Human coagulation factor XI (FXI) is a plasma serine protease composed of 2 identical 80-kd polypeptides connected by a disulfide bond. This dimeric structure is unique among blood coagulation enzymes. The hypothesis was tested that dimeric conformation is required for normal FXI function by generating a monomeric version of FXI (FXI/PKA4) and comparing it to wild-type FXI in assays requiring factor IX activation by activated FXI (FXIa). FXI/PKA4 was made by replacing the FXI A4 domain with the A4 domain from prekallikrein (PK). A dimeric version of FXI/PKA4 (FXI/PKA4-Gly326) was prepared as a control. Activated FXI/PKA4 and FXI/PKA4-Gly326 activate factor IX with kinetic parameters similar to those of FXIa. In kaolin-triggered plasma clotting assays containing purified phospholipid, FXI/PKA4 and FXI/PKA4-Gly326 have coagulant activity similar to FXI. The surface of activated platelets is likely to be a physiologic site for reactions involving FXI/FXIa. In competition binding assays FXI/PKA4, FXI/PKA4-Gly326, and FXI have similar affinities for activated platelets (Kᵢ = 12-16 nM). In clotting assays in which phospholipid is replaced by activated platelets, the dimeric proteins FXI and FXI/PKA4-Gly326 promote coagulation similarly; however, monomeric FXI/PKA4 has greatly reduced activity. Western immunoblot analysis confirmed that activated monomeric FXI/PKA4 activates factor IX poorly in the presence of activated platelets. These findings demonstrate the importance of the dimeric state to FXI activity and suggest a novel model for factor IX activation in which FXIa binds to activated platelets by one chain of the dimer, while binding to factor IX through the other. (Blood. 2001; 97:3117-3122)
described above. In this study we describe the preparation and characterization of a monomeric version of FXIa and demonstrate that FXIa must be a dimer to properly promote coagulation in the presence of activated platelets. The findings suggest a novel model for a factor IX activation complex on platelets in which one heavy chain of FXIa binds to the platelet, and the other binds to factor IX.

**Materials and methods**

**Preparation and activation of recombinant proteins**

Expression constructs for wild-type FXI and for chimeras FXI/PKA4 and FXI/PKA3 (FXI with the A4 or A3 domain, respectively, replaced with the corresponding domain from PK) have been described. A dimeric version of FXI/PKA4 (FXI/PKA4-Gly326) was created by changing Cys326 to glycine, using a Chameleon site-directed mutagenesis kit (Stratagene, LaJolla, CA). FXI with Cys321 replaced by alanine (FXI-Ala321) was made in a similar manner. Proteins were expressed in 293 fibroblasts and purified from 500-2000 mL serum-free conditioned media by affinity chromatography. Proteins were checked for purity by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), and protein concentration was determined by dye binding assay (BioRad, Hercules, CA). Proteins were activated by diluting to 100-200 μg/mL in 2.5 mM Tris-HCl pH 7.4, 100 mM NaCl (TBS) containing 5 μg/mL human FXIa, and incubating at 37°C. Conversion of the 80-kdzymogen to the 45- and 35-kd chains of FXIa was followed by reducing SDS-PAGE.

**Plasma proteins**

FXI and HK were purified from human plasma by published methods. All proteins appear as single bands on Gelcode Blue (Pierce, Rockford, IL)-stained gels. Specific activity of human FXI (230 U/mg) was determined by aPTT assay, using FXI-deficient plasma as substrate and pooled normal plasma as standard (1 U FXI activity/mL plasma). Human plasmas were from George King Biomed (Overland Park, KS). For competition binding assays, human FXI was labeled with 125 I by a modification of the Iodogen method. A specific activity of 125 I-FXI was 29.4 Ci/mmol.

**Platelet-binding experiments**

Gel-filtered human platelets were prepared from fresh blood as previously described. Thrombin receptor agonist SFLLRN-amide was prepared at the Protein Chemistry Facility of the University of Pennsylvania. Binding experiments were performed by a modification of published methods. Briefly, platelets (10^9/mL) were activated by 5 μM SFLLRN-amide for 5 minutes at 37°C and then supplemented with ZnCl2 (25 μM), CaCl2 (2 mM), HK (50 nM), and 125 I-labeled plasma-derived FXI (22 nM) either in the presence or absence of recombinant proteins. Incubation was continued for 30 minutes at 37°C. Aliquots (100 μL) were layered on Dow Corning methyl silicon oil (3 parts 550 density oil:2 parts 200 density oil) and platelets were separated from unbound protein by centrifugation in a microfuge.

**Activity of recombinant proteins in plasma clotting assays**

Coagulant activities for zymogen FXI/PKA4 and FXI/PKA4-Gly326 were determined by aPTT assay. HEPES-Tyrode buffer pH 7.4 (100 μL) containing 1 nM FXI/PKA4 or FXI/PKA4-Gly326 was mixed with 50 μL FXI-deficient plasma. To this mixture was added 50 μL kaolin (5 mg/mL) in HEPES-Tyrode buffer pH 7.4 containing either phospholipid (inosithin 0.04%; Accurate Chemicals, Westbury, NY) or activated platelets (10^9/mL). Incubation was for 5 minutes at 37°C, followed by addition of 50 μL 50 mM CaCl2. Time to fibrin clot formation was determined on a photometer. All proteins were tested in triplicate and were compared to a standard curve prepared with wild-type FXI. One nanomolar wild-type FXI was assigned an activity of 1.00 (100%). FXIa and activated chimeric enzymes were tested in a similar manner, except that phospholipid or platelet suspensions did not contain kaolin, and incubation at 37°C was for 60 seconds prior to addition of CaCl2. A standard curve was prepared with wild-type FXIa (1 nM FXIa was assigned an activity of 1.00 or 100%).

**Western immunoblot analysis of factor IX activation by FXIa**

Human factor IX (150 nM) was incubated at 37°C with 1 nM wild-type FXIa or activated FXI/PKA4 in TBSA that contained 2 nM CaCl2. At various time points, 10 μL samples were removed into 5 μL SDS-sample buffer (500 mM Tris-HCl pH 6.8, 40% glycerol, 10% SDS). A second set of experiments was carried out under similar conditions, except that reactions included HK (50 nM), ZnCl2 (25 μM), and activated platelets (0.5 × 10^9/mL). Samples were size-fractionated on 12% polyacrylamide gels, followed by transfer to nitrocellulose membranes. Blots were developed with a goat antihuman factor IX polyclonal immunoglobulin G (Affinity Biologicals, Hamilton, Ontario, Canada), using an enhanced chemiluminescence Western blotting detection kit (Amersham Pharmacia Biotech). From www.bloodjournal.org by guest on October 22, 2017. For personal use only.
Results

Recombinant proteins

Human FXI is a 160-kd disulfide bond-linked dimer comprised of 2 identical 80-kd polypeptides (Figure 1A, lanes 1 and 4).18,20 The A4 domain mediates dimer formation, with Cys321 in A4 forming the inter-chain disulfide bond.34 FXI in which Cys321 is replaced by alanine (FXI-Ala321, Figure 1A, lane 3) is an 80-kd protein on nonreducing SDS-PAGE; however, gel filtration experiments performed under conditions of physiologic salt concentration and pH demonstrate that it is the same size as plasma factor XI (Figure 1B). This suggests that the protein is a noncovalently associated dimer. These data confirm earlier work, demonstrating that an inter-chain disulfide bond is not required for dimer formation.34 In contrast, PK (Figure 1A, lane 5), which is structurally homologous to FXI,20,22 has a higher retention time on gel filtration (molecular mass ~90 kd; Figure 1), demonstrating it is a monomer.34

To prepare monomeric FXI, the A4 domain was replaced with PKA4. The resulting protein, FXI/PKA4, as expected, is an 80-kd protein on nonreducing SDS-PAGE (Figure 1C, lane 2). In gel filtration experiments, FXI/PKA4 has a similar retention time to PK, indicating it is a monomer (Figure 1D). FXI/PKA4 is expressed poorly by 293 fibroblasts (<100 ng/mL conditioned media).37 This is consistent with data showing that mutations interfering with FXI intracellular dimerization result in poor protein expression.35,36 Dimeric FXI/PKA4 (FXI/PKA4-Gly326) was made by replacing Cys326 in FXI/PKA4 with glycine. Cys326 in PKA4 is normally paired with Cys321 to form an intra-chain disulfide bond. Its removal leaves Cys321 free to form an inter-chain disulfide-link with Cys321 on another polypeptide. FXI/PKA4-Gly326 is expressed by 293 cells at similar levels to wild-type FXI (data not shown), and the expressed protein is entirely dimeric (Figure 1C, lane 4, and 1D). Parenthetically, this suggests that elements in FXI distinct from the A4 domain are involved in promoting dimer formation.

Table 1. Activities of activated proteases in chromogenic substrate assays

<table>
<thead>
<tr>
<th>S-2366</th>
<th>Factor IX</th>
</tr>
</thead>
<tbody>
<tr>
<td>K_m (μM)</td>
<td>k_cat (sec^{-1})</td>
</tr>
<tr>
<td>FXIa</td>
<td>500</td>
</tr>
<tr>
<td>FXIa/PKA4</td>
<td>450</td>
</tr>
<tr>
<td>FXIa/PKA4-Gly326</td>
<td>590</td>
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</tbody>
</table>

Recombinant activated proteases were tested as described in “Materials and methods.” Results represent the averages of duplicate experiments.

K_m indicates Michaelis-Menten constants; k_cat, turnover number; FXIa, activated factor XI; FXIa/PKA4, monomeric version of FXIa; FXIa/PKA4-Gly326, dimeric version of FXIa/PKA4.

FXI activity in purified protein assays

Activated proteases were studied in 2 purified protein systems. In the first system, the capacities of the proteases to cleave the chromogenic substrate S-2366 were tested. Kinetic parameters for S-2366 cleavage are similar for all proteins tested (Table 1), indicating that the catalytic domains are intact. Kinetic parameters for activation of factor IX by recombinant proteases were determined by a 3-stage assay.23,27 The results demonstrate that activated FXI/PKA4 and FXI/PKA4-Gly326 activate factor IX similarly to FXIa (Table 1). This indicates that both chimeric molecules bind normally to, and have normal catalytic activity toward, factor IX when activation takes place in solution.

FXI binding to platelets

FXI/PKA4 and FXI/PKA4-Gly326 were tested for their capacity to compete with 125I-labeled FXI for binding to activated platelets (Figure 2). Results were compared to those for plasma-derived FXI (positive control) and to FXI/PKA3 (negative control). FXI/PKA3, a chimera consisting of FXI with the A3 domain replaced by the PKA3 domain,27 binds poorly to platelets because PKA3 lacks critical amino acids required for platelet binding.15,25 The K_D for FXI/PKA4 (14 nM) and FXI/PKA4-Gly326 (16 nM) are similar to the value for plasma FXI (12 nM), indicating the 3 proteins bind to platelets with similar avidity. In contrast, the K_D for FXI/PKA3 is more than 500 nM, a result similar to reported values for PK binding to platelets.12

Activity of FXI and FXIa in plasma clotting assays

In contact activation-initiated clotting assays, such as the aPTT, a negatively charged substance is used to initiate coagulation, and...
phospholipid is required for several enzymatic steps. Activated platelets may serve as a source of phospholipid. Clot formation in this type of assay depends on factor IX activation by FXIa. In aPTT assays using purified phospholipid (inosithin), FXI/PKA4 and FXI/PKA4-Gly326 correct the defect in FXI-deficient plasma similarly to wild-type FXI (Table 2, column 1). In contrast, when the phospholipids are replaced by activated platelets, only the dimeric protein FXI/PKA4-Gly326 shows significant activity (Table 2, column 2). In the presence of activated platelets, the activity of monomeric FXI/PKA4 is below the lower limit of detection of the assay.

These data indicate that activated FXI/PKA4 bound to platelets does not properly activate factor IX. Alternatively, zymogen FXI/PKA4 may not be activated well in this system. To distinguish between these possibilities, a modified clotting assay was performed in which coagulation is initiated by FXIa or activated chimera rather than by kaolin. Poor activation of FXI/PKA4 is not an issue in this case as the protease is added in the active form. Activated FXI/PKA4 and activated FXI/PKA4-Gly326 demonstrate significant activity in the presence of phospholipid (Table 2, column 3). However, consistent with the results obtained usingzymogens in the aPTT assay, activated FXI/PKA4 has little activity in the presence of activated platelets compared to its dimeric counterpart FXI/PKA4-Gly326 (Table 2, column 4).

**Factor IX activation by FXIa in the presence of platelets**

During activation of factor IX (molecular mass 55 kd), an approximately 11-kd activation peptide is released to generate the active enzyme, factor IXaβ (45 kd). Therefore, the activation of factor IX by FXIa can be directly observed by Western immunoblot assay under nonreducing conditions. Wild-type FXIa and activated FXI/PKA4 activate factor IX to factor IXaβ similarly in the absence of platelets (Figure 3A). In contrast, and consistent with the results of the clotting assays, activation by activated FXI/PKA4 is significantly reduced compared to wild-type FXIa and activated XI/PKA4-Gly326 when activated platelets are included in the reaction (Figure 3B). It is not clear if the relatively small amount of factor IXaβ generated by FXI/PKA4 represents enzyme activity on the platelet surface, or activation in solution phase by FXI/PKA4 that has not bound to the platelet.

**Discussion**

The formation of protease-substrate complexes on activated platelets and damaged tissue is crucial for normal hemostasis. In complexes involving vitamin K–dependent proteases (prothrombin and factors VII, IX, and X), both protease and substrate bind to phospholipid in an interaction involving the N-terminal “Gla-domain” of each protein. Gla-domains contain 10 to 12 glutamic acid residues that undergo post-translational modification by addition of a carboxyl group to the γ-carbon. This modification is necessary for calcium-dependent protein binding to phospholipid. FXI is the only serine protease required for normal coagulation that lacks a Gla-domain. This may explain why phospholipid has little effect on factor IX activation by FXIa. Although FXI may not interact with purified phospholipid, it is clear that FXI and FXIa

Table 2. Activities of FXI/PKA4 chimeras in plasma coagulation assays

<table>
<thead>
<tr>
<th></th>
<th>Zymogen*</th>
<th>Activated enzyme†</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Phospholipid</td>
<td>Platelets</td>
</tr>
<tr>
<td>FXI/PKA4</td>
<td>0.80 ± 0.05</td>
<td>&lt; 0.05</td>
</tr>
<tr>
<td>FXI/PKA4-Gly326</td>
<td>0.81 ± 0.04</td>
<td>0.83 ± 0.01</td>
</tr>
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</table>

Recombinant proteins were tested as described in “Materials and methods.” Results represent the means for 3 separate experiments.

*Activities determined by kaolin-initiated aPTT assay.
†Activities determined by modified clotting assay in the absence of kaolin.

Figure 3. Western immunoblot analysis of factor IX activation by FXIa in the presence of platelets. (A) Factor IX (150 nM) was incubated with 1 nM wild-type FXIa or activated FXI/PKA4 in TBSA containing 2 mM CaCl2. At the indicated time points, samples were removed into sample buffer and processed as described in “Materials and methods.” (B) Reactions were run in the same manner as in panel A with the following additions: HK 50 nM, ZnCl2 (25 μM), and activated platelets (0.5 × 109/mL). The positions of zymogen (FIX) and activated (FIXaβ) factor IX are shown to the right of each blot. Time in minutes is shown across the top of each panel.
bind to activated platelets. Amino acids in the C-terminus of the A3 domain are required for platelet binding. Curiously, this region may overlap with the factor IX binding site. Data suggest that the platelet surface is a physiologic site for FXI activation and activity, this finding raises a question as to the manner in which FXI would bind simultaneously to its substrate and a platelet surface. A possible answer may be found in the dimeric structure of FXI.

Human FXI is a disulfide-bond linked homodimer, a unique feature among coagulation proteases. Meijers et al demonstrated that the A4 domain is involved in dimer formation, with Cys321 involved in the inter-chain disulfide bond. To test the significance of the dimeric state to FXI function, a FXI monomer is required for comparison. Taking advantage of the homology between FXI and PK (a monomeric protein), we generated the monomeric chimera FXI/PKA4. As in FXI, there is a cysteine at position 321 in PKA4; however, it is involved in an intra-chain disulfide bond. Several assumptions were made in preparing the model that require further testing and may, therefore, not be accurate. For example, it is likely that factor IX is bound to the platelet through its Glu-domain during activation, rather than being in solution as shown in Figure 4. In addition, it is not clear if one or both FXIa catalytic domains interact with the substrate. A study by Wolberg et al suggested that the 2 proteolytic cleavages made in factor IX during activation by FXIa may require both FXIa catalytic domains. Finally, for most reactions involving vitamin K-dependent coagulation proteases, phospholipid or activated platelets are both suitable surfaces. In contrast, our results strongly indicate that FXIa behaves differently in the presence of activated platelets compared to purified phospholipid. This suggests that FXIa may be interacting with a platelet membrane protein rather than the phospholipid component of the platelet membrane as shown in Figure 4. In this regard, preliminary data indicating that FXI binds to glyocalcin, the extramembrane portion of glycoprotein Ib, are of interest. Additional work will be required to validate this model; however, it offers a reasonable explanation as to why FXI, alone among coagulation proteases, is dimeric.

Acknowledgments

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Model for a factor IX activation complex on blood platelets: dimeric conformation of factor Xla is essential

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