Identification of a Factor IX Binding Site on the Third Apple Domain of Activated Factor XI*

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Activated factor XI (factor XIa) participates in blood coagulation by activating factor IX. Previous work has demonstrated that a binding site for factor IX is present on the noncatalytic heavy chain of factor XIa (Sinha, D., Seaman, F. S., and Walsh, P. N. (1987) Biochemistry 26, 3768–3775). Recombinant factor XI proteins were expressed in which each of the four apple domains of the heavy chain (designated A1 through A4) were individually replaced with the corresponding domain from the homologous but functionally distinct protease prekallikrein (PK). To identify the site of factor IX binding, the chimeric proteins were activated with factor XIIa and tested for their capacity to activate factor IX in plasma coagulation and purified protein assays. The chimera with the substitution in the third apple domain (factor XI/PK A3) had <1% of the coagulant activity of wild type factor XI a in a plasma coagulation assay, whereas the chimeras with substitutions in A1, A2, and A4 demonstrated significant activity (68–140% of wild type activity). The Km for activation of factor IX by factor XIa/PKA3 (12.7 μM) is more than 30-fold higher than the Km for activation by wild type factor XIa or the other factor XI/PK chimeras (0.11–0.37 μM). Two monoclonal antibodies (2A12 and 11AE) that recognize epitopes on the factor XI A3 domain were potent inhibitors of factor IX activation by factor XIa, whereas antibodies against the A2 (1A6) and A4 (3G4) domains were poor inhibitors. The data indicate that a binding site for factor IX is present on the third apple domain of factor XIa.

Factor XI is the zymogen of a plasma serine protease which is a critical component of the intrinsic pathway of blood coagulation (1). Human factor XI is comprised of two identical 80-kDa polypeptides connected by a disulfide bond (2, 3). A single proteolytic cleavage in each polypeptide converts the zymogen to the active form of the enzyme, factor XIa. Activated factor XII (factor XIIa), thrombin, and factor XIa have all been shown to activate factor XI in vitro; however, the physiologic mechanisms responsible for conversion of factor XI to the active protease are not certain (2, 4, 5). Regardless of the mechanism of activation, it is generally agreed that factor XI contributes to coagulation by activating factor IX by limited proteolysis in the presence of calcium ions (6, 7). The factor XIa molecule is comprised of a pair of 35-kDa trypsin-like catalytic light chains and two 45-kDa non-catalytic heavy chains (8). The heavy chain appears to be required for factor XI specific activities including the activation of factor IX (9, 10). Indeed, isolated factor XIa light chains have less than 5% of the factor IX activating activity of intact factor XIa, even though both molecules cleave a chromogenic substrate similarly (9, 11).

The human factor XI heavy chain is comprised of four apple domains repeated in tandem (designated A1, A2, A3, and A4), a feature shared with the structurally homologous but functionally distinct plasma protease prekallikrein (PK) (12). Using synthetic peptides based on the factor XI and prekallikrein amino acid sequences, Baglia and colleagues (13) demonstrated that a peptide representing amino acids Asn145 to Ala176 of the A2 domain of factor XI interferes with the activation of factor IX by factor XIa, whereas a peptide representing the corresponding area of the prekallikrein A2 domain does not. As activated prekallikrein (kallikrein) activates factor IX poorly, these results indicate the presence of a binding site for factor IX on the factor XI A2 domain. In this report we describe the preparation and characterization of recombinant factor XI molecules in which the individual apple domains of the heavy chain have been replaced with the corresponding domain from prekallikrein. The chimeric proteins were activated with factor XIIa and were tested for their capacity to activate factor IX in plasma coagulation assays and a purified protein system. The chimeric protein with the prekallikrein substitution in the A3 domain demonstrated a marked reduction in capacity to activate factor IX, indicating that the A3 domain of factor XI is involved in interactions with factor IX.

EXPERIMENTAL PROCEDURES

Materials—Chromogenic substrates S-2238 (β-phenylmalonyl-arginine-p-nitroaniline), S-2302 (H-β-phenylalanyl-arginine-p-nitroaniline), and S-2765 (Nα-benzoylarginyl-L-β-phenylalanyl-L-arginine-p-nitroaniline) were obtained from Chromogenix (Molndal, Sweden). Factor XI-deficient plasma was purchased from George King Biomedical (Overland Park, KS). Affi-Gel 10 was obtained from BioRad. Cellgro complete medium was purchased from Fisher Scientific; Dulbecco’s modified Eagle’s medium and G418 (geneticin) were from Life Technologies, Inc. Recombinant human factor VIII (Recombinate) was purchased from Baxter/Hyland. Purified factors IX, X, IXa, and XIa; anti-human factor XI murine monoclonal antibody 11AE; and an affinity column of IgG11AE coupled to Sepharose were purchased from Enzyme Research Laboratories (South Bend, IN). Soybean trypsin inhibitor, lima bean trypsin inhibitor, aprotinin, benzamidine, nitro blue tetrazolium, 5-bromo-4-chloro-3-indolyl phosphate, bovine serum albumin (BSA), and rabbit brain cephalin were purchased from Sigma Chemical Co. A 1:10 dilution of the rabbit brain cephalin stock was found to be optimal for clotting assays and was used in all subsequent experiments. A crude preparation of corn trypsin inhibitor (CTI) was prepared from corn meal (14).

Preparation of Human Factor XI/PK Chimeric Complementary DNAs—To introduce prekallikrein domains into the factor XI cDNA

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1 The abbreviations used are: factor XIa, XIIa, IXa, and Xa, activated factor XI, XII, IX, and X, respectively; PK, prekallikrein; BSA, bovine serum albumin; CTI, corn trypsin inhibitor; TBS, Tris-buffered saline.

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unique restriction endonuclease sites were required between the apple domain (factor XI) and the 5′ heavy chain cDNA to transform wild type cDNA for human factor XI between the first and second apple domains (Eco47III) and between the third and fourth apple domains (PstI) (Fig. 1). Two modified cDNAs were prepared using the site-directed mutagenesis technique of Kunkel et al. (15), which contain an introduced unique NheI site between the second and third apple domains (factor XI-NheI) or an SpeI site at the 3′ end of the fourth apple domain (factor XI-SpeI). The oligonucleotides used to create these restriction sites are 5′-GCCAGATTAGCATAGCAGAGGTTTT-3′ for factor XI-NheI and 5′-TTACACAAACTACTAGTGATCTGAC-3′ for factor XI-SpeI (the underlined base pairs indicate the introduced mutations). The wild type factor XI cDNA, factor XI-NheI, and factor XI-SpeI were ligated into the EcoRI site of a modified pUC19 vector in which two unique restriction enzyme sites in the polylinker were eliminated except for the NheI site, which had been removed. The DNA sequences representing the individual apple domains were then removed from the construct by digestion with the appropriate restriction enzymes as follows. For the A1 domain, wild type XIPUC19 was digested with SalI (cuts in the pUC19 vector) and Eco47III; for the A2 domain, factor XI-NheI/pUC19 was digested with Eco47III and NheI; for the A3 domain, factor XI-NheI/pUC19 was digested with PstI and SpeI; and for the A4 domain, factor XI-SpeI/pUC19 was digested with PstI and SpeI. The apple domains of prekallikrein were amplified by the polymerase chain reaction using primers that introduce appropriate restriction endonuclease sites at the ends of the apple domain to allow introduction into the corresponding factor XIPUC19 construct. The oligonucleotide primers used were as follows (underlined base pairs indicate the introduced mutations introduced to create restriction endonuclease sites, shown in parentheses): for the A1 domain, 5′-AACAGCTATAGCATCTG-3′ (M13/pUC reverse sequencing primer) and 5′-ATTAGCAACGCTTTATTGATG-3′ (Eco47III); for the A2 domain, 5′-ATCTAAATAGGCGCTGCATCG-3′ (Eco47III) and 5′-CAATCTCTGACGCAAGCCTT- CAG-3′ (NheI); for the A3 domain, 5′-AGGCCGTTCGCTGCTCAGA- AATTGTCG-3′ (NheI) and 5′-TAAAGTTCTTCTGCAGGTTAA-3′ (PstI); and for the A4 domain, 5′-CTTTAATCCTGCGAAGAATTTACC-3′ (PstI) and 5′-ACACAAATCTACTAGTGATCAGACCTG-3′ (SpeI). Polymerase chain reaction products were precipitated with 10.0 volume of 3 M sodium acetate, pH 4.0, and ethanol to a final volume of 70% and then taken up in 10 μl Tris, pH 8.0, 1 m EDTA. After digestion with the appropriate restriction endonuclease, polymerase chain reaction products were size fractionated on a 1.5% agarose gel and then purified using a Qiagen gel extraction kit (Qiagen). The purified apple domains were ligated into the appropriate factor XI cDNA using T4 DNA ligase. Chimeric cDNAs and proteins will be referred to as factor XI/PKA as, where n is the number of the substituted apple domain. The ligations reached completion after 16 h at 16°C. Chimeric cDNAs containing an apple domain inserted were expanded and the DNA purified by conventional techniques. The DNA representing the introduced PK apple domain was sequenced for each construct to determine that the polymerase chain reaction had not introduced mutations. The cDNAs for factor XI, prekallikrein, and the factor XI/PK chimeras (Fig. 1) were cloned into the EcoRI site of a mammalian expression vector (pJVCVM) which contains the cytomegalovirus promoter. Large scale DNA preparations of cDNA/pJVCVM constructs were prepared using a Qiagen plasmid kit according to the manufacturer's recommendations.

Monoclonal Antibodies to Human Factor XI and Prekallikrein—50 μg of purified human factor XI or prekallikrein in Freund's complete adjuvant was injected intraperitoneally into BALB/c mice followed by two intraperitoneal booster immunizations in Freund's incomplete adjuvant administered 3 and 6 weeks after the initial injection. Three days after the final immunization the spleens were harvested, and hybridoma cell lines were prepared as described previously (16). The culture supernatant of hybridoma clones was screened for binding to the appropriate antigen using an enzyme-linked immunosorbent assay. 1 × 10⁹ hybridoma cells from each positive clone were injected into the peritoneal cavities of pristane-primed BALB/c mice and ascites collected 10 days later. The IgG was purified by passing ascites over a staphylococcal protein A-Sepharose column, and protein concentration was determined by measuring absorbance at 280 nm (extinction coefficient of 14.0 for factor XI, 1.8 for factor XI/PK chimera at 5 μg/ml in TBS with 0.1% BSA (TBSA)) was mixed with 75 μl of TBSA and 5 μl of CTI and incubated for 20 min at room temperature. CTI inhibits the factor XIIs used to activate the factor XI and does not inhibit factor XIAs (14). The mixture was diluted to 900 μl with TBSA and 100 μl of chromogenic substrate S-2366 at varying concentrations (50–1,000 μM final concentration) was added. Cleavage of S-2366 was followed by measuring the change in absorbance at 405 nm with a Beckman DU-640 spectrophotometer. An identical procedure was performed for kallikrein using the chromogenic substrate S-2302 (50–1,000 μM final concentration). Michaelis-Menten constant (Kₘ) and Vₘₐₓ for the cleavage of the chromogenic substrates were determined by standard methods. The value for Vₘₐₓ was converted to nm/p-nitroanilide generated per s using an extinction coefficient for p-nitroanilide of 9,800 optical density (OD) units (405 nm/μmol of p-nitroanilide). Turnover number (kcat) was calculated from the ratio of Vₘₐₓ (in s⁻¹) and kcat (in s⁻¹). Clotting Assays for Factor XIX Activity and Antibody Inhibition Assays—Serial 1:2 dilutions of enzyme (factor XIX, kallikrein, or activated chimera) at an initial concentration of 5 μg/ml (30 nM) were made in TBSA. 60 μl of each dilution was mixed with 60 μl of factor XI-deficient human plasma and 60 μl of a 1:10 dilution of rabbit brain cephalin and incubated for 30 s at 37°C. 60 μl of 25 mM CaCl₂ was added, and the
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time to fibrin clot formation was determined on a Dataclot 2 fibrometer (Helena Laboratories, Beaumont, TX). Experiments were run in duplicate for each enzyme, and the results were averaged. Enzyme concentration was plotted against clotting time in seconds on log-log paper, and the factor XIa activity of each preparation was determined by comparison with a control curve constructed with plasma factor XIa. To determine the effects of murine anti-human factor XI monoclonal antibodies on factor XIa activation of factor IX, plasma factor IXa at 0.5 μg/ml (3 nM) in TBSA was incubated for 30 min at room temperature with various concentrations of monoclonal antibodies. The residual factor XIa activity in the mixture was measured by the clotting assay described above.

Activation of Factor IX by Factor XIa and Chromogenic Assay for Factor XIa—Activation of factor IX by factor XIa was determined by a modification of the method of Wagenvoord et al. (18). Purified human factor IX at various concentrations (0.05–10 μg/ml) was incubated with 5 mM CaCl$_2$ and 0.5 mM factor Xa, kallikrein, or factor XIa/PK chimeras for 60 s at 37°C. The reaction was stopped by the addition of EDTA to a final concentration of 25 mM and chilling to 4°C. The reaction mixture was diluted 1:100 in TBSA, and 10 μl of the diluted was mixed with 50 μl of human factor VIII (5 units/ml), 10 μM CaCl$_2$, and rabbit brain cephalin (1:5 dilution of stock), and 10 μl of human thrombin (0.6 units/ml). After incubation at 37°C for 60 s to allow the thrombin to inactivate residual factor XIIa, Aprotinin at 20 antitrypsin units/ml was added, and the incubation was continued for an additional 5 min. The activation of factor X by factor XIa was stopped by adding EDTA to a 25 mM final concentration and placing the reaction on ice. The final concentration of the reagents during factor X activation were 135 nM factor XI (1 unit/ml), 4 units/ml factor VIII, 5 mM CaCl$_2$, and 1:10 dilution of rabbit brain cephalin. 50 μl of the reaction was diluted into 490 μl of TBSA; 60 μl of 5 mM chromogenic substrate S-2765 was added, and the change in absorbance at 405 nm was followed. The results were compared with a control curve constructed with known concentrations of factor IXa to determine the extent of factor IX activation by factor XIa. There was a linear correlation between the results of this assay and factor IX concentration at factor XIa concentrations between zero and 100 ppt. Michaelis-Menten kinetic parameters were determined as described above using the average of two sets of experiments for each enzyme.

Determination of Epitopes on Factor XI Recognized by Monoclonal Antibodies by Western Blot—0.5 μg of factor XI, prekallikrein, and each chimeric protein were run on a 10% polyacrylamide-SDS gel under nonreducing conditions. Proteins were transferred to a nitrocellulose membrane using a Bio-Rad mini-protean II electrophoresis apparatus in transfer buffer (20% methanol, 192 mM glycine, 25 mM Tris, pH 8.3) at 100 volts for 75 min, and then nitrocellulose was blocked by incubation in 10% milk in 10% milk and then for 2 h with goat anti-murine IgG for the cleavage of the chromogenic substrate S-2366 by plasma factor XIa, wild type recombinant factor XIa, and each of the chimeric proteins was determined. The same parameters were also calculated for plasma and wild type recombinant kallikrein using the chromogenic substrate S-2366. Results are shown in Table I. All chimeric proteins had kinetic parameters similar to those of wild type recombinant and plasma-derived factor XIa, demonstrating that the catalytic domains of the molecules are intact. Similar results were obtained for plasmaderived and recombinant kallikrein. These studies demonstrate that the chimeric molecules are recognized by a factor XI activator (factor XIIa) and have intact catalytic domains.

Expression and Purification of Recombinant Factor XI, Prekallikrein, and Factor XI/Prekallikrein Chimeras—293 fetal kidney fibroblasts were chosen for stable transfections because, when transfected with the appropriate construct, they readily expressed and secreted wild type factor XI and prekallikrein and did not constitutively express either protein. The amino acid sequences of factor XI and prekallikrein are 90 or 91 amino acids in length and are separated by linking regions of six or seven amino acids. The restriction sites in the linking regions were used to construct the chimeric cDNAs. An NheI site between A2 and A3 and an SpeI site at the 3’ end of A4 were introduced for this purpose (Fig. 1). These endonuclease restriction sites were selected because they involved the least alteration of the factor XI cDNA. Both restriction sites result in a Leu to Ser mutation (position 177 for NheI and 353 for SpeI) which is not found in either the factor XI or prekallikrein wild type sequence. To determine if these changes were deleterious to factor XI function, factor XI cDNAs containing either the NheI or SpeI mutation were expressed in 293 cells and purified as described under “Experimental Procedures.” Both recombinant mutant proteins corrected the clotting time of factor XI-deficient plasma in a partial thromboplastin time assay with specific activities that were the same as wild type factor XI (200 units/mg), indicating that the amino acid substitutions did not alter factor XI function.

The concentration of wild type and chimeric proteins in the conditioned medium of transfected 293 cells was 1–3 μg/ml with the exception of the factor XI/PK A4 chimera, which was expressed at approximately 100 μg/ml. It has been shown that the A4 domain is required for proper formation of the factor XI homodimer and that failure to form a proper dimer results in decreased expression of the molecule in a mammalian expression system (19, 20). All proteins were purified by monoclonal affinity chromatography. Factor XI and factor XI/PK chimeras appeared as single bands on an SDS-polyacrylamide gel run under nonreducing conditions (Fig. 2). Prekallikrein appeared as two bands between 80 and 85 kDa. Note that factor XI/PKA4 runs as an 75–80-kDa monomer rather than a 150–160-kDa dimer because the cysteine involved in the interchain disulfide bond in wild type factor XI has been removed by the replacement of the fourth apple domain. Factor XI and factor XI/PK chimeras appeared as single approximately 80-kDa bands and prekallikrein as an 85-kDa band on SDS-polyacrylamide gels run under reducing conditions, indicating that significant activation or degradation had not occurred during the purification process (data not shown).

Activation of Recombinant Proteins with Factor XIIa and Activity of Activated Recombinant Proteins in a Chromogenic Substrate Assay—All wild type and chimeric proteins were completely activated when incubated with human factor XIIa (determined by the disappearance of the 80-kDa zymogen band on SDS-polyacrylamide gels run under reducing conditions), indicating that epitopes required for interactions with factor XIIa are present on the chimeric molecules. The $K_m$ and $k_{cat}$ for wild type factor XIa, wild type recombinant factor XIa, and each of the chimeric proteins was determined. The same parameters were also calculated for plasma and wild type recombinant kallikrein using the chromogenic substrate S-2366. Results are shown in Table I. All chimeric proteins had kinetic parameters similar to those of wild type recombinant and plasma-derived factor XIa, demonstrating that the catalytic domains of the molecules are intact. Similar results were obtained for plasmaderived and recombinant kallikrein. These studies demonstrate that the chimeric molecules are recognized by a factor XI activator (factor XIIa) and have intact catalytic domains.

Activity of Activated Recombinant Proteins in a Plasma Clotting Assay—The capacity of recombinant factors to activate factor IX in plasma was determined by a modified partial thromboplastin time assay in which factor XIa, kallikrein, or activated chimera was added to factor XI-deficient plasma supplemented with rabbit brain cephalin as a phospholipid source, followed by reconstitution with calcium. Results are shown in Table I. Factor XIa/PKA1, PKA2, and PKA4 demonstrated substantial coagulant activity (68, 140, and 80% of wild type factor XIa activity, respectively). In contrast, the factor XIa/ PKA3 chimera exhibited <1% of the activity of wild type factor XIa, a result similar to that obtained for kallikrein. Kallikrein
would be expected to be a poor activator of factor IX in this system. These data implicate the third apple domain of factor XI in binding interactions with factor IX.

**Activation of Factor IX by Activated Recombinant Proteins in a Purified System**—Although the results in the plasma assay strongly indicate that the third apple domain contains a factor IX binding site, other explanations are possible. For example, the introduction of the prekallikrein A3 domain may have enhanced the affinity of the molecule for other plasma components such as factor XII (a substrate of kallikrein during contact activation), resulting in smaller amounts of enzyme available for factor IX activation. Similar interactions may explain the moderate reduction in coagulant activity of factor XIa/PKA4 compared with wild type factor XIa. To examine directly the capacity of the chimeric proteins to activate factor IX, a system using purified proteins was used. Unfortunately, there is no readily available chromogenic substrate for factor IXa, therefore a two-stage assay was developed in which factor IX is activated by activated recombinant factor XI proteins in the first step, and the resulting factor IXa is used subsequently to activate factor X in the presence of phospholipid, calcium, and factor VIIIa. Factor Xa is then measured by amidolytic assay using the synthetic substrate S-2765, and the results are compared with a control curve constructed with known concentrations of factor IXa.

The kinetic parameters for the activation of factor IX by each of the recombinant proteins are shown in Table II. The $K_m$ and $k_{cat}$ for factor XIa/PKA1, PKA2, and PKA4 are of the same order as values for plasma-derived factor XIa, recombinant wild type factor XIa, and those published for plasma-derived factor XIa by Sinha and co-workers (9). The result for factor XIa/PKA3 is consistent with the data from the clotting assay. The derived catalytic efficiency ($k_{cat}/K_m$) for factor XIa/PKA3 is at least 35-fold lower than that for wild type factor XIa or the other chimeras. This is due to the markedly increased $K_m$ for the reaction with factor XIa/PKA3 compared with the other proteins (12.7 μM and 0.11–0.37 μM, respectively) as would be expected with the loss of a factor IX binding site. The $K_m$ values for the reactions with factor XIa/PKA3 and kallikrein should be considered approximations, as the actual $K_m$ exceeded achievable factor IX concentrations.

**Effect of Monoclonal Antibodies Directed against the Factor XI Heavy Chain on Factor XI Activity in Plasma**—A panel of antibodies that bind to the factor XI heavy chain was evaluated for inhibitory activity in the factor XI clotting assay. All antibodies have suitable affinity for factor XI to immunoprecipitate the protein from plasma. The apple domain to which each antibody binds was determined using the monoclonal antibody as the primary detection IgG in Western immunoblots of a panel of wild type and chimeric proteins (Fig. 3). The antibodies did not recognize wild type prekallikrein (data not shown). The effects of the antibodies on factor XI activity in the clotting assay are shown in Fig. 4. Antibodies 2A12 and 11AE, which recognize sites on the A3 domain, were effective in interfering with factor XIa activity. IgGs directed against the A2 domain (1A6) and A4 domain (3G4) had little effect, even at concentrations 200-fold greater than factor XIa. It should be noted that antibody 2A12 does not recognize either factor XIa/PKA3 or FXI/PKA4 on the immunoblot. It is not known if part of the binding epitope is actually on the A4 domain, or if the antibody requires dimeric factor XI for binding (factor XI/PKA4 is...
monomeric).

Inhibition of Wild Type Factor XIa and Factor XIa/PKA3 by Aprotinin—Factor XIa/PKA3 is activated by factor XIa and cleaves a chromogenic substrate in a manner similar to that of wild type factor XI and the other factor XI/PK chimeras, suggesting that it is structurally similar to factor XI and that its decreased capacity to activate factor IX is due to the absence of a factor IX binding site. It is possible, however, that the protein may have a peculiar structure, unrelated to the factor IX binding site, which is responsible for the decreased coagulant activity. As a further test of structural similarities between wild type factor XIa and factor XIa/PKA3, experiments were performed to assess inhibition of the enzymes by the prototype Kunin inhibitor, aprotinin. Data are shown in Fig. 5. The progressively greater inhibition of wild type factor XIa cleavage of S-2366 with increasing concentrations of the inhibitor are mirrored in the study with factor XIa/PKA3, indicating that the conformation required for aprotinin mediated inhibition is present in both proteins.

**DISCUSSION**

The proteases of the coagulation cascade are comprised of noncatalytic portions that appear to confer factor specific functions on the molecules, and trypsin-like catalytic domains (1). Activation of a coagulation protease requires at least one proteolytic cleavage in the peptide chain between the catalytic and noncatalytic domains. The cleavage presumably results in conformational changes that expose catalytic and substrate binding sites. The active form of factor XI, factor XIa, is comprised of two 35-kDa catalytic light chains and two 45-kDa heavy chains (8). Previous work by Sinha and colleagues (9) and by van der Graaf et al. (11) have demonstrated that although the active site resides within the light chain, optimal activation of factor IX by factor XIa requires the heavy chain and calcium ions. Furthermore, a monoclonal antibody directed against the factor XI heavy chain was demonstrated to be a competitive inhibitor of factor IX activation (9).

The amino acid sequence and disulfide bond structure of factor XI and the related protease prekallikrein have been determined and show that the heavy chain of both proteins is comprised of four in tandem repeated structures called apple domains (3, 12). Indeed, the two proteins are so similar that they are very likely the result of a gene duplication event. Despite this high degree of homology, kallikrein has different substrate specificities compared with factor XI and is a poor activator of factor IX (21). Baglia and co-workers (13) prepared a panel of polypeptides representing sequences within the four apple domains of factor XI and prekallikrein. They demonstrated that a polypeptide representing Asn145 through Ala156 within the A2 domain of factor XI was a potent competitive inhibitor of factor IX activation by factor XIa, whereas a peptide representing the corresponding area of the prekallikrein A2 domain was a poor inhibitor, indicating the presence of a

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**TABLE II**

Kinetic parameters for the activation of factor IX by factor XIa, kallikrein, and activated factor XIa/PKA chimeras

| Enzyme                        | $K_a$ (µM) | $k_{cat}$ (min$^{-1}$) | $k_{cat}/K_a$ (min$^{-1}$·µM$^{-1}$) |
|-------------------------------|------------|------------------------|-------------------------------------|
| Plasma factor XIa             | 0.16       | 11.0                   | 69                                  |
| Recombinant factor XIa        | 0.37       | 12.0                   | 32                                  |
| Factor XIa/PKA1               | 0.14       | 3.6                    | 26                                  |
| Factor XIa/PKA2               | 0.25       | 7.8                    | 31                                  |
| Factor XIa/PKA3               | 12.70      | 8.9                    | 0.7                                 |
| Factor XIa/PKA4               | 0.11       | 3.4                    | 31                                  |
| Recombinant kallikrein        | >20.00     | ND                     | ND                                  |

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**Fig. 4.** Inhibition of activated factor XI coagulant activity by anti-human factor XI monoclonal antibodies. 0.5 µg/ml plasma-derived factor XIa was incubated at room temperature for 30 min with varying concentrations of murine monoclonal anti-human factor XI IgGs and then assayed for residual factor XIa activity with the coagulation assay described under “Experimental Procedures.” The monoclonal antibodies tested are 11AE (○), 2A12 (●), 1A6 (△), and 3G4 (●).

**Fig. 5.** Inhibition of factor XIa and factor XIa/PKA3 by aprotinin. Wild type recombinant factor XIa (○) or factor XIa/PKA3 (●) (0.3 nM final concentration) was added to 0.3 nM chromogenic substrate S-2366 and various concentrations of aprotinin. Residual factor XIa activity was determined by following the change in absorbance at 405 nm on a spectrophotometer.
factor IX binding site on the factor XI A2 domain. A similar strategy was employed to assign binding sites for high molecular weight kininogen and thrombin to the factor XI A1 domain (22, 23), a platelet binding site to the A3 domain (24), and a factor XII binding site to the A4 domain (25).

We have taken advantage of the structural homology between factor XI and prekallikrein to generate a panel of factor XI/PK chimeric proteins. The molecules are activated by factor XIXa, cleave a chromogenic substrate, and are active in plasma and purified protein coagulation assays (with the exception of factor XI/PKA3), indicating that they are structurally similar to wild type factor XI. Factor XIXa/PKA3, although deficient in coagulant activity, interacts with factor XIIa properly, appears to have an intact catalytic domain, and is recognized by a factor XIXa inhibitor (aprotinin) in a manner similar to wild type protein, indicating that severe structural abnormalities were not introduced by the substitution of the A3 domain. The set of factor XI/PK chimeric proteins and their wild type counterparts, therefore, are a useful tool for evaluating the functions of the factor XI apple domains.

The data obtained with these proteins clearly point to a factor IX binding site on the factor XI A3 domain. Factor XIXa/PKA3 has little activity in a factor XIa coagulation assay. The $K_m$ for activation of factor IX by factor XIXa/PKA3 chimera is more than 30-fold greater, whereas the $k_{cat}$ is similar, to the values obtained for wild type factor XIXa and factor XIXa/PKA1, PKA2, and PKA4. This is consistent with the loss of a substrate binding site rather than an alteration of the catalytic properties of an enzyme. The $K_m$ for the activation by factor XIXa/PKA3 is similar to the value reported by Sinha et al. (9) for factor IX activation by isolated factor XIXa light chain lending further credence to the hypothesis that the A3 domain is involved in factor IX binding (9, 11). Finally, two monoclonal antibodies that recognize epitopes on the A3 domain were potent inhibitors of factor IX activation by factor XIXa. The reasons for the disparity between our results and those of the previously published peptide inhibition studies are unclear. The studies with the chimeras do not rule out a site on the A2 domain contributing to the factor IX binding site. However, it is likely that if a binding site exists on factor XI A2, then a similar site is also present on prekallikrein A2, as the factor XI/XIXa/PKA2 chimera was at least as active as wild type factor XI in functional assays. It is conceivable that the factor IX binding site encompasses more than one apple domain. Herwald and co-workers (26) recently presented evidence that the binding site for high molecular weight kininogen on prekallikrein involves components of the A1 and A4 domains.

Factor XI is unique among coagulation proteases in that it is a dimer with two catalytic active sites. Interestingly, it has been noted that point mutations in the A4 domain are associated with congenital factor XI deficiency (27). Meijers and colleagues have demonstrated that the A4 domain is involved in proper formation of the homodimer and subsequent protein secretion in an in vitro mammalian expression system (19, 20). Our difficulty in expressing factor XI/PKA4 is consistent with this observation. The function of the fourth apple domain in prekallikrein appears to differ from that of factor XI, as prekallikrein circulates as a monomer. It is possible that binding sites for factor IX, high molecular weight kininogen, or one of the enzymes responsible for factor XI activation may involve sites on both polypeptides of the dimer. Attempts to create monomeric factor XI by mutating the cysteine residue involved in the interchain disulfide bond were unsuccessful, as noncovalent interactions between A4 domains are sufficient for dimer formation (19). Factor XI/PKA4 is probably monomeric but activates factor IX in a manner similar to wild type factor XI, suggesting that a single third apple domain is sufficient to bind factor IX. It is not known if wild type factor XIXa binds two molecules of factor IX simultaneously.

In conclusion, studies with a panel of recombinant factor XI/PK chimeric proteins indicate that a site or sites on the third apple domain of factor XI are required for proper binding to factor IX. Further work will be required to identify the specific amino acid residues involved in the binding site.

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