Factor Xla (FXIa) is a serine protease important for initiating the intrinsic pathway of blood coagulation. Protease nexin 2 (PN2) is a Kunitz-type protease inhibitor secreted by activated platelets and a physiologically important inhibitor of FXIa. Inhibition of FXIa by PN2 requires interactions between the two proteins that are confined to the catalytic domain of the enzyme and the Kunitz protease inhibitor (KPI) domain of PN2. Recombinant PN2KPI and a mutant form of the FXI catalytic domain (FXIac) were expressed in yeast, purified to homogeneity, co-crystallized, and the structure of the complex (5) on the plasma membranes of activated human platelets, where it can be incorporated into platelet membrane microdomains (i.e. lipid rafts) (6) for efficient activation by thrombin or by FXIa (7–10). The resulting enzyme, FXIa, can then activate the vitamin K-dependent protein, FIX, to initiate the consolidation phase of blood coagulation (1).

A variety of important control mechanisms exist for regulating the activity of coagulation proteases in plasma. Several members of the serpin family have been proposed as physiological regulators of FXIa activity in plasma, including C1 inhibitor (11, 12), α-1-protease inhibitor (13, 14), antithrombin III (15), α-2-antiplasmin (16), and protease nexin 1 (17). However, a platelet secretory protein and member of the class of Kunitz-type inhibitors, protease nexin 2 (PN2), has recently been shown to be a much more potent and physiologically relevant FXIa inhibitor based on detailed kinetics studies (18–23). PN2 is a ~120-kDa isoform of the Alzheimer β-amyloid protein precursor (APP) that contains a Kunitz-type serine protease inhibitor (PN2KPI) domain (22). Platelets are an important source of several isoforms of APP, including the APP751 isoform of PN2 (23, 24). Full-length APP is membrane-associated (25) and is processed by proteases in platelets (26, 27). Upon platelet activation by physiological stimulators, PN2 is secreted from α-granules into plasma and inhibits FXIa (22–24), suggesting a role for this protein in blood coagulation. PN2 is a slow, tight binding inhibitor of FXIa with Ki of ~300–500 pM (18, 20, 22). The KPI domain of PN2 is 57 amino acids in length (Glu286–Ile343) in the 751-amoic acid isoform of PN2) and is known to contain the entire FXIa inhibitory function of PN2 (19–21, 28). Similarly, all of the information required for PN2KPI inhibition of FXIa is contained within the catalytic domain, residues 370–607 (19–21, 28).

We have determined the crystal structures of the catalytic domain of FXIa (rhFXIa-(370–607)) with ecotin, an Escherichia coli serine protease inhibitor (29), as well as benzamidine, a small molecule inhibitor of trypsin-like serine proteases. In the present study, we report the crystal structure of a complex of FXIac (FXI-370–607)-S434A,T475A,C482S: PN2, protease nexin 2; KPI, Kunitz protease inhibitor; PN2KPI, Kunitz protease inhibitor domain of PN2; APP, Alzheimer amyloid protein precursor; BPTI, bovine pancreatic trypsin inhibitor; APTT, activated partial thromboplastin time; APP, amyloid β-protein precursor; WT, wild type.

EXPERIMENTAL PROCEDURES

Preparation of the Recombinant FXI Catalytic Domain—The recombinant human FXI catalytic domain, amino acid residues 370–607 (in
FXI numbering) with three mutations (S434A, T475A, and C482S in FXI sequence numbering; S75A, T115A, and C123S in chymotrypsin sequence numbering), was expressed as a secreted protein in the methylotrophic yeast *Pichia pastoris*. For ease of discussion, recombinant human FXI-(370–607)-S434A,T475A,C482S will be termed FXIac, whereas the unmutated form will be referred to as FXI-(370–607), consistent with our previous publication (29). The protein was cloned, expressed, and purified as described previously. Throughout this paper, the chymotryptsin sequence numbering system is used for FXIac in order to be consistent with other published data on trypsin-like serine proteases (see supplemental material in Lin et al. (29) for the corresponding residue numbers for the FXI sequence and chymotrypsin numbering system)5.

Recombinant PN2KPI Wild Type (WT) Domain Gene Segment and Its Mutant Gene Constructs—The full-length human PN2 gene in pcDNA3.1 vector (kind gift from William E. Van Nostrand, SUNY, Stony Brook, NY) served as template for the synthesis by PCR of the WT PN2KPI domain. WT PN2KPI 5’ primers had an XhoI signal cleavage site, and 3’ primers had stop codons followed by a NotI site. This WT PN2KPI PCR product was restriction-digested with XhoI and NotI (New England BioLabs, Inc.), ligated into the similarly treated yeast expression vector, pPICZαA (Invitrogen), and propagated in XL1-Blue bacteria. PCR-based site-directed mutagenesis (QuickChange; Stratagene), using mutagenic primers, was used for incorporating desired mutations by inserting codons preferentially used by yeast for appropriate amino acids in the WT PN2KPI sequence. The pPICZαA plasmids containing WT PN2KPI and mutant inserts were sequenced from 5’ to the α-mating factor secretion signal using an AOX5 primer to confirm mutations at desired sites as well as the reading frame integrity.

Recombinant PN2KPI Expression in Yeast—pPICZαA plasmid constructs containing WT PN2KPI and mutant gene inserts were treated with the Scl (New England BioLabs, Inc.) restriction enzyme. The linearized plasmids were incorporated into the methylotrophic yeast P. pastoris X33 (Invitrogen) competent cell genome (Invitrogen) by recombinational cloning. Selected yeast clones were lysed either by repeated heat-shock cycles or by lyticase (Sigma), and the lysates were analyzed by PCR using PN2KPI-specific forward and reverse primers for correct insert size. Successful clones were grown in yeast growth medium containing glycerol, BMGY (buffered medium containing glycerol and yeast nitrogen base) at 30 °C until obtaining sufficient cell density. These cells were transferred to methanol-containing expression medium BMMY (buffered medium containing methanol and yeast nitrogen base) and incubated at 30 °C for 96 h under vigorous agitation, supplemented with 0.5% methanol every 24 h.

Purification of Recombinant WT and Mutant PN2KPI Domains—Yeast cultures containing WT PN2KPI domain and mutant proteins were centrifuged to remove yeast cells. The supernatant was precipitated at room temperature using saturating concentrations of ammonium sulfate and centrifuged at 11,000 × g for 30 min. Pellets were resuspended onto large desalting columns (G25 16/60; Amersham Biosciences) followed by anion exchange chromatography on 20-ml columns (HiTrap Q HP; Amersham Biosciences) in 50 mM Tris, pH 7.8. The eluted samples were concentrated in dialysis tubing (Float-a-lyser, Spectrum Laboratories, Inc.) using cross-linked sodium polycrylate gel (Spectragel; Spectrum Laboratories, Inc.) as an external dehydrant at 4 °C. Concentrated samples were loaded onto gel filtration columns (HiLoad 30, 16/60; Amersham Biosciences). Recovered samples were pooled, and the protein concentration was estimated by the bicinchoninic acid assay (Pierce). All PN2KPI proteins were analyzed by SDS-PAGE to determine the size followed by Western blotting and probing blots using rabbit anti-APP polyclonal antibody (Chemicon International). To confirm mutations at desired sites, purified PN2KPI proteins were sequenced from the N terminus by Edman degradation using a PerkinElmer Life Sciences protein sequencer and amino acid analyzer (protein facility, Iowa State University).

Crystallization and Structure Determination of the FXIac-PN2KPI Complex—The FXIac-PN2KPI complex was formed by incubating stoichiometric quantities of FXIac and PN2KPI at 4 °C and isolated by Superose 12 column chromatography (HR10/30; Amersham Biosciences) at a flow rate of 1 ml/min in a buffer containing 20 mM Tris-HCl, pH 7.8, and 0.1 M NaCl on a BioCAD 700E. The FXIac-PN2KPI complex was concentrated using a Centricon YM-10 concentrator (Amicon) to an absorbance of 36 at 280 nm with a 1-cm path length. The complex was crystallized at 10 °C by hanging drop vapor diffusion from 4 M sodium formate (Crystal Screen 33; Hampton Research). Rodlike crystals were obtained in 2–3 days with dimensions of 0.2 × 0.2 × 0.5 mm. Diffraction data, to 2.6 Å resolution, were measured on an R-AXIS IV++ imaging plate detector with an RU-H3R generator and an X-stream™ 2000 low temperature system (Rigaku/MSC) and processed using HKL2000 (30). The crystal belongs to the space group P3221 with unit cell parameters of a = b = 92.8 Å and c = 107.0 Å. There was one FXIac-PN2KPI complex in the asymmetric unit.

The structure of FXIac-PN2KPI was solved by molecular replacement using AMoRe (31). The initial search model was the catalytic domain of FXI from the FXIac-benzamidine structure (Protein Data Bank code 1ZHM) with all of the side chain atoms beyond Cα and all water molecules truncated. A clear solution was obtained using data of 10 to 5 Å resolution with a correlation coefficient of 0.591 and R factor.

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**TABLE ONE**

Data collection and refinement statistics

| Parameters                        | Values                  |
|-----------------------------------|-------------------------|
| Resolution (Å)                    | 30–2.6 (last shell 2.69–2.60) |
| Space group                       | P3,21                   |
| Unit cell dimensions (Å)          | a = b = 92.8; c = 107.0 |
| Completeness (%)                  | 99.4 (last shell 100)    |
| Redundancy                        | 7.2                     |
| Rmerge (%)                        | 0.080 (last shell 0.358) |
| (I/|<i>σ</i>(I))                  | 21.7 (last shell 5.6)    |

Rmerge: average intensity for multiple measurements.

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**Data (no cut-off)**: 16312

**Protein/solvent atoms**: 2321/62

**Redundancy**: 7.2

**Rmerge(%)/No. of reflections**: 21.6/14,673 (last shell 32.4/1158)

**Rmerge(%)/No. of reflections**: 25.5/1639 (last shell 33.8/170)

**r.m.s.d. in bond lengths (Å)**: 0.007

**r.m.s.d. in bond angles (degrees)**: 1.1

**r.m.s.d. in dihedrals (degrees)**: 25.13

**Mean B factors (Å²)**: 61.7

**Ramachandran plot**

**Favored (%)**: 79.9

**Additionally allowed (%)**: 20.1

**Generously allowed (%)**: 0.0

**Disallowed (%)**: 0.0

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**Crystallization and Structure Determination of the FXIac-PN2KPI Complex**—The FXIac-PN2KPI complex was formed by incubating stoichiometric quantities of FXIac and PN2KPI at 4 °C and isolated by Superose 12 column chromatography (HR10/30; Amersham Biosciences) at a flow rate of 1 ml/min in a buffer containing 20 mM Tris-HCl, pH 7.8, and 0.1 M NaCl on a BioCAD 700E. The FXIac-PN2KPI complex was concentrated using a Centrifcon YM-10 concentrator (Amicon) to an absorbance of 36 at 280 nm with a 1-cm path length. The complex was crystallized at 10 °C by hanging drop vapor diffusion from 4 M sodium formate (Crystal Screen 33; Hampton Research). Rodlike crystals were obtained in 2–3 days with dimensions of 0.2 × 0.2 × 0.5 mm. Diffraction data, to 2.6 Å resolution, were measured on an R-AXIS IV++ imaging plate detector with an RU-H3R generator and an X-stream™ 2000 low temperature system (Rigaku/MSC) and processed using HKL2000 (30). The crystal belongs to the space group P321 with unit cell parameters of a = b = 92.8 Å and c = 107.0 Å. There was one FXIac-PN2KPI complex in the asymmetric unit.

The structure of FXIac-PN2KPI was solved by molecular replacement using AMoRe (31). The initial search model was the catalytic domain of FXI from the FXIac-benzamidine structure (Protein Data Bank code 1ZHM) with all of the side chain atoms beyond Cα and all water molecules truncated. A clear solution was obtained using data of 10 to 5 Å resolution with a correlation coefficient of 0.591 and R factor.
Structure refinement was carried out using CNX (Accelrys), and model building was performed in Quanta (Accelrys). The electron density map clearly showed additional densities for PN2KPI. When the catalytic triad of FXIa (His413, Asp462, and Ser557 in FXI numbering; His57, Asp102, and Ser195 in chymotrypsin numbering) was superimposed with that of thrombin, derived from the structure of thrombin in complex with bovine pancreatic trypsin inhibitor (BPTI) (Protein Data Bank code 1BTH) (32), the BPTI backbone fitted into the PN2KPI density. Clear densities for the side chains of PN2KPI were observed after changing the BPTI sequence to alanine, combining the polyalanine coordinates with the coordinates of FXIa and further refining with CNX. The structure of the FXIa-PN2KPI complex was obtained after several rounds of refinement and model building. Detailed data and refinement statistics are listed in TABLE ONE.

**RESULTS**

**FXI Catalytic Domain Structure in the FXIa-PN2KPI Complex**—In order to identify the key interactions between PN2KPI and the FXIa catalytic domain, we determined the structure of the FXIa-PN2KPI complex to 2.6 Å resolution. The recombinant catalytic domain of FXI used for the crystallization experiment contains the following mutations: S75A (S434A, FXI sequence number in parenthesis) and T115A (T475A) to remove the glycosylation sites and C123S (C482S) to remove an unpaired Cys residue.5 When the Cα atoms of residues of 0.495. Structure refinement was carried out using CNX (Accelrys), and model building was performed in Quanta (Accelrys). The electron density map clearly showed additional densities for PN2KPI. When the catalytic triad of FXIa (His413, Asp462, and Ser557 in FXI numbering; His57, Asp102, and Ser195 in chymotrypsin numbering) was superimposed with that of thrombin, derived from the structure of thrombin in complex with bovine pancreatic trypsin inhibitor (BPTI) (Protein Data Bank code 1BTH) (32), the BPTI backbone fitted into the PN2KPI density. Clear densities for the side chains of PN2KPI were observed after changing the BPTI sequence to alanine, combining the polyalanine coordinates with the coordinates of FXIa and further refining with CNX. The structure of the FXIa-PN2KPI complex was obtained after several rounds of refinement and model building. Detailed data and refinement statistics are listed in TABLE ONE.

**Determination of IC₅₀ Values of FXIa Inhibition by PN2KPI**—Increasing concentrations of the WT PN2KPI domain or its mutants (0–100 nM) in 50 mM Tris, 150 mM NaCl, 0.5% bovine serum albumin, pH 7.5, were incubated with FXIa (0.1 nM) in a 285-μl volume for 30 min at 37 °C in a microtiter plate for establishing equilibrium between the inhibitor and the enzyme. To this preincubation mixture, 15 μl of substrate i-pyr-Glu-Pro-Arg-p-nitroanilide-HCl (S-2366; Chromogenix) was added to a final concentration of 0.25 mM. Initial reaction velocity readings for 20 min at 37 °C in a microplate reader (Molecular Devices Thermo Max) were converted to the fraction of amidolytic activity remaining. Values of IC₅₀ were determined using KaleidaGraph version 3.5 software.

**Activated Partial Thromboplastin Time (APTT) Assay**—In plastic microcuvettes (Sigma), 50 μl of normal pooled plasma and 25 μl of APTT reagent (Alexin; Sigma Diagnostics) were incubated for 15 min at 37 °C with 50 μl of Tris-buffered saline containing increasing concentrations of WT PN2KPI or mutants. Twenty-five μl of CaCl₂ was added (6.7 mM, final concentration) to initiate clot formation, and clotting time was determined in an Amelung KC4 microcoagulometer (Amelung GmbH, Germany).

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**FIGURE 1. The superimposition of the catalytic domain of FXIa in FXIac-PN2KPI and FXIa-benzamidine structures.** A, a ribbon representation of the superimposition of the catalytic domain of FXIa in FXIac-PN2KPI (green) and FXIa-benzamidine (blue) structures. PN2KPI is colored in pink. The arrows indicate the loop containing residues 125–133 that adopt different conformations in each structure. B, a close-up view of only the superimposed catalytic domains of FXIa with several key residues that are discussed is shown in a ball-and-stick representation. The atoms of the FXIac-PN2KPI structure are colored as carbon atoms in green, nitrogen atoms in blue, oxygen atoms in red, and sulfur atoms in yellow. The FXI-benzamidine structure is colored in blue.
16–123 and 135–240 of FXIac in the FXIac-PN2KPI structure are superimposed with those in FXIac-benzamidine and in rhFXI-(370–607)-ecotinM84R structures, the root mean square deviations are 0.34 and 0.43 Å, respectively. The core structure of FXIac in the FXIac-PN2KPI complex is very similar to that in the FXIac-benzamidine and rhFXI-(370–607)-ecotinM84R complexes (Figs. 1 and 2A), with only small differences in the surface regions. The largest difference is found in the loop containing residues 125–133 (the 130/H11032s loop); this is due to different crystal packing environments in all three crystal structures.

Structural Comparison of PN2KPI with the Structures of Other Alzheimer Amyloid β-Protein Precursors (APPs)—The main-chain conformation of PN2KPI, except for the N and C termini, is very similar to that of the structures of other protease inhibitor domains of APP deposited in the Protein Data Bank: APP1 dimer (Protein Data Bank code 1AAP) (33), trypsin-APP1 (Protein Data Bank code 1TAW) (34), and chymotrypsin-APP1 (Protein Data Bank code 1CA0) (34) as well as the other KPIs, such as BPTI. Fig. 3A presents the Cα atom superimposition of PN2KPI with all the APP1 structures including a few side chains (Arg15, Thr11, Met17, Arg20, and Asp46) that are located at the interface with FXIac and are in different conformations in PN2KPI than in the other APPs.

Interactions between FXIac and PN2KPI—PN2KPI has extensive interactions with FXIac via two loops (Fig. 4A), those containing residues 11TGPCRAMISR20 (P5–P5/H11032) and residues 34FYGGC38, linked by a disulfide bond between Cys14 and Cys38 (Fig. 4B). The equivalent two loops of BPT1 are residues 11TGPCARKIIR20 and 34VYGGC38. The general orientation of the two loops in PN2KPI is similar to the orientation of the corresponding loops in BPT1. Although the primary binding site of ecotin also interacts with FXIac via two loops (29), the orientation of the two loops in PN2KPI and ecotin is different relative to the active site of FXIa, with parts of the loops (P1’–P3) overlapping (Fig. 2).

Selection of PN2KPI Amino Acids for Mutation Based on the X-ray Crystal Structure of the FXIac-PN2KPI Complex—As depicted in Fig. 4, Arg15 of PN2KPI (P1) extends into the S1 pocket of FXIac and interacts with Asp189 of FXIac at the bottom of the pocket. Pro3 (P2) bends the backbone of PN2KPI, allowing Lys192 of FXIac to form hydrogen bonds with the backbone carboxyl oxygen atoms of Cys14 (P2) and Gly12 (P4) of KPI. Met17 (P2/H11032) and Ser19 (P4/H11032) are in contact with Arg37D (the fourth residue in an insert in the catalytic domain of FXIa following residue 37, numbered in accordance with the standard chymotrypsin numbering system) of FXIac. Arg20 and Tyr35 of PN2KPI interact with Tyr59A of FXIac (a residue unique to FXI). Phe34 is in van der Waals contact with the side chain of Met17 of PN2KPI as well as Tyr43 of FXIac. On the basis of these observed interactions, residues Pro13, Arg15, Met17, Ser19, Arg20, Phe34, and Tyr35 were chosen for mutation (TABLE TWO).
Purification and Characterization of PN2KPI WT and Mutant Domains—All mutants and WT PN2KPI domains were expressed in a Pichia yeast expression system and purified as described under “Experimental Procedures.” The expression levels of all mutants and WT PN2KPI were between 7 and 10 mg/liter, with the exception of the mutations at Tyr35 (Y35A and Y35I), which yielded negligible quantities of proteins and hence were insufficient for detailed kinetic studies. Purified proteins gave a sharp, single band at 6.3 kDa by silver-stained SDS-PAGE in the presence of reducing agent, \( \beta \)-mercaptoethanol. Edman degradation results confirmed the expected amino acid sequences of these recombinant proteins.

The WT PN2KPI was characterized by a prolonged transient phase for inhibition of FXI\(_{\text{ac}}\), typical of slow, tight binding Kunitz inhibitors (20). Increasing concentrations of either WT PN2KPI or mutant PN2KPI domains, incubated with 0.1 nM FXI\(_{\text{ac}}\), resulted in plots of fractional amidolytic activity versus the inhibitor concentration (Figs. 5–8), which correlated well with the results of an assay to measure the \( \text{in vitro} \) prolongation of clotting time (APTT) by WT and mutant PN2KPI domains (Fig. 9).

**P1 Site (Arg\(^{15}\)) PN2KPI Mutations (Fig. 5)**—The IC\(_{50}\) value for WT PN2KPI domain inhibition of FXI\(_{\text{ac}}\) was 1.28 nM. When the P1 site amino acid (Arg\(^{15}\)) was mutated to Ala (R15A), the mutant protein completely lost its inhibitory activity (Fig. 5A). However, a substitution with Lys in the P1 site (R15K) resulted in only partial loss of inhibitory activity (IC\(_{50}\) of 42 nM). This substitution resulted in a 33-fold reduction in inhibitory activity as compared with WT PN2KPI. Similarly, in the APTT assay, the R15A mutant was inert (i.e., it failed to prolong the clotting time), whereas R15K mutant showed a partial loss of inhibitory activity (Fig. 5B).

**P3 Site (P13A, P13E, P13K, P13Q, and P13R) PN2KPI Mutations (Fig. 6)**—Proline is a structurally important amino acid. An Ala substitution at this location resulted in a 5-fold loss of inhibitory activity (IC\(_{50}\) of 5.92 nM) compared with WT PN2KPI, whereas replacements with Arg (IC\(_{50}\) of 1.71 nM) or Lys (IC\(_{50}\) of 2.25 nM) for Pro\(^{13}\) rescued the loss of function...
(Fig. 6A), in agreement with the results of the APTT assay (Fig. 6B). On the contrary, Glu (IC50 of 13.5 nM) and Gln (IC50 of 11.79 nM) mutations disrupted the function 11- and 9-fold, respectively (Fig. 6A), results also confirmed by those of the APTT assay (Fig. 6B).

**TABLE TWO**

PN2KPI amino acids that have apparent interactions with FXIac observed in PN2KPI-FXIac co-crystal structure

| PN2KPI mutation | IC50 | Activity loss |
|------------------|------|---------------|
| IC50 | Activity loss |
| P13A | 5.92 | 4.6 |
| P13E | 13.5 | 10.6 |
| P13Q | 11.79 | 9.2 |
| P13K | 2.25 | 1.8 |
| P13R | 1.71 | 1.3 |
| R15A | No activity |
| R15K | 42.02 | 32.8 |
| M17A | 1.62 | 1.3 |
| S19A | 1.86 | 1.5 |
| R20A | 5.67 | 4.4 |
| F34A | 9.85 | 7.7 |

| Select amino acids from FXIac that are in contact with PN2KPI amino acids. FXIac numbering follows the chymotrypsin numbering system. |
|Fold activity loss compared with FXIa inhibition by WT PN2KPI (1.28 nM) in kinetic assays. |

(Fig. 6A), in agreement with the results of the APTT assay (Fig. 6B). On the contrary, Glu (IC50 of 13.5 nM) and Gln (IC50 of 11.79 nM) mutations disrupted the function 11- and 9-fold, respectively (Fig. 6A), results also confirmed by those of the APTT assay (Fig. 6B).
similar results in the APTT assay (Fig. 7B). In contrast, mutation at the P5' site with an Ala replacement for Arg (R20A) resulted in a 4-fold loss of activity (IC50 of 5.67 nM) compared with the WT PN2KPI (Fig. 7A) and a similar observation of loss of activity in the APTT assay (Fig. 7B).

F34A PN2KPI Mutation in Loop 2 (Fig. 8)—In addition to PN2KPI loop 1 amino acids (Figs. 5–7), the loop 2 region amino acid, Phe34, was also replaced with Ala, resulting in an 8-fold loss of activity (IC50 of 9.85 nM) as compared with WT PN2KPI (IC50 of 1.28 nM), which agrees with the loss of activity in the APTT assay (Fig. 8, A and B).

Correlation between Results of Kinetic Assays and APTT Results (Fig. 9)—The results of our in vitro kinetic assays to measure the inhibitory properties of PN2KPI mutants were compared with the prolongation of clotting times measured by APTT assays (Fig. 9). The APTT assay monitors the activity of intrinsic pathway of blood coagulation, in which the primary target for PN2KPI, FXIa, is located. These two methods of monitoring the inhibitory properties of PN2KPI mutants were found to correlate well (r = −0.8).

DISCUSSION

The Kunitz-type inhibitor, PN2, is postulated to have an important role in the regulation of FXIa at the site of blood vessel injury where platelets form a hemostatic thrombus and play an essential part in the activation of FXI by thrombin (19, 20, 22, 23). Thus, it has been observed that normal human plasma contains very little (<60 pM) PN2 (i.e. concentrations well below the reported Kij value) (300–500 pM) for FXIa inhibition by PN2 (19, 20, 22–24). However, the protein is secreted from platelet α-granules in sufficient quantities (2–30 nM) to result in rapid and complete inhibition of FXIa in solution (20, 23). In contrast, the FXIa bound to the GPIb-IX-V complex on the activated platelet surface (5, 6) is completely protected from inactivation by PN2 (21, 35). Therefore, it is likely that PN2 has an important role in regulating the initiation of the consolidation phase of blood coagulation and localizing it to the hemostatic thrombus. These facts emphasize the importance of understanding the mechanism and structural determinants of FXIa inhibition by PN2.

Previously in this laboratory, a detailed quantitative study (21) of FXIa and FXIac with PN2 and PN2KPI suggested that the totality of interactions that result in inhibition reside within the catalytic domain of FXIa and the KPI domain of PN2. Thus, in the current study, it is assumed that co-crystals of PN2KPI and FXIac domains will yield as much information as studies of the respective complete molecules.

FXI Catalytic Domain Structure and Interactions between FXIac and PN2KPI—Binding of PN2KPI causes a slight enlargement of the active site of FXIac compared with the active site containing benzamidine (Fig. 1B). This is similar to the enlargement observed when the structures of rhFXI-(370–607)-ecotinM84R (Protein Data Bank code 1XX9) and FXIac-benzamidine (Protein Data Bank code 1ZHR)5 were compared. The catalytic triad and Asp189 at the bottom of the S1 pocket superimpose well in the two structures (Fig. 1B), whereas Lys is in different
conformations. Lys192 in the FXIac-benzamidine structure interacts with a sulfate molecule from the crystallization medium. It forms hydrogen bonds with PN2KPI in the FXIac-PN2KPI structure (see below) (Fig. 4B). The hydrogen bond between Glu98 and His174 in FXIac-benzamidine structure is absent in the FXIac-PN2KPI structure. The aliphatic portion of the Glu98 side chain is in van der Waals contact with Trp215 as it is in the FXIac-benzamidine and the rhFXI-(370–607)-ecotinM84R structures, whereas the carboxylic acid group is packed against Cys38 and Pro13 of PN2KPI. Glu217 and Arg224 point away from the S4 pocket, similar to their orientation in the rhFXI-(370–607)-ecotinM84R structure; however, they bend inward in the FXIac-benzamidine structure. All other residues surrounding the active site do not move significantly, including Tyr59A, although the latter interacts with PN2KPI (see below).

The P1 residue (Arg15) of PN2KPI extends into the S1 pocket of FXIac. One nitrogen atom of the guanidinium group forms a hydrogen bond with Asp189 of FXIac, whereas the other nitrogen atom forms a hydrogen bond with the main-chain carbonyl oxygen of Gly218 in FXIac (Fig. 4B). The main-chain nitrogen of PN2KPI Arg15 forms a weak hydrogen bond (3.3 Å) with the carbonyl oxygen of Ser214 of FXIac. The carbonyl oxygen of PN2KPI Arg15 is located in the oxyanion hole, forming hydrogen bonds with the main-chain nitrogen atoms of Gly193 and Ser195. The carbonyl carbon of PN2KPI Arg15 is 2.7 Å away from the Oγ of Ser195, forming the Michaelis complex. The side chain of PN2KPI Arg15 fills the space in the S1 pocket, as well as forming hydrogen bonds with Asp189 of FXIa. Therefore, PN2KPI Arg15 makes important contributions to the affinity for the PN2KPI inhibition of FXIa. The disulfide linkage between Cys194 (P2) and Cys39 in PN2KPI occupies the S2 pocket of FXIac and is surrounded by Tyr59A, Met96, and Glu98 (Fig. 4B). Most interestingly, Pro15 (P3) of PN2KPI bends the main chain of PN2KPI. That prevents Gly12 (P4) from interacting with the S4 pocket of FXIac.
formed by Trp\textsuperscript{15}, His\textsuperscript{174}, and Glu\textsuperscript{98}. Fig. 2B clearly shows that the Cα atoms of the P3 residue from PN2KPI and ecotin are located close to each other; PN2KPI Pro\textsuperscript{13} bends the loop away from the direction that the ecotin loop is heading. Other members of the KPI family, such as BPTI (32) and soybean protease inhibitor (36), also have proline at the P3 position and adopt the same backbone conformation as PN2KPI. PN2KPI forms a turn around Pro\textsuperscript{13} and allows Lys\textsuperscript{192} of FXIa to insert into PN2KPI and form two hydrogen bonds with carboxyl oxygen atoms of Cys\textsuperscript{14} (P2) and Gly\textsuperscript{12} (P4) (Fig. 4B). Both FXIa and FVIIa contain a lysine at position 192, whereas that position is either Glu or Gln in the other serine proteases in the coagulation cascade. After surveying all of the serine protease structures in complex with APPi and BPTI in the Protein Data Bank, we observed that Glu/Gln\textsuperscript{192} of the serine protease forms either no hydrogen bond or one hydrogen bond with the carbonyl oxygen atom of Cys\textsuperscript{14} (P2) and Gly\textsuperscript{12} (P4) (Fig. 4B). This hydrogen bond network as well as the side chain van der Waals contact with the conserved disulfide linkage between Cys\textsuperscript{40} and Cys\textsuperscript{17} of PN2KPI. 

**TABLE THREE**

| Number of residues of two loops in different serine proteases | FXIa Trypsin Chymotrypsin FXa FIXa FVIIa Thrombin |
|---------------------------------------------------------------|-----------------------------------------------|
| 36–37D loop                                                  | 6 2 4                                         |
| 59–64 loop                                                   | 9 4 5                                         |

Mutation Studies on PN2KPI Domain—Selective Ala mutations at P3, P1, P2, P4, and P5' sites of loop 1 and Phe\textsuperscript{38} of loop 2 of PN2KPI domain were studied, and extended mutations at P3 and P1 sites were also done for understanding the molecular mechanism of FXIa inhibition by PN2KPI. All mutants (except the P1 site mutant, R15A) were found to exhibit varying degrees of inhibitory activity against FXIa (TABLE TWO). 

P1 Site (Arg\textsuperscript{15}) of PN2KPI—Among trypsin-like serine proteases that can be inhibited by KPIs, the Thr site in the binding pocket of FXIa can accommodate preferentially either Arg or Lys from the P1 site of the inhibitor. The primary interaction between Arg\textsuperscript{15} of the PN2KPI and Asp\textsuperscript{189} of FXIa is an optimal fit. This observation is substantiated by our experimental evidence that an Ala replacement for Arg at position 15 (R15A) in PN2KPI resulted in complete loss of inhibitory activity and that the R15K mutation resulted in a ~33-fold reduction in inhibitory activity. In the R15A mutant, the side chain is shortened as well as lacking the positive charge, thus disrupting the primary interaction. However, in the R15K mutant, a shortened side chain (compared with Arg) may not directly interact with the carbonyl group of Asp\textsuperscript{189} but could still have an interaction with Asp\textsuperscript{189} using a water molecule as a bridge, similar to the P1 Lys interaction in BPTI-trypsin complex (34). Both FXIa and plasma kallikrein have Ala residue 190 in the S1 pocket instead of Ser as in trypsin, lacking an additional hydrogen-bonding interaction with the P1 residue. A Lys mutation at Arg\textsuperscript{15} in the inhibitor combined with the absence of a Ser at position 190 in FXIa could pose severe constraints in their interactions, similar to plasma kallikrein, which less favorably accommodates R15K compared with trypsin (39). This may partly explain why R15K still retains its FXIa-inhibitory function, albeit with a ~33-fold reduction compared with the WT PN2KPI molecule.

Earlier studies have shown that the P1 site mutations of PN2KPI result in enhanced potency toward other proteases. Two separate single amino acid substitutions at the Arg\textsuperscript{15} position of PN2 (R15K and R15V) resulted in increased inhibitory potency for human plasmin (40) and human neutrophil elastase (41), with decreased inhibitory activity against FXIa. BPTI has a Lys at position 15 (P1 site). 

Comparison of PN2KPI Structure with Other APPi Structures—Superimposition of Cα atoms of PN2KPI with all of the APPi crystal structures reveals that Arg\textsuperscript{15} (P1) inserts into the S1 pocket of serine proteases. The different conformations of Arg\textsuperscript{15} are dictated by the different environment of the S1 pocket in different serine proteases. Thr\textsuperscript{11} (P5) is a different rotamer in PN2KPI than the other APPis. Thr\textsuperscript{11} does not form polar interactions with other residues. It is not clear why Thr\textsuperscript{11} adopts a different conformation in PN2KPI. It is close to Lys\textsuperscript{192} (Fig. 4B), a residue that is unique in the active site of FXIa. PN2KPI Met\textsuperscript{17} (P2') is in different conformation in all four structures compared herein. The most interesting changes are in PN2KPI Arg\textsuperscript{20} and Asp\textsuperscript{38}, which move in a concerted fashion away from FXIa relative to those in the APPi structures (Fig. 3B). These changes are clearly caused by the presence of Tyr\textsuperscript{192} of FXIa; therefore, those are unique features of the PN2KPI interaction with FXIa.
Therefore, P1 of PN2KPI is important for its selectivity and potency in the inhibition of FXIa.

**P3 Site (Pro**²¹ **of PN2KPI)—There are two Pro residues in PN2KPI, one at position 13 (P3 site) and the other at position 32, each located at the beginning of characteristic loop regions that are involved in interaction with FXIac as observed in the PN2KPI-FXIac co-crystal structure. Pro²¹ is a highly conserved residue among the Kunitz family of protease inhibitors. The β carbon in Pro²¹ is oriented toward a solvent-exposed region; therefore, this site could accept a variety of amino acids (34). A study utilizing phage display mutagenesis, where a large number of random mutations are generated within the PN2KPI domain, showed that amino acids found at position 13 were highly dependent upon the amino acid at position 39 (43). Having Gly at 39 allowed several substitutions at position 13, whereas having a non-Gly residue at 39 rendered Pro the requisite residue at position 13. In the current study, having Gly at position 39, varieties of mutations at position 13 should be tolerated. Here, the Pro²¹ site was substituted individually with Ala, Glu, Gln, Lys, or Arg. The substrates of FXIa have either Asp or Lys at the P4 position. The charged residues were chosen to mimic FXIa substrate.

The P13A mutant showed a 4.6-fold decrease in IC⁵₀ against FXIa, whereas P13R and P13K had a marginal decrease of 1.3- and 1.8-fold, respectively. Without structures of the mutants, we speculate that a larger drop of IC⁵₀ with P13A might be caused by the lack of bulk of Ala compared with Pro to occupy the space and/or slight change of backbone conformation. Because the S4 pocket of FXIa has Gly⁹⁸ and Glu²¹⁷, P13R and P13K mutants might interact with the negatively charged residues and therefore rescue the inhibition function of PN2KPI. However, P13E and P13Q are less favored at the P3 site, resulting in a 9–11-fold (IC⁵₀ of 13.5 and 11.8 nm, respectively) reduction in activity against FXIa compared with the WT PN2KPI. In the crystal structure of FXIac in complex with ecotin that has P2’ to P5 mutated to FXIa substrate sequence (29), the Asp of P4 is interacting with His²¹⁴ in the S4 pocket. Because the Cβ of Pro²³ in PN2-KPI points toward S4 in the FXIa-PN2KPI structure but its Cα locates further away from His²¹⁴ compared with P4 of ecotin, we chose to mutate Pro²³ to the longer Glu in order to mimic FXIa substrate Asp at P4. Apparently, Glu failed to orient as Asp and therefore FN2KPI was not as effective as PN2KPI in inhibiting FXIa.

**P5’ (Ser**²⁸ **of PN2KPI) Interactions**

Because the Cγ of Ser²⁸ of PN2KPI interacts with Tyr⁵⁹ of FXIa, the co-crystal structure appears to be required for the inhibitory function. The side chain of Phe³⁴ also packs closely to the side chain of Met¹⁷. The crystal structure of FXIac-PN2KPI provides detailed structural information for the interaction between FXIa and PN2KPI. Our current studies delineate details of some of the molecular interactions between PN2KPI and FXIa. Not all of the PN2KPI amino acids identified from the co-crystal structure appear to be required for the inhibitory function. For example, mutating Met¹⁷ and Ser²⁸ to Ala had no major effect on their inhibition of FXIa. Among the mutants tested, the P1 site Arg⁵ is the most important in FXIa inhibition, followed by Phe³⁴, Pro¹³, and Arg⁹⁰. Additional mutagenesis studies are currently in progress in our laboratory to identify residues within FXIa that interact with PN2KPI.

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Structural and Mutational Analyses of the Molecular Interactions between the Catalytic Domain of Factor XIa and the Kunitz Protease Inhibitor Domain of Protease Nexin 2

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