antibodies HKL16 and HKL22 directed to domains D1 and D4 of human HK were used (24). To produce active forms of kalikrein, PPK was incubated with FXIIa at a molar ratio of 100:1 in PBS (6.5 mM Na2HPO4, 1.5 mM KH2PO4, 2.7 mM KCl, 150 mM NaCl, pH 7.4) at 37 °C for 2 h to give a-kaIkirein or for 72 h to yield β-kaIkirein. For biotinylation, 100 μg of HK was incubated with 10 μg of biotin-e-amino-caproyl-N-hydroxysuccinimide (biotin-X-NHS, Pierce, St. Augus-

tin, Germany) in 0.1 M NaHCO3 for 4 h at 4 °C. The buffer was changed to 1% bovine serum albumin, serial dilutions (2n) of the supernatants were applied, and bound PPK was detected by antibody PKL16 to the PPK light chain followed by a horseradish peroxidase-coupled secondary antibody against mouse immunoglobulin and the chromogenic substrate. A competitive ELISA was established to test for the interference of unlabeled antibodies with HK-PPK complex formation. PPK was coated at 4 μg/ml (45.4 nm) and serial dilutions (2n, starting concentrations 180 μg/ml = 1.2 μM) of antibodies to PPK including 1 μg/ml (8.3 nm) biotinylated HK were applied. Bound biotinylated HK was probed with streptavidin-peroxidase followed by the chromogenic substrate. Values of IC50 were calculated by the KaleidaGraph 3.05 algorithm (Synergy Software, Reading, PA).

Recombinant Expression of PPK Domains in Escherichia coli—The pMAL-c2 expression system (New England Biolabs, Bad Schwalbach, Germany) was used for expression and purification of fusion proteins consisting of the bacterial maltose-binding protein (MBP) and PPK apple domains in E. coli strain BL21. Polymerase chain reaction (PCR) with Taq polymerase (Amersham Pharmacia Biotech) generated cDNA fragments encoding single PPK apple domains with the following upstream and downstream primers (Roth): 5′-gattgataagattttagga-gaagc-3′ and 5′-catgaggaagattttatgagc-3′ (for domain A1), 5′-cat-aaaaatagttcctacagc-3′ and 5′-catggttcgaaatctagc-3′ (A2), 5′-gctttagttatgtggc-3′ and 5′-gaacatgcagttgagattggc-3′ (A3) and 5′-etctagatagtccttactccg-3′ and 5′-gaaacatgccgattttgccg-3′ (A4), respectively. Plasmid pPK was used as the template in a polymerase chain reaction that comprises 40 cycles of denaturation at 95 °C for 45 s, annealing at 50 °C for 45 s, and extension at 72 °C for 90 s in a thermal cycler (Biometra, Göttingen, Germany). Before ligating the constructs into the pMAL-c2 vector using T4 DNA ligase (New England Biolabs), the vector and the isolated PCR products were cleaved with restriction enzymes HindIII and XhoI (A1), EcoRI and XhoI (A2), and XhoI (A3 and A4). HindIII-digested A3 and A4 were, respectively, amplified by polymerase chain reaction using primers introducing a HindIII site at the 5′ end and an XhoI site in the 3′ end of the constructs. The PCR products were ligated into the pMAL-c2 vector and the constructs were confirmed by sequencing. Recombinant plasmids were propagated in E. coli XL1-blue strain; vectors were isolated by a plasmid DNA isolation kit (Qiagen, Hilden, Germany) before transfection into E. coli BL21 strain for expression. Exponentially growing cultures containing the relevant constructs were stimulated for 2 h with 0.5 mM isopropyl-β-D-thiogalactopyranoside (Roth), and the cells were harvested by centrifugation at 4,000 × g for 15 min at 4 °C. The pellets were resuspended in 2 times PBS supplemented with a protease inhibitor mixture (10 μg/ml each soybean trypsin inhibitor, benzamidine, leupeptin (Sigma), and 0.1 mM Pefabloc SC), put on ice, and lysed by repeated brief ultrasonic pulses for 3 min. Following centrifugation at 20,000 × g for 15 min at 4 °C to remove the cell debris, the supernatants containing the MBP-PPK apple fusion proteins were applied to an amylose resin. After extensive washing with PBS, bound proteins were eluted with PBS, 20 mM maltose, followed by gel filtration over a Sephadex 200 column (Amersham Pharmacia Biotech) in PBS. Pooled fractions of the fusion proteins were characterized by SDS-PAGE and Western blotting using domain-specific antibodies (see below).

Expression of Single Apple Domains Fused to pI-A—The cDNA encoding single PPK apple domains, A1 (Gly3-Ser25), A2 (Ala1-Ine180), A3 (Gly181-Glu271), and A4 (Pro272-Ser326) were amplified by Taq polymerase PCR with primers introducing a BglII site and a XhoI site at the 5′- and 3′-ends, respectively, of the amplified DNA. PCR products were cloned into the TA-cloning vector pCR2 (Invitrogen, San Diego, CA), and subjected to dyeoxygen sequencing. The cDNAs were excised from pCR2 by BglII and XhoI digestion and downstream of the corresponding sites of the expression vector pZLP7(Ser77-Ala) modified as described (16, 26). The corresponding constructs encode fusion proteins with the pre-pro sequences of pTA, followed by a single PPK apple domain each, kringles 1/2, and the active site-mutated Ser77-Ala catalytic domain of pTA. The expression plasmids were transfected into BHK cells, and the corresponding fusion proteins expressed in serum-free medium (Opti-MEM, Life Technologies, Inc.)

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were purified by immunoabsorption using a monoclonal antibody to tPA, as described (16).

Expression of PPK Deletion Mutants and PPK-FXI Chimeras—To clone the PPK cDNA as an EcoRI unit, an internal EcoRI site encompassing codon GAA for Glu

were eliminated by mutation to GAG using overlap extension with PCR (27) in the plasmid vector pZEM229R (a gift from Donald Foster, ZymoGenetics, Inc.). In these studies, the 5’- and 3’-noncoding sequences in the native PPK cDNA were also removed, and EcoRI sites were introduced immediately before the initiator codon and after the stop codon. In the construction process, a silent mutation in the codon for Thr

(referred to as A1 to A4) were coated with antibodies to PPK (AS176) or FXI (AS199) using the Amine Coupling Kit provided by the manufacturer. Serial dilutions (2

were precipitated at 8,000 g for 2 min at 4 °C, and the supernatant was discarded by centrifugation. The recombinantly expressed proteins were essentially free of other contaminants.

RESULTS

Our strategy to identify relevant interaction site(s) of the PPK heavy chain with the corresponding acceptor site on the HK light chain comprised (i) the analysis of various kallikrein forms generated by limited proteolysis, (ii) the study of HK binding to recombinantly single apple domains of PPK, (iii) competition studies with antibodies interfering with PPK-HK complex formation, (iv) the construction and HK binding analysis of PPK mutants where single apple domains had been deleted, (v) the construction and binding analysis of PPK-FXI chimeras where relevant apple domains had been exchanged, and (vi) the study of effects of antibodies directed to the identified interaction sites of PPK and HK on FXIIa-mediated kinin liberation.

Binding of HK to Various Kallikrein Forms—Initially the binding of HK to three forms of kallikrein, namely PPK, α-kallikrein, and β-kallikrein was determined. The extent of conversion of PPK to α-kallikrein by FXIIa, and the autocatalytic conversion of α-kallikrein to β-kallikrein, was confirmed by SDS-PAGE and Western blot analyses using PKH1 and PKH119 antibodies, specific for the A4 and A1 domains, respectively (data not shown). The binding affinity of the three forms of kallikrein for HK was assessed in direct binding assay, in which increasing concentrations of PPK, α-kallikrein, and β-kallikrein were immobilized on a microtiter plate, and exposed to 8.3 nM biotinylated HK. Bound HK was detected by the streptavidin-peroxidase system. As shown in Fig. 1, biotinylated HK bound with high affinity to PPK (apparent Kd = 6 nM), and α-kallikrein (4 nM), whereas the affinity of β-kallikrein was drastically reduced (90 nM). Since β-kallikrein differs from α-kallikrein in having a single peptide bond cleavage at Lys

in having a single peptide bond cleavage at Lys140 in the A2 domain (9), this result indicates that the integrity of A2 is important to the high affinity binding of HK.

HK Binding to Single PPK Apple Domains—To further investigate the role of PPK apple domain A2 for HK binding we cloned and recombinantly expressed single apple domains as fusion proteins with tPA in eukaryotic BHK cells (Fig. 2, left panel), and as fusion proteins with MBP in prokaryotic E. coli cells (Fig. 2, right panel). The purity of the affinity-purified fusion proteins was monitored by SDS-PAGE under reducing conditions. The recombinantly expressed proteins were essen-
Single apple domains bind differentially to HK. Single PPK apple domains were recombinantly expressed in BHK cells as fusion proteins with tPA (left) or E. coli fused to MBP (right). A, following purification, 100 ng (1.8 pmol) of domains A1, A2, A3, A4, and unfused protein were separated by SDS-PAGE under reducing conditions and visualized by silver staining technique. B, microtiter plates were coated with 100 nM (5 µg/ml) of recombinant constructs, followed by incubation with HK (33 nM = 4 µg/ml). The complex formation between HK and the fusion proteins was probed by HKH14 antibody directed to the heavy chain of HK and a horseradish peroxidase-coupled secondary antibody, followed by the chromogenic substrate. Mean ± S.D. from three independent experiments are presented. The set-up of the assay is given on the right; the first antibody is marked “*,” and the second horseradish peroxidase-labeled antibody identified by an asterisk. Others symbols are as in Fig. 1.

Interference of Antibody PKH6 with HK-PPK Complex Formation—In a previous study we have developed a panel of 20 monoclonal antibodies to PPK, of which 11 are directed to the HK heavy chain, we sought to correlate epitope classes A-D with interaction with PPK. To this end we constructed PPK mutants in which each epitope class was deleted. Antibodies PKH1, PKH6, PKH19, and α-rA3 to PPK. B, microtiter plates coated with 4 µg/ml (45.4 nM) PPK were incubated with 1 µg/ml (8.3 nM) biotinylated HK and serial dilutions (2ⁿ) of antibodies PKH19 (A), PKH6 (●), α-rA3 (■), PKL16 (●), and PKH1 (●). Bound HK was detected by the streptavidin-peroxidase system. A representative result of three independent experiments is shown. The setup of the assay is outlined on the top; the symbols as described in the legends of Figs. 1 and 2 are used.

Using the panel of antibodies to each of the four apple domains of PPK, we measured the relative potency of each antibody to interfere with PPK-HK complex formation. To this end we set up a competitive ELISA in which native PPK was bound to a microtiter plate, and serial dilutions of antibodies PKH19 (A1), PKH6 (A2), α-rA3 (A3), PKH1 (A4), and PKL16 (directed to the PPK light chain) in the presence of 1 µg/ml biotinylated HK were added. Of the five antibodies tested PKH6 efficiently blocked HK binding to PPK with an apparent IC₅₀ of 8 nM (Fig. 3B). Antibodies PKH19 to A1 and PKH1 to A4 inhibited with IC₅₀ values of 1 µM and 40 nM, respectively, whereas α-rA3 and PKL16 (IC₅₀ > 3 µM) failed to interfere with HK-PPK complex formation. Hence A1, A2, and A4 seem to mediate HK docking to PPK although to different extents, whereas A3 is likely not involved. Antibody PKH6 is most potent in inhibiting HK-PPK complex formation; this finding is underlined by the previous observation that the loss of the PKH6 epitope, e.g. by limited proteolysis of α-kallikrein, is paralleled by a complete loss of the HK binding capacity of kallikrein (22).

HK Binding to PPK Deletion Mutants—To further analyze the contribution of the single apple domains to HK binding in the context of the PPK molecule, we employed a loss-of-function model. To this end we constructed PPK mutants in which each of the apple domains was deleted by a PCR-based gene excision technique (Fig. 4A). We transiently transfected the constructs into HEK293t cells which do not endogenously express PPK (30). After 72 h culture supernatants were collected and analyzed by SDS-PAGE and Western blotting. Immunoprinting proved functional expression of the various mutants (Fig. 4B): antibody PKH1 directed to apple domain A4 readily recognized the Δ1, Δ2, and Δ3 deletion constructs lacking apples 1, 2, and 3, respectively, but not the Δ4 mutant devoid of apple 4, while cross-reacts with native PPK (not shown).
antibody PKH6 directed to A2 recognized the Δ1, Δ3, and Δ4 mutants, but not the Δ2 construct. Full-length PPK and an unrelated protein served as positive and negative controls, respectively (Fig. 4B). The concentration of the deletion mutant proteins in cell supernatants was determined by sandwich ELISA and biospecific interaction analysis (data not shown).

Next, we tested for the HK binding capacity of the various deletion mutants. Recombinant proteins (22 nM) were coated on microtiter plates, followed by incubation with biotinylated HK and the streptavidin-peroxidase complex and the substrates. Relative HK binding capacity of the constructs is defined as the percentage relative to that obtained with wild-type PPK, where the HK binding capacity is assigned a value of 100%. Mean ± S.D. of three independent experiments are shown. Top right, schematic set-up of the assay; symbols are as described in the legend to Fig. 1.

**HK Binding to PPK-FXI Chimeras**—Since apple domain A2 proved to be crucial for HK binding in a loss-of-function model, we wondered whether this domain could serve to transfer high-affinity HK binding in a gain-of-function model. To maintain the structural context, we chose FXI the only other human protein known to contain tandem apple domains, and constructed four FXI-PPK chimeras (Fig. 5A) in which the A2 domain (6.2 versus 6.3) or the complete heavy chains (7.1 versus 7.2) had been homologously exchanged between PPK and FXI. HEK293t cells were transiently transfected with pcDNA3(+) vectors encoding wild-type FXI, PPK-FXI chimeras, or unrelated protein for control (cont). Cells were metabolically labeled with [35S]Cys/Met and the supernatants precipitated using a mixture of antibodies to PPK and FXI. Immunoprecipitates were resolved by reducing SDS-PAGE and visualized by autoradiography. C, microtiter plates were coated with 0.5 μg/ml wild-type proteins, recombinant chimeras, and an unrelated protein. Following incubation with 1 μg/ml biotinylated HK bound was detected by the streptavidin-peroxidase system. The HK binding capacity of the constructs is expressed as the percentage of that of wild-type PPK (100%). Mean ± S.D. of three independent experiments are given.

Fig. 4. Deletion of apple domain A2 reduces PPK binding to HK. A, schematic diagram of the PPK deletion mutants. The Δ symbol followed by a number identifies the apple domain that has been deleted. The preformed heavy and light chain portions of single-chain PPK are indicated by lines above the constructs. B, HEK293t cells were transiently transfected with pcDNA3(+) vectors encoding wild-type PPK, the indicated deletion mutants, or an unrelated protein for control (cont). The supernatants (25 μg/ml each) were subjected to SDS-PAGE under reducing conditions, followed by Western blotting with antibody PKH1 to apple domain A4 or with antibody PKH6 to A2. C, microtiter plates were coated with 22 nM recombinant proteins, and incubated with 1 μg/ml streptavidin-peroxidase complex and the substrates. Relative HK binding capacity of the constructs is defined as the percentage relative to that obtained with wild-type PPK, where the HK binding capacity is assigned a value of 100%. Mean ± S.D. of three independent experiments are shown. Top right, schematic set-up of the assay; symbols are as described in the legend to Fig. 1.

Fig. 5. Transfer of PPK apple domain A2 increases HK binding activity of FXI. A, scheme of the domain structures of PPK (black), FXI (white), and of PPK-FXI chimeras. B, HEK293t cells were transiently transfected with pcDNA3(+) vectors encoding wild-type FXI, PPK-FXI chimeras, or unrelated protein for control (cont). Cells were metabolically labeled with [35S]Cys/Met, and the supernatants precipitated using a mixture of antibodies to PPK and FXI. Immunoprecipitates were resolved by reducing SDS-PAGE and visualized by autoradiography. C, microtiter plates were coated with 0.5 μg/ml wild-type proteins, recombinant chimeras, and an unrelated protein. Following incubation with 1 μg/ml biotinylated HK bound was detected by the streptavidin-peroxidase system. The HK binding capacity of the constructs is expressed as the percentage of that of wild-type PPK (100%). Mean ± S.D. of three independent experiments are given.
Antibodies to mutual HK/PPK-binding sites interfere with BK liberation. Fresh human citrate plasma samples were preincubated with 450 nM each of antibodies PKH19 (to A1 of PPK), PKH6 (A2), α-rA3 (A3), PKH1 (A4), PKL16 (light chain), or antibodies HKL16 and HKL22 (to domain D6α of HK). Following incubation with 20 nM FXIIIa for 1 h at 37°C, 0.1 μl of plasma was separated by SDS-PAGE and analyzed for the presence of uncleaved HK (116 kDa) by Western blotting using MBK3 to the kinin moiety of HK. Inset, relative inhibition of kinin release is defined as the percentage of intact HK in the samples relative to intact HK in native plasma (not shown) which is assigned a value of 100%. The target epitopes of the antibodies are indicated; D6α antibodies: HKL16 (left), HKL22 (right).

Effect of Apple-directed Antibodies on BK Release from HK—Does the structural importance of apple domain A2 for HK binding bear functional implications on the kallikrein-mediated kinin release from HK? To address this question, we tested the effect of apple domain-specific antibodies on FXIIα-induced kinin release in human plasma. Samples of plasma were preincubated with antibodies directed to PPK apple domains A1 (PKH19), A2 (PKH6), A3 (α-rA3), and A4 (PKH1), and to the light chain portion (PKL16); the latter antibody does not interfere with the catalytic activity of the enzyme (22). We also included an antibody to HK domain D6α (HKL16) directed to the PPK-binding site on the HK light chain portion, and an antibody to a neighboring epitope on D6α (HKL22) which does not overlap the PPK-binding site. Following preincubation with the respective antibodies, FXIIα was added for 1 h, and then the reaction was stopped. The samples were probed for the presence of uncleaved HK using Western blotting with MBK3 antibodies directed to the kinin moiety (Fig. 6). Antibodies interfering with PPK-HK complex formation, i.e. PKH19, PKH6, PKH1, and HKL16 protected HK from cleavage and therefore prevented BK release. We quantified the amount of intact HK in the various samples and found that the protective effect of PKH6 is equivalent to that of antibody HKL16 directed to the PPK-binding site on HK. Antibodies PKH1 to A4 and PKH19 to A1 were less effective, and antibodies α-rA3 to A3 and HKL22 to an irrelevant epitope of D6α failed to attenuate kinin release. Together these results underline the unique role of apple domain A2 for HK binding to PPK, and stress the importance of physical association of zymogen and prohormone for efficient effector release.

H-kininogen circulates in plasma in the form of binary complexes with plasma prekallikrein or factor XI. A considerable fraction of plasma HK docks via a continuous site present in domain D6α of its light chain portion. Apple domain A2 considered the major platform for HK is flanked by apple domains A1 and A4 which also contribute to the discontinuous binding site.

DISCUSSION

Our studies with recombinantly expressed single, combined, and chimeric apple domain constructs bear important structural and functional implications for the PPK-HK interaction. First, the finding that single apple domains bind to HK may indicate that each domain serves as a module that adopts correct folding spontaneously and independently of the other domains of the heavy chain. This notion is corroborated by the finding that deletion mutants lacking single apples retain their HK binding capacity, although at a reduced level. Second, binding of HK was observed independently of the source of the
expression system, i.e. eukaryotic versus bacterial cells. This finding suggests that glycosylation (39) does not play a critical role in the complex formation between HK and PPK. Third, the combined data of this study indicate that A3 is not accessible to HK in the native conformation of the PPK heavy chain. This finding is well reflected by the observation that among the 22 distinct monoclonal antibodies produced against native PPK (22), none binds to A3. Fourth, transplantation of PPK to the FXI heavy chain raises the HK binding capacity of the resultant chimera almost to the level of wild-type PPK.

One of the limitations of the present approach is that we have strictly focused on intact apple domains, and therefore we cannot entirely exclude the possibility that inter-domain sequences may also contribute to HK binding. Indeed it has been shown that peptide PK251 (Thr251-Gly280) which covers the linker region between A3 and A4, and further extends into the HK-binding site of apple domain A4 (Lys266-Gly280) competes with PPK for binding to HK, although with low efficiency (IC50 = 1.4 mM; note that, the Kf for the native complex is 12 nM) (18). On the other hand, structural analyses with whole apple domains which likely have an “intact” disulfide bridge pattern may help avoid problems encountered in studies using linear peptides. For example, previous work (18) has demonstrated that peptide PK143 (Tyr143-Ala176) encompassing the carboxyl-terminal portion of A2 fails to compete with PPK binding to HK, whereas or fusion proteins overlapping the entire A2 domain sequence has been unequivocally identified, we have been unable to pinpoint the corresponding target sequence(s) by linear peptides or fusion proteins overlapping the entire A2 domain sequence demonstrating that the PKH6 epitope is highly discontinuous.2

Sequence analysis has revealed that PPK and FXI share 58% identity on the protein level, with a similar disulfide bond pattern in the apple domains except that FXI apple 4 has an unpaired Cys residue mediating homodimerization of the FXI heavy chain (11, 40). Although highly similar in its overall structure this “cousin” protease differs from PPK in some important functional aspects. The compound structure of FXI offers multiple target sites for interacting proteins, and Walsh and co-workers (41) have analyzed the interplay between FXI and various substrates and binding proteins in great detail. Their studies indicate that FXI apple domain A1 binds to HK via a sequence segment (41) that mirrors the corresponding HK attachment site of PPK A1 (15). In addition FXI apple A1 binds to thrombin (42), an important endogenous activator of FXI in blood coagulation (43, 44). FXI domain A3 has been shown to bind to platelets (45) and heparin (46), and domain A4 to FXIIa (47). Using a synthetic peptide approach Walsh and co-workers (48) demonstrated that FXI domain A2 binds FIX although this conclusion has been challenged by the recent study of Sun and Gailani (30) who used recombinant FX-PPK chimeras to show that the FIX-binding site is located in A3 rather than in A2. At present it is unclear whether FXI apple domain A2 has a prime role in HK binding. Our data indicate that incorporation of FXI A2 in construct 6.2 (Fig. 5) increases the affinity for HK by a small but significant increment as compared with deletion mutant Δ2 (Fig. 4) lacking this domain. The recent advent of a FXI RNA splice variant (49) characterized by a deletion within apple A2 may help to address this intriguing question.

Mapping studies defining interaction sites between mole-

2 T. Renne, unpublished observations.

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