Factor XIa Dimer in the Activation of Factor IX†

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ABSTRACT: Factor XI, unlike other coagulation proteins, is a homodimer of two identical subunits linked by a single disulfide bond formed by Cys321. The present study was undertaken to understand the physiological significance of the dimeric nature of factor XI. We have expressed a mutant FXI/G326C in which the Gly326 residue of factor XI has been mutated to Cys326, reasoning that Cys321 would form an intrachain disulfide bond with Cys326 as in prekallikrein, a plasma protein that exists as a monomer even with 58% amino acid sequence identity and a domain structure very similar to factor XI. No free thiol could be detected in the expressed protein, and it migrated as a monomer on nonreduced SDS−PAGE. In physiological buffer, however, the protein was found to exist in a state of monomer−dimer equilibrium as assessed by gel-filtration chromatography and ultracentrifugation studies ($K_d \sim 36$ nM). Functional studies revealed that FXI/G326C was indistinguishable from plasma factor XI in a plasma-clotting assay and in a factor IX activation assay both in the presence and absence of activated platelets even at concentrations at which less than 5% of the mutant exists as dimers. We conclude that, for optimal function in the presence of activated platelets, a preformed dimer of factor XI is not required.

Human factor XI (FXI),[1] a serine protease precursor of 160 kDa, has an essential function in the process of blood coagulation (*1*, *2*). It circulates in plasma in complex with high molecular weight kininogen (HK) at a concentration of 30 nM (*3*) and is activated by either FXIIa or thrombin (*1*, *2*). The activated form of FXI (FXIa) activates FIX and thereby initiates the intrinsic or consolidation phase of coagulation. FXI is a homodimer of two identical subunits held together by a single disulfide bond (*4*). The amino acid composition of FXI is 58% identical with another plasma protein, prekallikrein (PK), and the N-terminal regions of both proteins consist of four homologous domains called Apple domains (designated A1, A2, A3, and A4), whereas the catalytic triad is located within the C-terminal region of the molecules (*4*−*7*). PK unlike FXI, however, exists as a monomer in plasma. The Cys residue at position 321 that is involved in the formation of the interchain disulfide linkage in FXI forms an intrachain linkage with Cys326 in the A4 domain of PK. In FXI, the residue at position 326 is a glycine. The dimeric feature is unique to FXI and distinguishes it from other coagulation factors such as FIX, FX, and prothrombin. The physiological significance of this dimeric nature of FXI is not clear. Meijers et al. (*8*) postulated that FXI needs to be a dimer for proper secretion based on their studies on the mutant FXI/F283L (type-III mutation) that causes FXI deficiency. Recently, Gailani et al. (*9*) hypothesized that the dimeric conformation of FXI is essential for optimal activation of FIX on the platelet surface. The authors made this conclusion based on their observations that, in clotting assays using phospholipids as the surface FXI/PKA4, a monomeric version of FXI is very similar to a dimeric version of FXI/PKA4 (FXI/PKA4G326) and to plasma FXI, whereas the activity of the same monomeric protein is greatly reduced when activated platelets instead of purified phospholipids provide the surface.

The purpose of the present paper is to determine whether a stable, preformed, FXIa dimer is required for optimal rates of FIX activation in solution or on the physiologically relevant surface provided by activated platelets. To accomplish this aim, we describe the structure and function of a mutant FXI/G326C in which the Gly residue at position 326 of FXI was altered to Cys as in PK. We demonstrate that the two subunits of the mutant are not disulfide-linked and that this molecule, unlike either PK or FXI, exists in monomer−dimer equilibrium such that between 12 and 50% of the protein exists as dimer at 10 nM. In both clotting and FIX activation assays, the functional activity of the mutant was almost identical to that of plasma FXI in the absence or presence of activated platelets even at subnanomolar concentrations at which it predominantly exists as a monomer. Therefore, the present data suggest either that monomeric FXI/FXIa can function normally on the platelet surface or

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[1] Abbreviations: FXI, factor XI; HK, high molecular weight kininogen; PK, prekallikrein; A1, A2, A3, and A4, Apple domains; TBS, Tris buffer saline; BSA, bovine serum albumin.

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that dimerization of FXI/G326C is promoted by binding to platelets.

EXPERIMENTAL PROCEDURES

Pooled normal plasma and FXI-deficient plasma were from George King Biomedical (Overland Park, KS). Human FXI, human FXIa, FXIIa, FIX, FX, and FIIa were purchased from Enzyme Research Laboratories (South Bend, IN). Recombinant FVIII was generously provided as a gift from Baxter/Hyland (Glendale, CA). Chromogenic substrates S-2366 (L-pyroglutamyl-L-prolyl-L-arginine-*p*-nitroaniline) and S-2765 (*N*-α-benzoxycarbonyl-D-arginyl-glycyl-L-arginine-*p*-nitroaniline) were from DiaPharma (Westchester, OH). The thrombin receptor hexapeptide, SFLLRN-amide, was synthesized using (9-fluorenyl)methoxy-carbonyl (FMOC) chemistry on an Applied Biosystems 430A Synthesizer and reverse-phase HPLC purified to >99.9% homogeneity.

Expression, Purification, and Characterization of FXI/G326C. Substitution of the base G with T at position 1073 of human FXI cDNA (kindly provided by Drs. Kazuo Fujikawa, Dominic Chung, and Earl Davie, Department of Biochemistry, University of Washington, Seattle, WA) in the expression vector pJVCMV was made using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). This substitution replaces the glycine residue at amino acid position 326 with cysteine. Human embryonic kidney 293 (HEK293) cells were cotransfected with 2 μg of plasmid vector RSVneo containing a gene that confers resistance to neomycin and 40 μg of the mammalian expression vector pJVCMV containing the cDNA of either the wild type or the mutant FXI using lipofectamine (GIBCO, Carlsbad, CA). Cells were grown in DMEM with 10% fetal bovine serum for 48 h and then switched to the same medium supplemented with 500 μg/mL of G418. Stable clones expressing FXI protein as assessed by ELISA using a polyclonal goat anti-FXI antibody (Affinity Biologicals, Hamilton, Ontario, Canada) were expanded in roller bottles (NUNC, Naperville, IL). After confluence was reached, cells were washed and the media was replaced by serum-free Cellgro complete media (Mediatech, Herndon, VA) supplemented with soybean trypsin inhibitor (SBTI, 10 μg/mL) and lima bean trypsin inhibitor (LBTI, 10 μg/mL). Conditioned media was removed every 48 h and kept frozen after supplementing with benzamidine (5 mM) until it was ready to be purified.

Wild-type FXI (wtFXI) and FXI/G326C from the cell supernatant were purified using monoclonal antibody 5F7 according to the protocol described earlier (*10*). Briefly, the cell supernatant was passed through the affinity column at a rate of 0.5 mL/min. The column was washed with 10 column volumes of Tris buffer saline (TBS) followed by elution of the protein using 2 M KSCN. The eluted protein was dialyzed and concentrated using Centricon Plus-20, 30 000 NMWL (Millipore Corporation, Bedford, MA). The protein concentration was estimated using the BCA assay (Pierce, Rockford, IL). The purified proteins were size-fractionated on a 7.5% SDS−PAGE under nonreducing condition and stained using GelCode blue stain (Pierce, Rockford, IL).

Quantitation of Free Thiols in FXI/G326C. The Thiol and Sulfide Quantitation Kit from Molecular Probes (Eugene, OR) was used for estimation of free thiols in FXI/G326C. The assay is ~100-fold more sensitive than Ellman's method and is based on a report by Singh et al. (*11*). In this procedure, thiols reduce a disulfide-inhibited papain-releasing active papain, which is then detected using the chromogenic substrate *N*-benzoyl-L-arginine-*p*-nitroanilide (L-BAPNA). Briefly, 15 μL of BSA (14 μM), plasma FXI (6 μM), or FXI/G326C (6 μM) in triplicates were added to the wells of a microtiter plate containing 15 μL of cystamine that permits the detection of poorly accessible thiols on proteins. A total of 100 μL of disulfide-inhibited papain (papain-$SSCH_3$, 0.6 mg/mL) was added to each well and incubated for 1 h. The liberated enzyme was then allowed to react with 100 μL of L-BAPNA (4.9 mM) for another 1 h, and liberated *p*-nitroaniline was read at 405 nm using a plate reader. Free thiol in each sample was estimated from the standard curve made using known concentrations of L-cysteine.

Comparison of Plasma FXI and FXI/G326C in ELISA Assays. ELISA assays were performed using two different capture antibodies: (1) goat anti-FXI polyclonal antibody and (2) a monoclonal antibody directed against the A4 domain of FXI (a gift from Professor Werner Muller-Esterl, Johann Wolfgang Goethe University, Germany). The detecting antibody was peroxidase-conjugated goat anti-FXI polyclonal antibody from Enzyme Research Laboratories. In brief, wells of a microtiter plate were coated with either the polyclonal or the monoclonal antibody used as the capture antibody followed by blocking using bovine serum albumin (BSA, 2 mg/mL). Different concentrations of FXI or FXI/G326C were then added to the wells and incubated for 2 h at room temperature or overnight at 4 °C. The wells were thoroughly washed with TBS/BSA and then incubated with peroxidase-conjugated goat anti-FXI polyclonal antibody and incubated for 1.5 h at room temperature. At the end of the incubation, the wells were washed and the bound second antibody was detected using orthophenylenediamine as the substrate.

Size-Exclusion Chromatography. Size-fractionation was done on a Superose-12 gel-filtration column (Amersham Pharmacia Biotech, Piscataway, NJ) fitted to a BioLogic FPLC workstation (BioRad, Richmond, CA) as described earlier (*12*). The column was equilibrated with TBS (50 nM Tris at pH 7.4 containing 150 mM NaCl) before loading. Each protein in 200 μL of the equilibration buffer was loaded onto the column and chromatographed at a flow rate of 500 μL/min, and 500 μL fractions of the eluate was collected. Loading concentration of the proteins ranged from 32 to 500 μg/mL. Elution profiles and retention times were obtained from the optical density values at 280 nm.

Analytical Ultracentrifugation. Equilibrium sedimentation experiments were carried out in a Beckman XLA ultracentrifuge utilizing six-sector cells in an eight-cell rotor. Plasma FXI, PK, and FXI/G326C were extensively dialyzed versus TBS using Slide-A-Lyzer (Pierce). The sample cell was loaded with 100 μL of either FXI, PK, or FXI/G326C and the reference cell with TBS. The centrifuge was set at 25 000 rpm and run for 20−24 h. Molar extinction values used for FXI subunit and PK were 104 800 and 101 790, respectively (*13*, *14*). Partial specific volumes ($\bar{v}$) were calculated from the sequences using "Sednterp" (*15*) and adjusted (*16*) for glycosylation levels of 5% for FXI and FXI/G326C and 15.5% for PK (*7*).

Activation of FXI and FXI/G326C by FXIIa. FXI or FXI/G326C was incubated at 37 °C with FXIIa at a molar ratio of 20:1. At specified time intervals, aliquots were removed into the wells of a microtiter plate containing 5-fold molar excess of corn trypsin inhibitor to quench FXIIa, and generated FXIa or FXIa/G326C was estimated by its ability to hydrolyze the small peptidyl substrate pyroGlu-Pro-Arg-*p*-nitroanilide (S-2366) (*2*).

Activation of FXI and FXI/G326C by Thrombin and Dextran Sulfate. FXI or FXI/G326C (33 nM) was incubated at 37 °C with thrombin (1 nM) and dextran sulfate (1 μg/mL), and at specified intervals, aliquots were removed into wells of a microtiter plate containing hirudin to inactivate thrombin and polybrene to neutralize dextran sulfate. The generated enzyme was estimated as described above.

Hydrolysis of S-2366 by FXIa and FXIa/G326C. FXIa or FXIa/G326C (at a fixed concentration) was added to increasing concentrations (50−1000 μM) of S-2366 in the wells of a microtiter plate, and the change in absorbance at 405 nm was measured using a plate reader in the kinetic mode.

Comparison of FXI and FXI/G326C in Activated Plasma Thromboplastin Time (aPTT) Assay: (A) aPTT Assay Using Commercial aPTT Reagent. The specific activities of plasma FXI and FXI/G326C were determined using FXI-deficient plasma and commercial aPTT reagent (Sigma). Standard curves were generated using normal pooled plasma.

(B) aPTT Assay in the Presence of Cephalin and Kaolin. In this assay, cephalin and kaolin replaced the commercial aPTT reagent. FXI-deficient plasma was the source of the substrate as described in the previous procedure.

(C) aPTT Assay in the Presence of Activated Platelets and Kaolin. This assay was similar to the previous one except that the phospholipid source was activated platelets instead of cephalin.

When the proteins were used in their activated forms, the procedure was the same except that kaolin was omitted from the reaction mixture.

Activation of FIX by FXIa and FXIa/G326C. FIX was incubated with FXIa or FXIa/G326C at a substrate/enzyme ratio of 500:1 at 37 °C in TBS containing 5 mM $CaCl_2$, and aliquots were removed at specified intervals into SDS-containing buffer and size-fractionated on SDS−PAGE. The protein bands were visualized with gel code blue.

Activation of FIX by FXIa and FXIa/G326C in the Presence of Activated Platelets. The activation mixture in this assay contained FIX (1 μM), FXIa (4 nM), HK (50 mM), $ZnCl_2$ (25 μM), $CaCl_2$ (5 mM), and activated platelets (10^8 mL^{-1}) in TBS/BSA buffer. Platelets were activated by incubation with 5 μM SFLLRN amide at 37 °C for 5 min. As described in the previous paragraph, aliquots from the reaction mixture incubating at 37 °C were removed at specified intervals into SDS-containing buffer and fractionated on SDS−PAGE. Protein bands were visualized by Western blot analysis using a polyclonal antibody against human FIX (obtained from Enzyme Research Laboratories) as the primary antibody and peroxidase-conjugated goat anti-goat antibody (obtained from Sigma) as the secondary antibody. Blots were developed using an enhanced chemiluminescence Western blotting detection kit (Amersham Pharmacia Biotech).

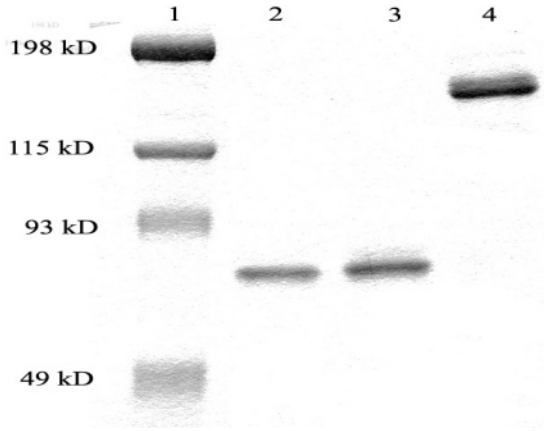

FIGURE 1: SDS−PAGE of FXI/G326C. Plasma FXI (lane 4), FXI/C321S (lane 2), and FXI/G326C (lane 3) were fractionated on a 7.5% SDS−polyacrylamide gel under nonreducing conditions, and the protein bands were visualized using Gel code blue. Protein markers are shown in lane 1.

RESULTS

Characterization of FXI/G326C. Size fractionation of FXI/G326C under nonreducing SDS−PAGE (lane 3 of Figure 1) clearly demonstrated that unlike plasma FXI (lane 4 of Figure 1) the two subunits of FXI/G326C are not disulfide-linked. Recombinant FXI/C321S (lane 2 of Figure 1) in which the Cys321 residue responsible for formation of the interchain disulfide bond in plasma FXI was mutated to Ser has been shown for comparison.

Free Thiol(s) in FXI/G326C. Using the enzyme-amplified assay of free thiols described in the Experimental Procedures, a value of 1 ± 0.15 mol of thiol/mol of BSA was obtained, while none could be detected on either FXI or FXI/G326C. Because cystamine that permits detection of poorly accessible thiols was used in the reaction mixture, it is reasonable to conclude that neither Cys321 nor Cys326 exists as a free thiol in FXI/G326C.

FXI/G326C was tested in an ELISA assay using both a polyclonal antibody and a monoclonal antibody raised against plasma FXI. K_d values of 8.4 and 4.5 nM were obtained for plasma FXI and FXI/G326C, respectively, when the monoclonal antibody directed against the A4 domain of FXI was used in the assay, whereas they were 0.4 and 0.5 nM, respectively, when a polyclonal antibody was used. Similar affinities of the two proteins toward the antibodies demonstrated that the antigenic determinants were not disrupted in the mutant.

Size-Exclusion Chromatography. Elution profiles and retention times of plasma FXI, PK, and FXI/G326C fractionated on a Superose 12 gel-filtration column are shown in Figure 2. It is interesting to note that, while retention times of plasma FXI, a dimer, and PK, a monomer, remained almost the same (10.59 ± 0.02 and 11.99 ± 0.03 min, respectively) over the range of concentrations from 32 to 500 μg/mL, FXI/G326C was concentration-dependent, with 500 μg/mL being the highest loading concentration at 10.94 min and 32 μg/mL being the lowest concentration at 11.67 min. These results strongly suggest a state of monomer−dimer equilibrium for FXI/G326C.

Analytical Ultracentrifugation of FXI/G326C. Plasma FXI, PK, and FXI/G326C were subjected to sedimentation equilibrium experiments to measure the equilibrium concentration

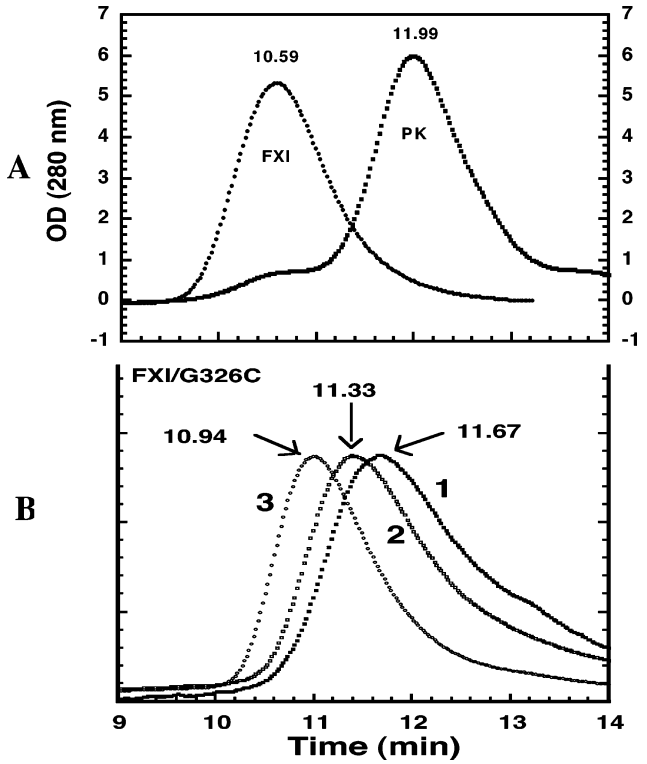

FIGURE 2: Size-exclusion chromatography. Plasma FXI, PK, and FXI/G326C at different loading concentrations were size-fractionated on a superose-12 gel-filtration column, and the retention time of the protein at each concentration was recorded. Retention times of FXI and PK were concentration-independent and are shown in A. (B) Retention times of FXI/G326C at (1) 32 μg/mL, (2) 64 μg/mL, and (3) 500 μg/mL of loading concentrations. The peaks in B are normalized for better visualization.

distribution of the macromolecules, and the results are shown in Figure 3. Each panel shows radial absorbance profiles (points) and fits (lines) to equations describing equilibrium sedimentation (*17*). Molecular weights from curve fits were consistent with 100% dimer for FXI (MW = 146 420 ± 2019, $\bar{\nu}$ = 0.735), a monomer for PK (MW = 70 848 ± 4045, $\bar{\nu}$ = 0.722) and a monomer−dimer equilibrium for FXI/G326C [MW = 72 626 (sequence-fixed), $\bar{\nu}$ = 0.735, pK_d = 7.44 ± 0.58 (pK_d is the negative base 10 logarithm of the monomer−dimer dissociation constant in molar units)]. Thus, sedimentation equilibrium data obtained for FXI, PK, and FXI/G326C were consistent with the gel-filtration data described in the previous section. A calculated curve showing the change in dimer fraction of FXI/G326C with the log concentration is shown in Figure 4. According to this result, only ~5% of the molecules exist as dimers at a concentration of 1 nM, whereas ~50% exist as dimers at the plasma concentration of FXI (30 nM), and the K_d for dimer dissociation is 36.3 ± 0.026 nM.

Activation of FXI and FXI/G326C by FXIIa and Thrombin. Generation of FXIa and FXIa/G326C by FXIIa and thrombin in the presence of dextran sulfate was measured by monitoring the rate of hydrolysis of the small peptidyl substrate S-2366 and is shown in Figure 5. Rates of activation of wild-type FXI and FXI/G326C were comparable in both of these assays. In the absence of dextran sulfate, thrombin activated neither FXI nor FXI/G326C.

Hydrolysis of Peptidyl Substrate S-2366 by FXIa and FXIa/G326C. The capacities of the two proteases to cleave the chromogenic substrate S-2366 were tested and compared in Figure 6. Kinetic parameters obtained from the double reciprocal plots were very similar (V_{max} = 187 and 161 mOD/min and K_m = 357 and 384 μM, respectively, for FXIa and FXIa/G326C) suggesting that mutating the Gly residue at position 326 to Cys in FXI did not alter the active-site architecture in the catalytic domain.

Comparison of FXI and FXI/G326C in Clotting Assays. Specific activities of FXI and FXI/G326C (assuming 5 μg/mL or 33 nM as 1 unit of FXI) obtained by the three different procedures were found to be very similar both as a zymogen and as activated proteins and irrespective of the surface (phospholipids versus platelets). The values obtained using cephalin versus activated platelets at two different concentrations of the proteins are compared and shown in Table 1. It is apparent that, even at a concentration of 0.025 μg/mL (or ~0.16 nM) when less than 5% of FXI/G326C exist as dimers, no significant difference in clotting activity was observed when activated platelets were used as the surface in the clotting assay instead of cephalin.

Activation of FIX by FXIa and FXIa/G326C. Cleavages at two sites, one at R145−A146 and the other at R180−V181, in FIX (56 kDa) by FXIa results in the generation of FIXaβ (45 kDa) with the release of the activation peptide of 11 kDa. Formation of FIXaβ could be demonstrated by fractionation on SDS−PAGE. The bands were visualized by staining when the reaction was carried out in the absence of platelets, and they were detected by Western blot analysis when platelets were present in the reaction mixture. Rates of formation of FIXaβ by plasma FXIa and by FXIa/G326C were almost identical both in the absence and presence of platelets as demonstrated in Figures 7 and 8, respectively.

DISCUSSION

Factor XI has an essential role in the consolidation phase of blood coagulation (*18*), and it is unique among coagulation proteins being a homodimer unlike FIX, FX, or prothrombin. The physiologic significance of the homodimeric structure of FXI is not clearly understood. Studies on the mutant FXI/F283L (type-III deficiency) expressed in BHK cells revealed that compared to wtFXI there was accumulation of the monomeric form of the protein within the cell (*8*), and this led the authors to hypothesize that for proper secretion the dimeric form of FXI is required. Several other mutants in addition to FXI/F283L have so far been reported to cause a reduction in the amount of FXI secreted from cells (*19*−*22*), and it has been hypothesized that, as in the case of FXI/F283L, the reduction in the protein level is the result of inadequate dimer formation. This hypothesis was further supported by the fact that deficiencies in FXI have been found predominantly to be the result of a lack of protein rather than the presence of a functionally impaired protein (*23*, *24*).

Gailani et al. (*9*) based on their studies on monomeric and dimeric forms of FXI/PK chimeras hypothesized that FXI needs to be a dimer for its proper function in the presence of activated platelets. In their study, a recombinant monomeric FXI molecule, FXI/PKA4, was constructed by replacing the A4 domain of FXI with that of PK. The functional properties of this protein were compared with those of a dimeric form of FXI/PKA4 (FXI/PKA4−Gly326) and wild-

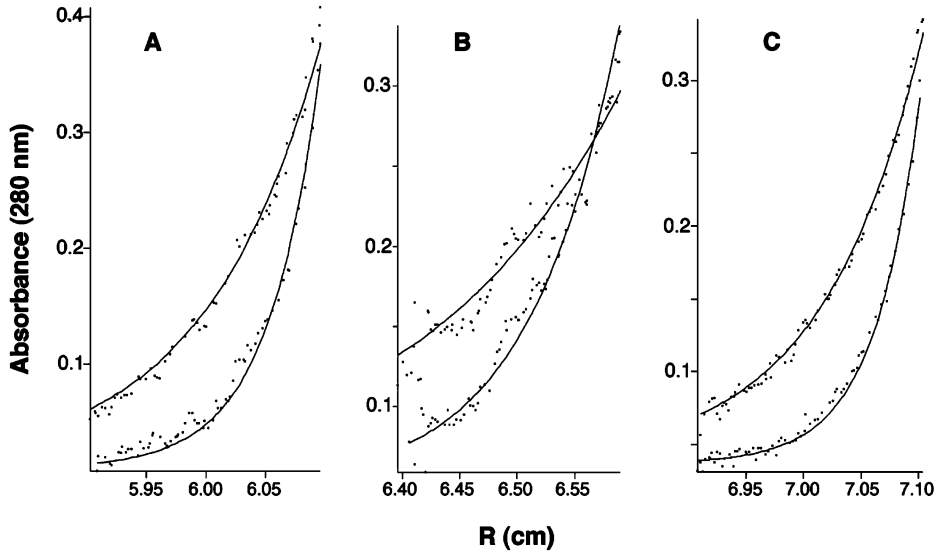

FIGURE 3: Sedimentation equilibrium analysis by analytical ultracentrifugation (AU). The optical density of Plasma FXI (A), PK (B), or FXI/G326C (C) at 280 nm along the radius of the centrifuge cell was measured after the proteins were subjected to centrifugation to equilibrium (14 h) at 10 (upper curve) and 15 (lower curve) KRPM using the Beckman XLA centrifuge. Each panel shows radial absorbance profiles (points) and fits (lines) to equations describing equilibrium sedimentation (see the Experimental Procedures and Results for details).

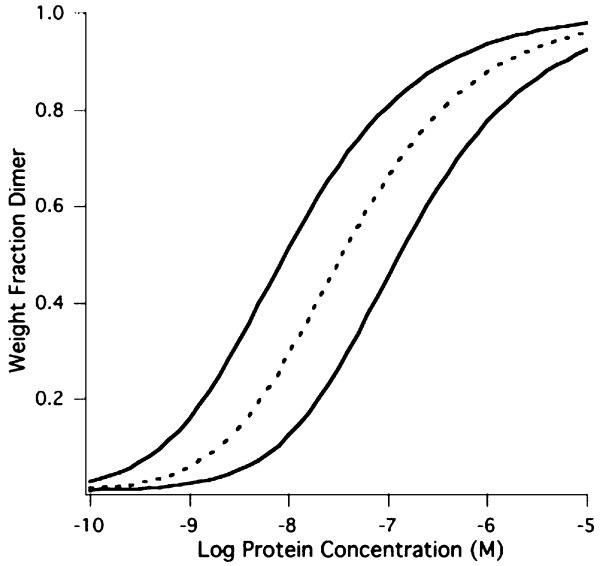

FIGURE 4: Weight fraction of FXI/G326C dimer species as a function of the protein concentration. The curves were calculated from the mass action expression for a monomer−dimer equilibrium using pK_d = 8.02 (upper), 7.44 (center), and 6.86 (lower) representing the average and 95% confidence limits of pK_d consistent with analytical ultracentrifuge data fitting.

type FXI. The monomeric and dimeric proteins were shown to have similar functional properties in solution. However, in the presence of activated platelets, monomeric FXIa/PKA4 was a poor activator of FIX, while its dimeric counterpart FXIa/PKA4−Gly326 and wild-type FXIa activated FIX normally. From these results, the authors reasoned that because FXIa binds to FIX and platelets both through its A3 domain (*25*, *26*) the monomer is unable to interact both with FIX and platelets at the same time. It was hypothesized that the dimeric conformation of FXIa is essential for optimal activation of FIX on the platelet surface. A model was proposed in which one subunit of FXIa is involved in binding to the platelet surface, while the other binds to and activates FIX (*9*).

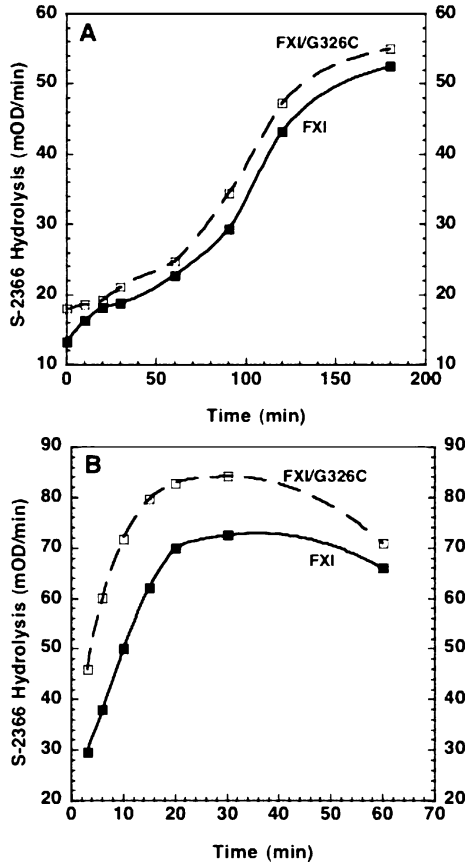

FIGURE 5: Activation of FXI and FXI/G326C by FXIIa (A) and thrombin (B). FXI or FXI/G326C was incubated with FXIIa at a molar ratio of 20:1 or with thrombin and dextran sulfate (5 μg/mL) at a substrate/enzyme ratio of 40:1. At specified intervals, aliquots were removed and analyzed for FXIa or FXIa/G326C generation using the small peptidyl substrate S-2366.

The question that we wished to address is whether a preformed covalent FXIa dimer is or is not required for normal catalysis of FIX activation in the presence of activated platelets. We have expressed a mutant FXI/G326C in which the Gly residue at position 326 of FXI was replaced with a

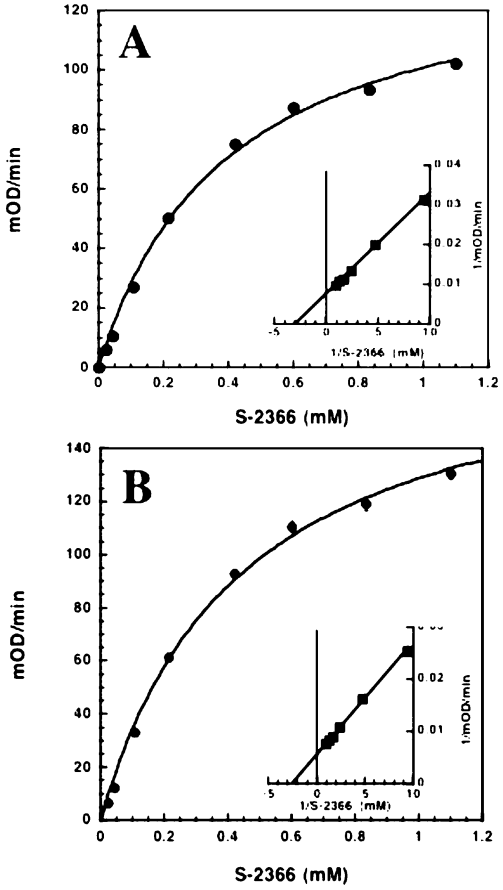

FIGURE 6: Hydrolysis of S-2366 by FXIa and FXI/G326C. FXIa or FXIa/G326C at a fixed concentration was added to increasing concentrations (50−1000 μM) of S2366, and the rate of hydrolysis was measured as described in the Experimental Procedures.

Table 1: Comparison of Plasma FXI and FXI/G326C in Clotting Assay

		specific activity (units/mg)			
		zymogen		activated protein	
protein	surface	1.6 nM	0.16 nM	1.6 nM	0.16 nM
plasma FXI	phospholipid	268	236	198	185
	platelets	200	196	211	200
FXI/G326C	phospholipid	240	240	225	290
	platelets	232	208	198	192

Cys to examine whether Cys321 can be forced to form an intrachain disulfide link with Cys326 as in PK. No free thiol could be detected in the expressed protein, and fractionation on SDS−PAGE demonstrated that there is no interchain disulfide linkage between the two subunits of the molecule (Figure 1). It is therefore reasonable to assume that Cys321 and Cys326 formed an intrachain disulfide bond in FXI/G326C as in PK. The affinity of the mutant for a monoclonal antibody specific for the A4 domain was very similar to that of plasma FXI, although the mutation G326C is located in the A4 domain, from which it can be inferred that the mutant protein is conformationally unperturbed.

The behavior of this mutant by gel filtration over a superose-12 column was distinctly different from that of FXI or PK. Whereas the retention time of either FXI or PK remained almost the same over a loading concentration range of 32−500 μg/mL, the retention time of FXI/G326C was found to be concentration-dependent. At the highest concentration of 500 μg/mL, the retention time of the mutant was closer to that of dimeric FXI, whereas at the lowest loading concentration studied, it was closer to that of monomeric PK. These results suggested a monomer−dimer equilibrium state for the mutant. This possibility was further investigated by studying macromolecular distributions in sedimentation equilibrium experiments using analytical ultracentrifugation. Both plasma FXI and PK were run for comparison. In this study, FXI was found to exist as a dimer and PK was found to exist as a monomer as expected, whereas FXI/G326C was found to exist as a mixture of monomer and dimer, confirming the results of our gel-filtration data. Analysis of the analytical ultracentrifugation (AU) data indicated that 12−50% of the protein exists as a dimer at 10 nM subunit concentration and 2−15% at a concentration of 1 nM. The K_d for dimer dissociation of FXI/G326C is 36.3 ± 0.026 nM.

Comparable functional activities of FXIa/G326C and plasma FXIa toward the small peptidyl substrate S-2366 demonstrated that mutation of Gly326 to Cys did not affect the active site within the catalytic domain. Clotting assays were performed over a wide range of concentrations (0.16−3

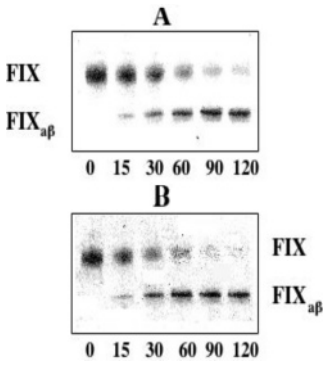

FIGURE 7: Activation of FIX by (A) FXIa and (B) FXIa/G326C in solution. FIX was incubated with FXIa or FXIa/G326C at a ratio of 1:500 at 37 °C in TBS/BSA supplemented with 5 mM $CaCl_2$, and at specified intervals, indicated (time in minutes shown across the bottom of each panel) aliquots were removed into SDS-containing buffer followed by size fractionation on SDS−PAGE. Protein bands were then visualized using Gel code blue.

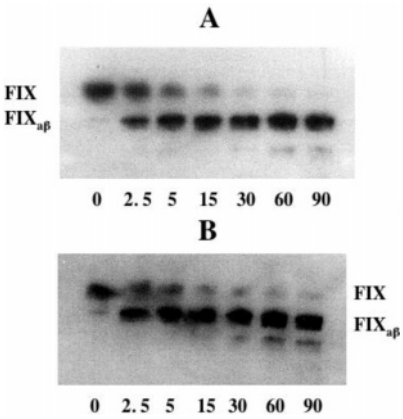

FIGURE 8: Activation of FIX by (A) FXIa and (B) FXIa/G326C in the presence of activated platelets. FIX was incubated with FXIa or FXIa/G326C at 37 °C in TBS/BSA supplemented with $CaCl_2$ (5 mM), $ZnCl_2$ (25 μM), high molecular weight kininogen (50 nM), and activated platelets (1×10^8 mL^{-1}), and as in Figure 7, aliquots were removed at indicated intervals (time in minutes shown across the bottom of each panel) into SDS buffer and fractionated on SDS−PAGE. The bands were visualized by Western blot analysis.

nM, assuming a M_r of 160 kDa) of the mutant, and its specific activity was found to remain unchanged at all concentrations tested even though from AU data FXI/G326C exists >99% as a monomer at 0.16 nM. The same observation was made when activated platelets were the source of phospholipids in the assay. This is in sharp contrast with the data reported for FXI/PKA4, a monomer that was almost inactive when the clotting assay was performed in the presence of activated platelets (*9*).

Activation of FIX by FXIa/G326C was carried out both in the presence and absence of platelets (Figures 7 and 8). As observed in the clotting assay, plasma FXIa and FXIa/G326C activated FIX almost identically both in the presence and absence of platelets. This observation also is in sharp contrast to what has been reported for FXI/PKA4 (*9*).

From the present studies in which it has been found that FXIa/G326C functions in the presence of activated platelets similarly to normal dimeric plasma FXIa and not like monomeric FXIa/PKA4, it can be concluded that a preformed, stable, covalent dimeric form of FXIa is not required for normal catalysis of FIX activation on the platelet surface. The present studies show that, even at concentrations at which FXIa/G326C exists predominantly as a monomer, its functional properties both in solution and in the presence of activated platelets are very similar to those of homodimeric plasma FXIa. What is not clear from the present study is the structural state of the platelet-bound mutant (FXIa/G326C) protein, i.e., monomeric versus dimeric. We are currently in the process of examining this question. If we find that the FXIa/G326C protein is monomeric on the platelet surface, then the hypothesis that dimeric FXIa is required for optimal rates of FXIa-catalyzed FIX activation (*9*) would require revision.

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