Activated coagulation factor XI (factor XIa) proteolytically cleaves its substrate, factor IX, in an interaction requiring the factor XI A3 domain (Sun, Y., and Gailani, D. (1996) J. Biol. Chem. 271, 29023–29028). To identify key amino acids involved in factor IX activation, recombinant factor XIa proteins containing alanine substitutions for wild-type sequence were expressed in 293 fibroblasts and tested in a plasma clotting assay. Substitutions for Ile183–Val191 and Ser195–Ile197 at the N terminus and for Ser258–Ser264 at the C terminus of the A3 domain markedly decreased factor XI coagulant activity. The plasma protease prekallikrein is structurally homologous to factor XI, but activated factor IX activity. Clotting and kinetics studies using these chimeras confirmed the results obtained with alanine substitutions for wild-type sequence were expressed in 293 fibroblasts and tested in a plasma clotting assay. Substitutions for Ile183–Val191 and Ser195–Ile197 at the N terminus and for Ser258–Ser264 at the C terminus of the A3 domain markedly decreased factor XI coagulant activity. The plasma protease prekallikrein is structurally homologous to factor XI, but activated factor IX poorly. A chimeric factor XIa molecule with the A3 domain replaced with A3 from prekallikrein (FXI/PKA3) activated factor IX with a Kₐ 35-fold greater than that of wild-type factor XI. FXI/PKA3 was used as a template for a series of proteins in which prekallikrein A3 sequence was replaced with factor XI sequence to restore factor IX activation. Clotting and kinetics studies using these chimeras confirmed the results obtained with alanine mutants. Amino acids between Ile183 and Val191 are necessary for proper factor IX activation, but additional sequence between Ser195 and Ile197 or between Phe260 and Ser265 is required for complete restoration of activation.

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EXPERIMENTAL PROCEDURES

Materials and Reagents

Molecular Biology—Human factor XI (10) and PK (14) cDNAs were gifts from Dr. Dominic Chung (University of Washington, Seattle). The Chameleon site-directed mutagenesis kit and plasmid pBluescript (SK⁺ version) were from Stratagene (La Jolla, CA).

Tissue Culture and Recombinant Protein Production—The human fetal kidney 293 fibroblast cell line was from American Type Culture Collection (ATCC CRL 1573, Manassas, VA). Dulbecco’s modified Eagle’s medium and G418 were from Mediatech (Herdon, VA). Soybean trypsin inhibitor, lima bean trypsin inhibitor, and benzamidine were from Sigma. Goat anti-human factor XI polyclonal IgG, with or without conjugated horseradish peroxidase, for enzyme-linked immunosorbent assays was from Enzyme Research Laboratories (South Bend, IN). Gelcode Blue stain for SDS-polyacrylamide gels was from Pierce.

Activation and Characterization of Recombinant Factor XI—Chromogenic substrates S-2366 (t-pyrrolatamyl-L-prolyl-L-arginine p-nitroaniline) and S-2765 (N⁺-benzoyloxycarbonyl-L-arginyl-L-arginine p-nitroaniline) were from Diapharma (West Chester, OH). Factor XI-deficient human plasma was from George King Biomedical (Overland Park, KS). Bovine serum albumin, nitro blue tetrazolium, and 5-bromo-4-chloro-3-indolyl phosphate were from Sigma. Polybrene was from Aldrich. Recombinant human factor VIII (Recombinate) was from Baxter/Hyland. Purified thrombin and factors IX, X, IXa, Xa, and XIIa were from Enzyme Research Laboratories. Rabbit brain cephalin was prepared from rabbit brain acetone powder (Sigma) by the method of Bell and Alton (16). A 1:10 dilution of this preparation was found to be optimal for clotting assays and was used in all subsequent experiments.

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FIG. 1. Comparison of amino acid sequences of the human factor XI and prekallikrein A3 domains and positions of amino acid substitutions for recombinant factor XI molecules. Amino acid sequences between Cys363 and Cys365, composing the A3 domains of factor XI (FXI) and PK are aligned. Areas in which the A3 domains have identical amino acid sequences are boxed. Numbered lines above the factor XI sequence indicate the positions of alanine substitutions in recombinant factor XI molecules. The amino acids at the carboxyl terminus labeled B indicate the amino acids in the chimeric protein FXI/PKA3-A that were converted from PK sequence to factor XI sequence to generate the molecule FXI/PKA3-B. The brackets labeled with I–III indicate the positions of PK amino acids that were changed to the corresponding amino acids for factor XI to create chimeric proteins FXI/PKA3-IA and FXI/PKA3-IB, FXI/PKA3-IIA and FXI/PKA3-IIIB, and FXI/PKA3-IIIA and FXI/PKA3-IIIB, respectively.

Unfractionated heparin was from Pharmacia Hepar, Inc. (Franklin, OH).

Preparation of Factor XI and FXI/PKA3 Chimeric Expression Constructs and Transfection of 293 Cells

Mutations to produce alanine substitutions were introduced into the wild-type factor XI cDNA in pBluescript using a Chameleon site-directed mutagenesis kit. Proper introduction of mutations was confirmed by DNA sequencing. Each mutant contains two to four alanine substitutions in adjacent amino acids (see Fig. 1). Factor XI cDNAs were introduced into a mammalian expression vector containing the cytomegalovirus promoter (pJVCVM) (9). Transfected 293 fibroblasts (5 x 10^6) were grown in Dulbecco's modified Eagle's medium with 5% fetal bovine serum for 24 h and then switched to the same medium containing the neomycin analog G418 (500 μg/ml). The medium was exchanged every 48 h for 14 days. G418-resistant clones were tested for protein expression by enzyme-linked immunosorbent assay. Transfection was by electroporation using an Ectorect Molecular Manipulator 600 (BTX, Inc., San Diego, CA). Transfected cells were grown in Dulbecco's modified Eagle's medium with 5% fetal bovine serum for 24 h and then switched to the same medium containing the neomycin analog G418 (500 μg/ml). The medium was exchanged every 48 h for 14 days. G418-resistant clones were tested for protein expression by enzyme-linked immunosorbent assay. Clones expressing high levels of protein were expanded in 175-cm² culture flasks. When cells reached 50% confluence, the medium was replaced with 75 ml of Cellgro complete medium containing soybean trypsin inhibitor (10 μg/ml) to prevent activation of factor XI. The medium was exchanged every 48 h. After collection, the conditioned medium was supplemented with 5 mM benzamidine and stored at −20 °C pending purification.

The chimeric FXI/PKA3 (human factor XI in which the A3 domain is replaced with the PK A3 domain) has been described (9). FXI/PKA3 cDNA was used as a template for a series of mutagenesis steps changing PK A3 sequence back to factor XI sequence (Fig. 1). Mutagenesis and expression of recombinant protein were carried out as described for alanine mutants. The original FXI/PKA3 construct is referred to as FXI/PKA3-A. A construct with Tyr299, Leu298, and Thr298 at the C terminus of the A3 domain changed to the corresponding amino acids for factor XI (Phe360, Gln363, and Ser365) is referred to as FXI/PKA3-B. Amino acids at positions 183–185 were changed at amino acids 188–190 to make FXI/PKA3-IIA and FXI/PKA3-IIIB, respectively. FXI/PKA3-IA and FXI/PKA3-IB were then changed at amino acids 183–185 to make FXI/PKA3-IIIA and FXI/PKA3-IIIB with amino acids 195–197 converted to factor XI sequence.

Purification and Activation of Recombinant Protein

The conditioned medium (500–2000 ml) was passed over a 3 ml murine anti-human factor XI monoclonal antibody 1G5.12 column (9). After loading, columns were washed with 10 volumes of 25 mM Tris-HCl (pH 7.4), 100 mM NaCl (TBS), and 5 mM benzamidine, followed by elution with 2 M sodium thiocyanate in 25 mM Tris-HCl (pH 7.4) and 5 mM benzamidine. Protein-containing fractions were pooled and concentrated in an ultrafiltration concentrator (Amicon, Inc., Beverly, MA), dialyzed against TBS, and stored at −80 °C. Proteins were analyzed by SDS-polyacrylamide gel electrophoresis for purity, and protein concentration was measured by dye binding assay (Bio-Rad). Activated recombinant factor XI (factor XIa) was prepared by diluting zymogen protein to 100–300 μg/ml in TBS containing 5 μg/ml human factor XIIa and incubating at 37 °C. The progress of conversion of the 80-kDa zymogen to the 45-kDa heavy chain and 35-kDa light chain of factor XIa was followed by SDS-polyacrylamide gel electrophoresis. Activated protein was stored at −80 °C.

Activity of Activated Recombinant Protein in a Modified Partial Thromboplastin Time Assay

The coagulant activity of recombinant factor XIa in a modified partial thromboplastin time assay was determined as follows. Factor XIa was diluted to 5 μg/ml (30 nM) in TBS containing 0.1% bovine serum albumin (TBSA), and 1:2 serial dilutions of these preparation were made in TBSA. Sixty microliters of each dilution was mixed with 60 μl of factor XI-deficient plasma and 60 μl of rabbit brain cephalin. After incubation at 37 °C for 30 s, 60 μl of 25 mM CaCl₂ was added, and the time to clot formation was determined on a Dataclot 2 fibrometer (Helena Laboratories, Beaumont, TX). The clotting time was compared with a standard curve prepared with wild-type factor XIa. Recombinant wild-type factor XIa has the same activity as plasma-derived factor XIa in this assay (9). Activities of recombinant proteins are given as a percentage of wild-type protein activity.

Cleavage of Chromogenic Substrate S-2366 by Recombinant Factor XIa

Factor XIa was diluted to 3.5 nM in TBS with 0.1% Tween 20 (17) containing S-2366 (50–1000 μM), and generation of free p-nitroaniline was followed by measuring the change in absorbance at 405 nm on a ThermoMax microtiter plate reader (Molecular Devices, Sunnyvale, CA). Reactions volumes were 100 μl. Michaelis-Menten constants (Kₘ and Vₘₐₓ) for cleavage of S-2366 were determined by standard methods. The value for Vₘₐₓ was converted to nM p-nitroaniline generated per s using an extinction coefficient of 2100 optical density units (405 nm)/mol of p-nitroaniline. Turnover number (kₘₐₓ) was calculated from the ratio of Vₘₐₓ to enzyme concentration.

Western Blot Assay for Heparin-induced Factor XI Autoactivation

Factor XI at 50 nM in TBS containing 25 μM ZnCl₂, was supplemented with heparin (1 unit/ml final concentration) and incubated at 37 °C. All reactions were 400 μl. At 10-min intervals, 30-μl samples were removed into 10 μl of SDS sample buffer (500 mM Tris-HCl (pH 6.8), 40% glycerol, 20% 2-mercaptoethanol, and 10% SDS). Samples were size-fractionated by electrophoresis on SDS-10% polyacrylamide gels and then transferred to nitrocellulose membranes using a Bio-Rad Mini-
Factor IX-binding Site on Factor XI

PROTEAN II apparatus. Membranes were blocked for 2 h with 5% powdered milk in TBS. The primary detection antibody was goat anti-human factor XI polyclonal IgG, and the secondary antibody was sheep anti-goat IgG conjugated to alkaline phosphatase. Blots were developed with a solution of 100 mM Tris-HCl (pH 9.0), 100 mM NaCl, 5 mM MgSO₄, 100 μg/ml nitro blue tetrazolium, and 50 μg/ml 5-bromo-4-chloro-3-indolyl phosphate.

**Determination of the \( K_{\text{m}} \) for Factor IX Activation by Activated Recombinant FXI/PKA3**

Activation of factor IX by factor Xa was assessed by a modification of the method of Wagenvoord et al. (18). All reagents are diluted in TBSA. Human factor IX (0.05–100 μM) was incubated with 5 mM CaCl₂ and 1.0 nm wild-type factor Xa or activated FXI/PKA3 chimera for 60 s at 37 °C in a 50-μl volume. The reactions were stopped by adding EDTA to 25 mM and chilling on ice. The reactions were diluted 1:100 or 1:10, and 10 μl of each dilution was added to 50 μl of a mixture of human factor VIII (8 units/ml), CaCl₂ (10 mM), and rabbit brain cephalin (1:5 dilution of stock). Ten microliters of human thrombin (0.6 units/ml) was added, and the sample was incubated at 37 °C for 60 s to allow the thrombin to activate factor VIII. Subsequently, 30 μl of human factor X (450 nM) was added, and incubation was continued for 10 min. The activated factor X by factor Xa was stopped by adding EDTA to 25 mM and placing on ice. The final concentrations of reagents were 135 nM factor X, 4 units/ml factor VIII, 5 mM CaCl₂, and 1:10 dilution of rabbit brain cephalin. Fifty microliters of each reaction was mixed with 50 μl of 1 mM S-2765, and the change in absorbance at 405 nm was followed on the ThermoMax microtiter plate reader. Results were compared with a control curve constructed with known amounts of purified factor IXa. There was a linear correlation between the results of the assay and the control curve constructed with known amounts of purified factor IXa. The assay is not sufficiently sensitive to detect subtle differences between proteins, it is a useful screen for identifying significant defects. Activity was compared with that of recombinant wild-type factor XI (9, 17). On SDS-polyacrylamide gels, the proteins ran similarly to wild-type factor XI (Fig. 2). Poor (<50 ng/ml) or no expression was obtained for alanine mutants 11–13, 15, and 18, implying that these proteins were either unstable or poorly secreted. Five proteins were not included in further analysis. Substitutions in mutants 11–13, 15, and 18 involve an area of the apple domain constrained by a pair of disulfide bonds with relatively high homology to the PK sequence (Figs. 1 and 3). Two proteins, mutants 7 and 20, were expressed at somewhat reduced amounts (100–200 ng/ml of conditioned medium). Substitutions in both proteins involve areas in which factor XI and PK are identical. On SDS-polyacrylamide gels, mutant 7 appears to contain additional bands compared with wild-type factor XI, implying altered protein structure or processing (data not shown).

**Expression and Purification of Recombinant Proteins**—Most alanine mutants and all FXI/PKA3 chimeras were expressed at 1–3 μg/ml of conditioned medium as determined by enzyme-linked immunosorbent assay. This is similar to expression of recombinant wild-type factor XI (9, 17). On SDS-polyacrylamide gels, the proteins ran similarly to wild-type factor XI (Fig. 2). Poor (<50 ng/ml) or no expression was obtained for alanine mutants 11–13, 15, and 18, implying that these proteins were either unstable or poorly secreted. These five proteins were not included in further analysis. Substitutions in mutants 11–13, 15, and 18 involve an area of the apple domain constrained by a pair of disulfide bonds with relatively high homology to the PK sequence (Figs. 1 and 3). Two proteins, mutants 7 and 20, were expressed at somewhat reduced amounts (100–200 ng/ml of conditioned medium). Substitutions in both proteins involve areas in which factor XI and PK are identical. On SDS-polyacrylamide gels, mutant 7 appears to contain additional bands compared with wild-type factor XI, implying altered protein structure or processing (data not shown).

**Activity of Factor XI Alanine Mutants in a Plasma Clotting Assay**—Alanine mutants were tested in a plasma clotting assay that requires activation of factor IX by factor Xa (9). Although the assay is not sufficiently sensitive to detect subtle differences between proteins, it is a useful screen for identifying significant defects. Activity was compared with that of recombinant wild-type factor Xa. A profound decrease in activity (10% of wild-type activity) was noted for mutants 1–3 and 5 at the N terminus of the A3 domain and for mutants 25 and 26 at the C terminus (Table I). These substitutions involve areas in which the amino acid sequences of factor XI and PK are significantly different (Fig. 1). More modest decreases in activity were noted for mutants 7, 10, and 20. As discussed above, mutants 7 and 20 were expressed at lower levels than wild-type factor XI, indicating that there may be some structural aberrations in the molecules. Mutants 7, 10, and 20 involve areas in which factor XI and PK are highly homologous and are therefore less likely to contain factor XI-specific elements required for factor IX activation. The data indicate that amino acids required for factor IX activation are located at the N and C termini of the factor XI A3 domain.

**Activities of Recombinant Factor XI Alanine Mutants in Factor Xa Chromogenic Substrate and Autoactivation Assays**—A major concern when using alanine-scanning mutagenesis is that proteins may be severely altered, resulting in a generalized rather than a specific defect in activity. The following studies were performed to demonstrate that the factor XI alanine mutants retained activities characteristic of wild-type factor XI (other than factor IX activation). All proteins were converted to the active two-chain form when incubated with factor Xla in a manner similar to recombinant wild-type factor XI (data not shown). To determine that the catalytic (light chain) domains of the recombinant molecules were intact, activated proteins were tested in a chromogenic substrate cleavage assay using the substrate S-2366 (9, 17). The kinetic parameters for the generation of free p-nitroaniline were similar for all proteins tested (Table I) and are comparable to previously published results for other recombinant factor XI proteins (9, 17). When exposed to certain negatively charged substances such as heparin or the synthetic polysaccharide dextran sulfate, factor XI undergoes autoactivation to factor Xla (17, 19, 20). This process involves a heparin-binding site in the A3 domain. We tested alanine mutants 1–6 and 22–26 in an autoactivation assay in the presence of heparin. All proteins activated at a rate similar to that of wild-type factor XI as determined by Western blotting, with the exception of mutant 23, which dem-
onstrated significantly delayed activation, and mutant 24, which had slightly delayed activation (data not shown). These two mutant proteins were previously shown to be defective in autoactivation, as they are missing parts of the heparin-bind-
ing site in the A3 domain (17). Although defective in the auto-
activation assay, mutants 23 and 24 have normal activity in
the plasma clotting assay. The observations that alanine mu-
tants are activated by factor XIIa, cleave a chromogenic sub-
strate, and autoactivate normally (except for mutants 23 and
24) indicate that substitutions in A3 have not caused severe
generalized abnormalities in factor XI function. This strength-
ens the premise that the defects observed for mutants 1–3, 5,
25, and 26 are specific for factor IX activation.

Activity of FXI/PKA3 Mutants in a Plasma Clotting
Assay—We previously demonstrated that factor XI with the PK
A3 domain (FXI/PKA3) functions poorly in a plasma clotting
assay (<1% of wild-type activity) (9). Guided by results with
the factor XI alanine mutants, we used FXI/PKA3 as a tem-
plate to reintroduce factor XI sequence into the A3 domain to
restore normal factor IX activation. This “gain-of-function”
strategy avoids some potential pitfalls of the “loss-of-function”
approach. Restoration of normal factor IX binding in this chi-
mera should be accompanied by a reduction in the Km for
factor IX activation. The Km values for activation of factor IX by
activated FXI/PKA3-A has been shown to be 35-fold greater than that for
wild-type factor XIa, whereas the turnover numbers (kcat) are
similar (9). These data are consistent with a defect in factor IX
binding. Restoration of normal factor IX binding in this chimer
should be accompanied by a reduction in the Km for factor
IX activation. The Km values for activation of factor IX by the
panel of activated FXI/PKA3 chimeras were determined by a
two-stage assay (9). In the first stage, factor IX was activated
by factor XIa or the activated FXI/PKA3 chimera in the pres-
ence of calcium. In the second stage, the resulting activated
factor IX was used to activate factor X in the presence of
calcium, phospholipid, and activated factor VIII. The amount of
activated factor X generated was measured with a chromogenic
substrate, and the amount of factor IXa generated in the first
stage of the assay was determined by comparison to a standard
curve. This complex assay is necessary because there is no
chromogenic substrate for factor IXa with sufficient sensitivity
to carry out kinetic studies.

The results of the study are shown in Table II. The Km values for
wild-type factor XIa (250 μM) is similar to published results (9,
22). Consistent with the clotting assay data, the Km values for
activation by FXI/PKA3-A and FXI/PKA3-B are substantially
greater (>10 μM) than those for wild-type protein. The Km values for these two proteins are approximations because the
The proteases of the plasma coagulation system are each composed of distinct domains (23, 24). The carboxyl-terminal domain resembles the pancreatic digestive enzyme trypsin. The N-terminal region is required for specific interactions with other components of the coagulation mechanism. The heavy (non-catalytic) chain of factor XI contains four repeats called apple domains (A1–A4) that are distinct from domains found in vitamin K-dependent coagulation proteases and factor XII (23, 24). Only plasma PK has been shown to contain similar structures (14). Using peptide mimicry techniques, Baglia et al. identified several prospective binding sites in the apple domains of human factor XI. Binding sites for high molecular weight kinogen (25) and thrombin (26) were localized to the A1 domain, for platelets to the A3 domain, (27) and for factor X to the A4 domain (13) based on the capacity of peptides to interfere with protein-protein or protein-surface interactions. Several peptide sequences were identified in the factor XI A2 domain that interfere with factor IX activation, whereas peptides from the PK A2 domain had little effect (11). This suggests a role for the A2 domain of factor XI in binding interactions with factor IX.

We employ site-directed mutagenesis and recombinant protein expression to investigate factor XI structure-function relationships (9, 17). Using recombinant factor XI proteins with individual apple domains replaced by the corresponding domains from PK, we demonstrated that substitution of factor XI A3 results in a protein with a severe defect in factor IX activation. Replacement of the A1, A2, or A4 domain did not alter factor IX activation (9). A kinetic analysis of factor IX activation by the chimeric proteins strongly suggested a role for the A3 domain in interactions with factor IX. The $K_m$ for activation of factor IX by activated FXI/PKA3 is 35-fold higher than that for plasma factor XI, whereas the $k_{cat}$ values are similar, indicating defective binding of factor IX. Furthermore, two monoclonal antibodies directed against the factor XI A3 domain block factor IX activation, whereas antibodies to the A2 and A4 domains do not (9).

The reasons for the discrepant results using two different techniques (peptide mimicry and recombinant chimeras) are not clear. Several possibilities must be considered. The results of the chimeric protein studies could be interpreted as demonstrating that a factor IX-binding site is present in the A3 domain. Alternatively, the A3 domain may be a component of a binding site requiring contributions from more than one domain or may be required for proper conformation of a binding site outside of the A3 domain (in the A2 domain, for example). The observation that factor XI chimeras with the PK A1, A2, and A4 domains activate factor IX normally indicates that these substitutions do not disturb factor IX binding. Components of a binding site provided by one of these three domains would presumably be available in both the factor XI and PK versions of these domains. If part of the A2 domain is required for interactions with factor IX, then the PK A2 domain provides this binding site in the context of the factor XI heavy chain. An additional possibility that must be considered is that the A2 domain is not required for factor IX interactions, but that PK peptides interfere with factor IX activation by binding directly to factor XI.

WORKING on the premise that part of the factor XI A3 domain is required for factor XIa to activate factor IX, a combination of alanine-scanning mutagenesis (loss-of-function studies) and replacement of PK sequence with factor XI sequence (gain-of-function studies) was used to study the A3 domain. Areas at the N and C termini of the A3 domain required for proper factor IX activation were identified (Fig. 3). Interestingly, these two area are probably in close proximity to each other in the native molecule due to the disulfide bond between Cys$^{182}$ and Cys$^{265}$.

**Table I**

| Factor XIa species | Clotting activity | Cleavage of S-2366 |
|-------------------|------------------|--------------------|
|                    | $K_m$ (μM) | $k_{cat}$ (s$^{-1}$) | % of plasma XIa |
| Wild-type         | 395 | 200 | 100$^a$ |
| Mutant 1          | <1 | 365 | 215 |
| Mutant 2          | <1 | 365 | 145 |
| Mutant 3          | 10  | 350 | 175 |
| Mutant 4          | 71  | 465 | 220 |
| Mutant 5          | <1 | 350 | 140 |
| Mutant 6          | 74  | 520 | 275 |
| Mutant 7          | 20  | 440 | 260 |
| Mutant 8          | 125 | 385 | 205 |
| Mutant 9          | 50  | 495 | 225 |
| Mutant 10         | 21  | 455 | 160 |
| Mutant 14         | 123 | 540 | 250 |
| Mutant 16         | 60  | 560 | 180 |
| Mutant 17         | 86  | 370 | 200 |
| Mutant 19         | 80  | 460 | 205 |
| Mutant 20         | 16  | 415 | 155 |
| Mutant 21         | 91  | 355 | 155 |
| Mutant 22         | 91  | 565 | 225 |
| Mutant 23         | 142 | 430 | 130 |
| Mutant 24         | 114 | 400 | 160 |
| Mutant 25         | <1 | 320 | 130 |
| Mutant 26         | 6   | 415 | 210 |

$^a$ The value for wild-type factor XIa was arbitrarily set at 100%.

**Table II**

| Factor XIa species | Cleavage of S-2366 | Clotting activity | Factor IX activation $K_m$ (μM) |
|-------------------|--------------------|------------------|-------------------------------|
|                    | % of plasma XIa | $K_m$ (μM) | $k_{cat}$ (s$^{-1}$) |
| Wild-type         | 395 | 200 | 100$^a$ |
| FXI/PKA3-A        | 485 | 205 | <1 |
| FXI/PKA3-B        | 395 | 170 | <1 |
| FXI/PKA3-IA       | 395 | 175 | 5 |
| FXI/PKA3-IB       | 345 | 120 | 40 |
| FXI/PKA3-IIA      | 505 | 260 | 15 |
| FXI/PKA3-IIIB     | 415 | 185 | 91 |
| FXI/PKA3-IIIB     | 520 | 240 | 140 |
| FXI/PKA3-IIIB     | 375 | 200 | 120 |

$^a$ The value for wild-type factor XIa was arbitrarily set at 100%.
and could represent a contiguous binding site. The sequence between Ile
183 and Val
191 is required for factor IX activation. The data indicate, however, that this region is not sufficient for normal interactions with factor IX, as additional sequence between Ser
195 and Ile
197 or between Phe
260 and Ser
264 is required for factor IX activation to be comparable to that of wild-type factor Xla. It must be noted that these studies do not determine if these areas of the A3 domain compose all or part of a factor IX-binding site or are required for conformation of a binding site at a different location. Resolution of this issue will require determination of the actual structure of the factor Xla-factor IX complex.

Recent work has implicated the factor XI A3 domain in binding interactions. Ho et al. (21) and Zhao et al. (17) independently identified a heparin-binding site involving lysines 252, 253, and 255 in the A3 domain of factor XI (Fig. 3B). There is also evidence for a platelet-binding site in this region (Fig. 3B) (27, 28). A recently proposed model of factor XI physiology suggests that the protein is activated on platelets and subsequently activates factor IX while bound to platelets (29). This implies that binding sites for platelets and for factor IX on factor Xla are distinct. The amino acid sequences we have identified in factor XI A3 may represent a factor IX-binding site. If this is the case, the close proximity to the proposed platelet-binding site raises questions regarding the mechanism by which factor Xla would bind simultaneously to factor IX and platelets through the A3 domain. A possible mechanism is suggested by the structure of the factor XI molecule. The human protein is unique among blood coagulation proteases in that it is a disulfide bond-linked homodimer. All other identified coagulation proteases are monomeric. Each factor Xla molecule therefore possesses two A3 domains (4). Bovine (30), porcine (31), and murine (22) factors XI have a similar structure. Rabbit factor XI is an 80-kDa monomer on SDS-polyacrylamide gels (32); however, it appears to be a dimer at physiologic pH and salt concentration. It is conceivable that factor Xla may use one A3 domain to interact with platelets and the other to bind to its substrate, factor IX, as activation of factor IX may require only a single A3 domain. A factor XI molecule in which the A4 domain is replaced with that of PK (FXI/PKA4) is a monomer because the A4 domain of factor IX contains critical elements for dimer formation (9, 33). FXI/PKA4 activates factor IX normally in a purified protein assay (9). Testing the hypothesis that dimeric factor XI is required for proper activation of factor IX on a platelet surface will be facilitated by preparation of significant amounts of a stable single chain variant of factor XI. We are currently working on such a protein.

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2 D. Gailani, unpublished observation.