Structure/Function Analyses of Recombinant Variants of Human Factor Xa: Factor Xa Incorporation into Prothrombinase on the Thrombin-Activated Platelet Surface Is Not Mimicked by Synthetic Phospholipid Vesicles†

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ABSTRACT: This report describes the expression, purification, and characterization of a series of recombinant factor Xa variants bearing aspartate substitutions for each of the glutamate residues which normally undergo γ-carboxylation. Factor X was expressed in human embryonic kidney cells and purified from conditioned media by immunoaffinity and hydroxylapatite chromatography. Factor X was activated with Russell's viper venom factor X activator, and single-chain unactivated factor X was removed from activated factor X by size-exclusion chromatography. Recombinant wild-type factor Xa had normal activity in a clotting assay, and mutants with aspartate substitutions for glas residues 16, 26, and 29 had no detectable clotting activity. In purified component assays, these gla variants had essentially no detectable activity in the prothrombinase complex assembled on synthetic phospholipid vesicles but had significant activity when the prothrombinase was assembled on thrombin-activated platelets. In addition, the gla 32 variant had normal activity in the platelet prothrombinase but diminished activity in prothrombinase assembled on synthetic PSPC vesicles. These differences were not accounted for by the total phospholipid composition of the thrombin-activated platelet membrane. We have produced fully active recombinant human factor Xa and demonstrated that gla residues 16, 26, and 29 are critical for normal activity of factor Xa. More importantly, this study provides an extensive characterization of macromolecular enzyme complex formation with gla variants of a vitamin K-dependent coagulation protein and provides evidence that prothrombinase complex assembly on thrombin-activated platelets is not equivalent to assembly on synthetic phospholipid vesicles. The data suggest that thrombin-activated platelets possess some element(s) (other than 30% phosphatidyl serine or factor Va), presumably either protein or phospholipid, that serves as a component of the factor Xa binding site.

Activated factor X (fXa)[1] is a vitaminK-dependent blood coagulation serine protease. Perturbed endothelial cells and activated platelets present at sites of vascular injury provide an activated phospholipid membrane surface that supports the binding of fXa and its protein cofactor, factor Va (fVa), in the presence of calcium. In this macromolecular complex, termed prothrombinase, fXa is the physiologically important enzyme that activates prothrombin. The amino terminal domain of all vitamin K-dependent coagulation proteins contains 9−12 glutamic acid residues that undergo a characteristic posttranslational carboxylation at the γ-carbon; this carboxylation is the hallmark of these proteins. These γ-carboxyglutamic acid (or gla) residues are necessary for calcium binding which induces a conformational change in the gla domain required for phospholipid binding (*1*). Phospholipid binding is essential for normal activity of the vitamin K-dependent proteins in coagulation. The crystal structure of calcium loaded bovine prothrombin fragment 1 (the amino terminal 156 residues of prothrombin which

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[1] The abbreviations used are the following: fX, factor X; fXa, activated fX; r-fX, recombinant fX; r-fXa, recombinant fXa; PD-fX, plasma-derived fX; WT r-fX, wild-type recombinant fX; fV, factor V; fVa, activated factor V; gla, γ-carboxyglutamic acid; fIX, factor IX; fVII, factor VII; CHO, Chinese hamster ovary cells; HEK293, human embryonic kidney cells; fIIa, thrombin; fII, prothrombin; ATIII, antithrombin III; RVV-Xa, fX activator from Russell's viper venom; DAPA, dansylarginine N-(3-ethyl-1,5-pentanediyl)amide; TLCK, Na-tosyl-Lys chloromethyl ketone; TPCK, Na-tosyl-Phe chloromethyl ketone; PPACK, D-Phe-Phe-Arg chloromethyl ketone; DEGR, 1,5-dansyl-Glu-Gly-Arg chloromethyl ketone; PMSF, (phenylmethyl)-sulfonyl fluoride; CMV, cytomegalovirus.

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contain the gla domain) predicts that the carboxylate oxygens of several of the gla residues are completely coordinated with calcium ions, while the carboxylate oxygens of others may be available for electrostatic interactions at the protein surface with negatively charged phospholipid headgroups via calcium ions (*2*, *3*). In addition, certain residues within the gla domain have been shown to affect the affinity of the vitamin K-dependent proteins for phospholipid membrane; these include residues equivalent to 10, 32, and 33 in fX (*4*). These residues cluster at the surface of the molecule (*5*) and form a pore that, in the presence of metal ions, may allow the insertion of a single phospholipid headgroup (*4*).

Several factors have conspired to make the expression of fX problematic, so that, in contrast to protein C, prothrombin, and factor IX (fIX), there are few published reports of the expression of human fX. The biosynthesis of fX requires a complex series of posttranslational steps that proceed more efficiently in hepatocytes than in most in vitro expression systems. Unlike normal hepatocytes, mammalian expression systems secrete recombinant proteins that are heterogeneous with respect to carboxylation (*6*−*9*). Calcium dependent antibodies to factor VII (fVII) and fIX (*10*, *11*) have greatly facilitated the separation of fully carboxylated recombinant proteins from those that are under- or uncarboxylated (and that have reduced activity), but such antibodies are not widely available for fX. Second, in the biosynthesis of vitamin K-dependent coagulation proteins, removal of the propeptide is an essential step (*12*, *13*) and does not occur accurately and efficiently in recombinant expression systems for proteins such as fX that lack paired dibasic residues at the cleavage site (*14*, *15*). Third, for both fX and protein C, complete processing also includes the removal of internal tribasic or dibasic peptides. Published reports of synthesis of r-fX (most of these are in Chinese hamster ovary cells) note contamination with single-chain fX, suggesting that the tripeptide may not always be cleaved out efficiently in these cells (*14*).

In this report we present strategies to overcome these limitations; these have allowed the determination of the functional roles of individual gla residues in activated fX. We describe the expression, purification, activation, and characterization of a series of recombinant human fX proteins bearing aspartic acid substitutions for each of the amino terminal 11 glutamic acid residues. The choice of aspartic acid as the substituted amino acid is a conservative one and was made to probe the effects of γ-carboxylation (at each of the 11 glutamic acid residues that normally undergo carboxylation) on the binding of fXa to membrane surfaces. Our findings demonstrate that glas 16, 26, and 29 are critical for activity. Substitution of aspartate for gla 32 (a conserved residue in prothrombin, fIX, and fX but not in protein C) results in 25% activity in a clotting assay but *normal* prothrombinase activity on the thrombin-activated platelet surface. This observation and kinetic analysis of the prothrombinase complex formed either on platelets or synthetic PSPC vesicles with fXa variants in which aspartate is substituted for gla 29 or gla 16 provide strong evidence for a qualitative difference in the binding of fXa in the prothrombinase complex formed on these two distinct phospholipid surfaces.

EXPERIMENTAL PROCEDURES

Proteins. Polyclonal fX antibody was obtained from Dako-Patts, Carpinteria, CA. Plasma-derived human fX and fXa, RVV-Xa, and biotin-EGR were obtained from Haematologic Technologies, Essex Junction, VT. Thrombin-titrated ATIII was a gift of Dr. F. Church, University of North Carolina, Chapel Hill, NC. Hirudin and soybean trypsin inhibitor were obtained from Sigma, St. Louis, MO. The calcium-dependent monoclonal human fX antibody (4G3) was obtained from Dr. Harold James, University of Texas, Tyler, TX.

Materials. *Vent* polymerase, *Sac II*, *Sma I*, and *Bgl II* were obtained from New England Biolabs, Beverly, MA. Sequenase II was obtained from USB, Cincinnati, OH. *Taq* polymerase and PCR Thermocycler were obtained from Perkin-Elmer Cetus, Norwalk, CT. Human embryonic kidney (HEK) 293 cells were obtained from ATCC, Rockville, MD. Lipofectamine and G418 were obtained from GIBCO-BRL, Gaithersburg MD. Complete protease inhibitor cocktail was obtained from Boehringer Mannheim. QAE-sepharose beads were obtained from Pharmacia Biotech, Uppsala, Sweden. Coomassie protein assay reagent was obtained from Pierce, Rockford, IL. Hydroxylapatite was obtained from BioRad Laboratories, Hercules, CA. TLCK, TPCK, PPACK, DEGR, and PMSF were obtained from Calbiochem, San Diego, CA. Centriprep 30 concentrators were obtained from Amicon, Beverly, MA. DC-4A cation-exchange resin was obtained from Dionex, Sunnyvale, CA. *o*-Phthalaldehyde was obtained from Pickering Laboratories, Mountain View, CA. Sephacryl 100 was obtained from Pharmacia Biotech, Uppsala, Sweden. S2765 and S2238 were obtained from Chromagenix, Mölndal, Sweden. Factor X deficient human plasma was the gift of HRF, Raleigh, NC. Thrombofax was obtained from Ortho Diagnostic Systems, Raritan, NJ. Thermomax spectrophotometric microtiter plate reader was obtained from Molecular Devices, Sunnydale, CA. Brain phosphatidyl choline, brain phosphatidyl serine, brain phosphatidyl ethanolamine, and brain sphingomyelin were obtained from Avanti Polar Lipids, Alabaster, AL, and Sigma, St. Louis, MO. NP-40 and streptavidin were obtained from Sigma, St. Louis, MO. Prism software was obtained from Graphpad Inc., San Diego, CA.

Construction of Expression Vectors. Specific mutagenic oligomers were designed to encode the substitution of aspartic acid for each of the 11 amino terminal glutamic acid residues of fX which normally undergo γ-carboxylation (glas 6, 7, 14, 16, 19, 20, 25, 26, 29, 32, 39). Site-directed mutagenesis was performed according to the method of Kunkel (*16*) using a phagemid vector containing the human fX cDNA (pMAC254). A 471 bp fragment including the coding sequence for the gla domain of fX was excised from the phagemid vector and subcloned into a cytomegalovirus promoter driven expression vector (pCMV4) (*17*) containing the fX cDNA. The entire 471 bp subcloned region was then sequenced in the expression vector to confirm the mutation and ensure that there were no polymerase-induced errors (*18*). This pCMV4-hfX was used to transfect human embryonic kidney (HEK) 293 cells.

Expression of rfX. HEK293 cells were transfected by calcium phosphate coprecipitation (*19*) or by Lipofectamine

Table 1: NH_2-Terminal Analysis of Recombinant fX and Recombinant fX (R-2)

	r-fX (pmol)			r-fX (R-2) (pmol)	
cycle	LC	HC	Pro P	LC	HC
1	Ala (119)	Ser (79)	Leu (38)	Ala (409)	Ser (294)
2	Asn (65)	Val (114)	Phe (62)	Asn (355)	Val (374)
3	Ser (53)	Ala (159)	Ile (43)	Ser (249)	Ala (318)
4	Phe (48)	Gln(114)	Arg (20)	Phe (344)	Gln(374)
5	Leu (28)	Ala (99)	Arg (21)	Leu (399)	Ala (317)
6		Thr	Glu		Thr
7		Ser	Gln		Ser
8	Met	Ser	Ala	Met	Ser
9	Lys	Ser	Asn	Lys	Ser
10	Lys	Gly	Asn	Lys	Gly
11	Gly	Glu	Ile	Gly	Glu
12	(His)	Ala	Leu	His	Ala
13	Leu	Pro	Ala	Leu (252)	Pro (118)
14		Asp	Arg		Asp (84)
15	Arg	Ser	Val	(Arg)	Ser

according to the manufacturer's instructions. Cotransfection with a plasmid containing the neomycin resistance gene (pSV2neo) was performed at a 1:10 molar ratio (pSV2neo/pCMV4.hfX). Transfectants were selected with the neomycin analogue G418, and resistant colonies were screened for fX production by sandwich ELISA using a rabbit polyclonal antihuman fX as the capture antibody and a peroxidase-labeled rabbit polyclonal antihuman fX as the detecting antibody. Selected clones were expanded into roller bottle culture and a total of 1.5−2 L of conditioned medium were collected daily over 10−14 days. Medium was passed over a 0.1 μm cellulose acetate filter to remove cellular debris, and benzamidine was added to a final concentration of 5 mM before storing at −20 °C. In initial expression studies using an unmodified wild-type fX cDNA, a substantial amount of expressed material was found by amino-terminal protein sequence analysis to have the propeptide attached (Table 1). This material could not be fully activated by RVV-Xa. Examination of the amino acid sequence of fX surrounding the propeptide cleavage site revealed that it differs from the other vitamin K-dependent proteases in that it lacks a basic residue (Arg or Lys) at P2; in fX this residue is Thr. Site-directed mutagenesis was employed to substitute Arg for Thr(−2) in the expression vector in order to create a dibasic sequence at the cleavage site for the processing protease. Amino-terminal sequence analysis of material expressed from this modified recombinant fX cDNA with the R(−2) substitution showed the absence of any forms with the propeptide attached; only the predicted sequences for the normal heavy and light chains were found (Table 1). This modified construct bearing Thr(−2) → Arg is henceforth referred to as wild-type fX. All mutant constructs also carry the Thr(−2) → Arg substitution. Expression levels were in the range of 0.5−9.0 μg/10^6 cells/24 h and varied with the construct being expressed.

Purification of r-fX. A novel purification scheme was developed to recover fully carboxylated recombinant fX from conditioned medium. Conditioned medium was thawed at 37 °C, EDTA was added to a concentration of 5 mM, soybean trypsin inhibitor was added to a concentration of 10 μg/mL, and 1 mL of a 25X stock protease inhibitor cocktail was added per liter of conditioned medium. The medium was diluted to bring the final NaCl concentration to 60 mM, and the mixture was then stirred at 4 °C for 30 min with 10 mL of equilibrated QAE-sepharose beads. Beads were washed before eluting isocratically using 20 mM Tris (pH 7.2)/700 mM NaCl. Fractions containing protein (Coomassie protein assay) were pooled and applied to a Ca^{2+}-dependent monoclonal human fX immunoaffinity column according to the method of Kim (*20*). Fractions eluted from the immunoaffinity column were screened for activity by incubating a 5 μL sample from each fraction with 1 nM Russell's viper venom-fX activator (RVV-Xa), and activated fX was detected spectrophotometrically with the chromogenic substrate, S-2765. Fractions containing fX were pooled and dialyzed into 1 mM Na_2HP0_4/NaH_2P0_4 (pH 6.8) and then applied to 5 mL of equilibrated hydroxylapatite. Factor X was eluted with a 1−400 mM Na_2HPO_4/NaH_2P0_4 (pH 6.8) gradient over 70 mL at a flow rate of 0.2 mL/min at 22 °C. This step yielded two distinct populations of r-fX; fractions from the later-eluting peak, which was found to be enriched for fully carboxylated material (see Figure 1B and gla analysis below), were pooled, and 1 μL of a protease inhibitor mix (1 mM each TLCK, TPCK, PPACK, DEGR, and PMSF) was added per mL of eluate. Purified fX was concentrated by ultrafiltration and dialyzed into 20 mM HEPES (pH 7.4)/150 mM NaCl, and the concentration of fX was determined by absorbance at 280 nm (extinction coefficient 1.16 mL mg^{-1} cm^{-1}). Aliquots were stored at −80 °C. This purification scheme resulted in a highly purified product that migrated as a single band on nonreducing polyacrylamide gel electrophoresis with a molecular mass of approximately 55 kD (Figure 2A). Protein yields of highly carboxylated fX after the final step were approximately 2% of starting material with significant losses at the antibody purification and hydroxylapatite steps (Table 2).

Table 2: Yield from Purification of r-fX

	total fX protein[a] (μg)	%
conditioned medium (vol = 2 L)	8387	100
Q-Sepharose	6043	72
immunoaffinity	2750	32
hydroxylapatite (vol = 19 mL)	176	2

[a] By ELISA.

Alkaline Hydrolysis Amino Acid Analysis for γ-Carboxyglutamic Acid and Aspartate/Asparagine (gla Analysis). A 20 μg amount of purified fX, without further purification, was subjected to alkaline hydrolysis. Approximately 5 μg was separated by HPLC on a DC-4A cation-exchange resin column according to the modified method of Price (*21*). Recombinant proteins were compared to plasma-derived fX purified in an identical fashion. A sample of highly purified commercially available plasma-derived fX was used as a standard for the method. In contrast to plasma-derived fX (Figure 1A), recombinant wild-type fX eluted from hydroxylapatite in two peaks (Figure 1B); the first peak had 0 mol of gla/mol of protein whereas the later eluting peak had a gla content comparable to plasma-derived fX (Table 3). Further analysis of fractions from the beginning, middle region, and the end of the second peak showed the same gla content for each fraction, demonstrating that material within the peak was homogeneous with respect to γ-carboxylation (data not shown). The overall gla content for (WT) r-fX and the variants was comparable to the gla content of plasma-derived material (Table 3). To confirm the results of gla analysis, amino terminal sequence analysis was carried out

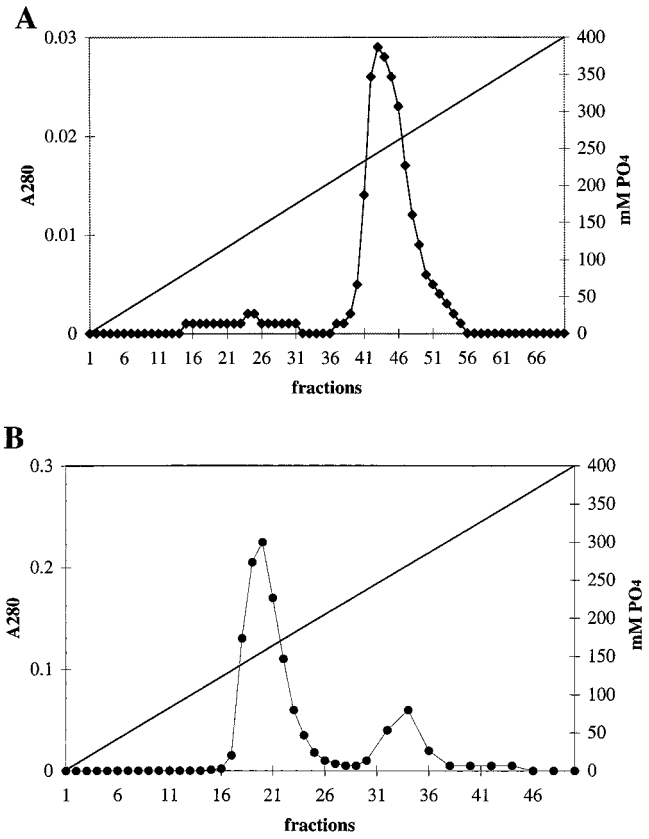

FIGURE 1: Hydroxylapatite elution profiles of plasma-derived and wild-type r-fX. Elution is monitored by absorbance at 280 nm. Panel A: Plasma-derived fX is recovered as a single peak when eluted from hydroxylapatite with a 1−400 mM gradient of phosphate buffer. Panel B: Recombinant wild-type fX elutes as two peaks, with fully carboxylated fX eluting at higher phosphate concentration (see text).

Table 3: Gla Analysis of Purified FX

fX	mol of Gla/mol of protein) (mean ± std dev)
PD-fX (IA)[a]	8.0 ± 0.2[b]
PD-fX (HA)[a]	7.9 ± 0.2
rWT-fX (HA peak 2)	8.9 ± 0.6
rWT-fX (HA peak 1)	0[c]
E6D	9.5 ± 0.2
E7D	8.2 ± 0.0
E14D	7.2 ± 0.4
E16D	9.5 ± 0.5
E19D	8.9 ± 0.5
E20D	8.7 ± 0.4
E25D	8.3 ± 0.1
E26D	6.1 ± 0.2
E29D	8.4 ± 0.0
E32D	8.8 ± 0.4
E39D	9.1 ± 0.6

[a] IA: immunoaffinity alone. HA: immunoaffinity followed by hydroxylapatite. [b] Although gla analysis yielded lower than the expected number of moles for all variants, gla analysis was not performed on reverse phase purified material. Full carboxylation was confirmed by amino-terminal sequence analysis (*9*, *44*) (see text). [c] No gla peak detectable.

on seven of the recombinant proteins. Gla is not detected with Edman degradation amino terminal protein sequence analysis and, therefore, appears as a blank in the sequence; significant contamination with non-carboxylated material will appear as a glutamic acid peak. All of the seven variants

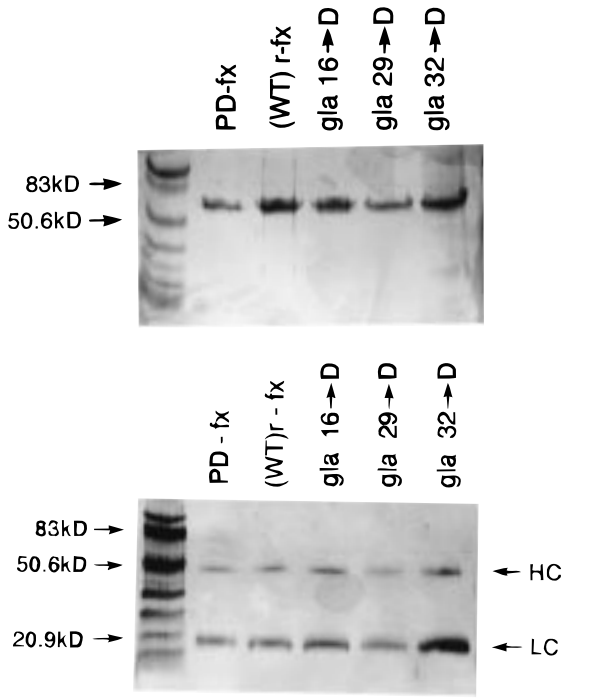

FIGURE 2: SDS−PAGE (8−25% gradient) of purified fX. Panel A (top): Nonreduced protein is visualized by silver staining. Lane 1: Low molecular weight markers. Lane 2: Plasma-derived fX. Lane 3: Wild-type recombinant fX. Lanes 4−6: Recombinant gla variants bearing aspartic acid substitutions for glas 16, 29, and 32, respectively. Panel B (bottom): Reduced protein is visualized by silver staining; lanes are identical to panel A.

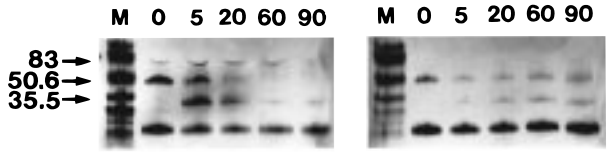

FIGURE 3: Time course of activation of plasma-derived and wild-type r-fX. A 10 μg amount of fX was activated with RVV in the presence of Ca^{2+}. SDS−PAGE (reducing conditions) of quenched aliquots from the activation reaction at 0, 5, 20, 60, and 90 min are visualized by silver stain. Left panel: Full activation of plasma-derived fX is evidenced by the disappearance of the ~50 kD band (unactivated heavy chain) by 90 min. Right panel: At 90 min recombinant wild-type fX is not fully activated.

subjected to amino terminal sequence analysis showed blanks at positions where gla residues were expected. The variant with a mutation at position 32 was taken through 42 cycles, and blanks were present at all residues that normally undergo carboxylation (data not shown).

Activation of Recombinant fX. In a reaction volume of 500 μL, 5.5 nm of plasma-derived and (WT) r-fX were each incubated with 0.1 μM of RVV-Xa in HEPES-buffered saline with 8 mM $CaCl_2$ at 25 °C for 90 min. A 10 μL amount of the reaction volume was removed at various time points and added to a reducing gel loading buffer containing β-ME and 8 mM EDTA. Samples were separated on an 8−25% gradient polyacrylamide gel. Bands were visualized by silver stain to follow the time course of activation. Figure 3 shows PAGE analysis of time points and demonstrates in the left panel that at 90 min plasma-derived fX is fully activated whereas (WT) r-fX (right panel) shows persistence of unactivated heavy chain and a second higher molecular weight band. Full activation of (WT) r-fX was not accomplished even after a 16 h of incubation (data not shown).

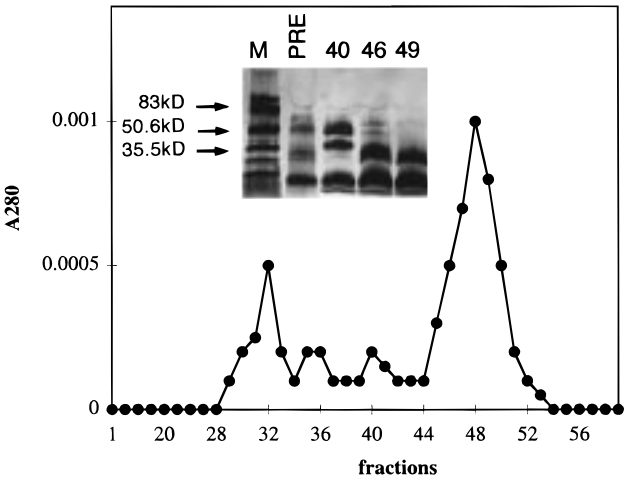

FIGURE 4: Separation of fXa from unactivated fX by size-exclusion chromatography (Sephacryl-100). Isocratic elution with 20 mM HEPES (pH 7.4)/150 NaCl mM is monitored by absorbance at 280 nm. Inset: SDS−PAGE (reducing conditions) in which protein is visualized by silver staining. Lane M: Low molecular weight markers. Lane PRE: Presample of purified recombinant human fX activated with Russell's viper venom for 16 h. Lane 46: Fraction 46 of isocratic elution from Sephacryl-100 showing a light chain (LC) and activated heavy chain (HC) along with contaminant unactivated heavy-chain (HC) and single-chain (SC) fX. Lane 49: Fraction 49 contains only fXa with activated heavy chain and light chain.

Separation of fX from fXa. This problem was resolved by separating activated fX from unactivated material by size exclusion chromatography. A 300 μg amount of fX was activated in a volume of 500 μL with RVV-Xa for 6 h at 37 °C as described above. A 16 mm × 60 cm column containing Sephacryl-100 was equilibrated in 20 mM HEPES (pH 7.4)/150 mM NaCl for 10 h at a flow rate of 0.4 mL/min at 4 °C. The 500 μL reaction mixture was then applied to the column and eluted with 70 mL of 20 mM HEPES (pH 7.4)/150 mM NaCl at a flow rate of 0.2 mL/min at 4 °C. The 1 mL fractions were screened by incubating a 5 μL sample with SpectrozymeFXa and measuring the rate of cleavage of the substrate by fXa. Factor Xa eluted between fractions 44 and 54 (Figure 4). These fractions were screened for purity by using heavily loaded samples on PAGE (inset to Figure 4), and only the later fractions which contained no higher molecular weight material (unactivated fX) were pooled. All mutant constructs were purified in this fashion for use in all clotting and purified component assays described in this paper**.**

Concentration Determinations of fXa Stocks. Concentrations of fXa stock solutions were determined by three independent methods in addition to absorbance: titration with ATIII, activity with the chromogenic substrate S2765, and Coomassie protein assay. Protein concentrations were initially determined spectrophometrically and then diluted to approximately 30 nM. A 30 μL amount of the diluted stocks was incubated for 5 min at 22 °C with 30 μL of each of a series of dilutions of thrombin-titrated anti-thrombin III (ATIII) in HEPES buffered saline containing 2 μg/mL heparin. Uninhibited fXa was detected by the addition of S-2765, and concentrations were determined by plotting the fXa activity versus concentration of ATIII with which the fXa stock had been incubated; the *x*-axis intercept of these linear plots defined the concentration. In addition, concentrations were determined by the rate of cleavage of the small chromogenic substrate S-2765 from a standard curve derived from dilutions of commercially available fXa. S-2765 determined concentrations were found to correlate with ATIII titrations and the Coomassie protein assay. K_Ms of several fXa variants (including those with no activity in clotting) and r-(WT) and plasma-derived fXa were determined and were the same for S-2765, as was expected for these light chain mutants (~100 μM; data not shown). K_Ms for prothrombin were determined for the three fXa variants that were extensively characterized (gla16 → D, gla29 → D, and gla32 → D; vide infra) and were not different from wild-type r-fXa and plasma-derived fXa (~6 μM; data not shown).

Factor Xa Activated Clotting Assay. In a microtiter plate, serial dilutions of fXa from 0.75 to 0.047 nM were made into HEPES buffered saline containing 1 mg/mL albumin and $CaCl_2$ (final concentration 5 mM) and incubated for 5 min at 37 °C. In separate wells, 50 μL of fX-deficient plasma was mixed with 30 μL of a bovine brain cephalin source of phospholipid without activator or tissue factor (Thrombofax) and incubated for 5 min at 37 °C. Reactions were started by the addition of 20 μL of the fXa/$CaCl_2$ solution to the plasma/partial thromboplastin well. Absorbance was measured at 340 nm every 6 s using a Thermomax plate reader, and the time to maximal velocity was plotted against fXa concentration (*22*). A standard curve was generated using plasma-derived fXa, and the activities of the variants were determined from this curve. Each assay was performed in duplicate. For variants that had activities below the lowest dilution on the standard curve, serial dilutions from 1.5 nM to 0.094 nM were also tested.

Activation of fV. Factor V was isolated from human plasma as previously described (*23*). In a 900 μL volume, 500 nM fV was activated with 15 nM thrombin (IIa) for 15 min at 37 °C. Activation was terminated by the addition of 25 nM hirudin.

Functional Assessment of Prothrombinase Activity. For these assays phospholipid vesicles were prepared by extrusion through a 100 nm filter (*24*). The concentration of the phospholipid vesicles was determined by the phosphorus assay of Gomori (*25*). Human platelets were isolated from freshly collected whole blood according to the method of Mustard (*26*). Platelets were counted and used at a concentration of 1×10^8/mL in 20 mM HEPES−Tyrode's buffer (pH 7.4) for all experiments.

The formation of a functionally active prothrombinase complex on thrombin-activated platelets or synthetic vesicles using either normal or variant fXa was assessed using purified components as previously described (*27, 28*). For experiments in which platelets provided the membrane surface, platelets (1×10^8/mL) were activated with 2 NIH U of thrombin/mL for 3 min at ambient temperature followed by the addition of DAPA (3 μM), ±fVa (5 nM), and various amounts of normal or variant fXa. Initial screening experiments were performed with a single fixed limiting amount of normal or variant fXa (50 pM). To kinetically determine the binding interaction of fXa (normal or variant) with thrombin-activated platelets, experiments were performed by varying the fXa concentration (0.05−50 nM) and keeping the other components of prothrombinase fixed. For experiments in which synthetic phospholipid vesicles provided the procoagulant surface, PSPC vesicles or vesicles comprised

Table 4: Clotting Activity of r-fXa Gla Variants

variant	activity (% PD)[a]
(WT) r-fXa	98 ± 6
gla 6 → D	47 ± 8
gla 7 → D	10 ± 3
gla 14 → D	25 ± 4
gla 16 → D	ND[b]
gla 19 → D	28 ± 6
gla 20 → D	6 ± 2
gla 25 → D	7 ± 2
gla 26 → D	ND[b]
gla 29 → D	ND[b]
gla 32 → D	25 ± 7
gla 39 → D	32 ± 4

[a] PD = plasma-derived fXa. ND = not detectable. [b] Tested at twice the concentration of PD-fXa (1.5 nM). The standard curve was generated with dilutions of fXa from 0.75 to 0.047 nM.

of 11:41:30:17 PS/PC/PE/sphingomyelin (wt %) in 20 mM HEPES/0.15 M NaCl/5 mM $CaCl_2$/Tween-80 (pH 7.4) were incubated with DAPA (3 μM) and varying amounts of normal or variant fXa. All reactions using synthetic phospholipid vesicles contained 5 nM fVa. After a brief incubation, all reactions were initiated with the addition of prothrombin (1.4 μM), and rates at which thrombin was generated were monitored at 405 nm using the chromogenic substrate S2238.

Data Analysis. Curves were derived from least-squares fit of the data to the Michaelis−Menten equation, and affinity constants (K_D(app)s) were determined from these curves. Curve fits were performed using the Prism program.

RESULTS

Purification of Fully Carboxylated r fXa. Plasma-derived fXa, (WT) r-fXa, and variant fXas were purified as described in methods. The recombinant variants were indistinguishable by gel analysis, gla analysis, and amino terminal analysis from plasma-derived fXa. Gla analysis yielded values that were within 2 mol of gla/mol of protein from normal fXa (WT rfXa or PD fXa) for all variants. These differences likely represent experimental error. If the differences truly represent undercarboxylation, however, such undercarboxylation is unlikely to have an effect on activity as purified recombinant fIX and fVII molecules that lack either 1 or 2 gla residues have been shown to have normal activity (*29*, *30*).

Factor Xa-Activated Clotting. In a clotting assay in which fXa was used to initiate coagulation, recombinant wild type fXa had activity identical to that of plasma-derived fXa (Table 4). Substitutions of aspartic acid at residues 16, 26, or 29 resulted in undetectable activity even when used at twice the concentration (1.5 nM) of wild-type fXa. Aspartic acid substitutions for glas 7, 20, and 25 showed activities of 10% of normal or less. Aspartic acid substitution for gla 6 had approximately 50% activity of normal and was the variant with the highest activity. The other mutants had activities between 10 and 50% (glas 14, 19, 32 and 39).

Prothrombinase Activity of Variant fXas. Variants were screened for activity in purified component assays in which the prothrombinase complex was assembled either on activated platelets [with or without exogenous fVa (5 nM)] or synthetic phospholipid vesicles (with 5 nM fVa) (Figure 5). Our results using 0.05 nM normal or variant fXa showed that, in the prothrombinase complex assembled on synthetic phospholipid vesicles, fXa variants with aspartic acid substitutions for glas 16, 26, and 29 had essentially no activity consistent with the clotting data shown in Table 4. However with assembly into the prothrombinase complex on platelets, significant activity (up to 15%) was detected with these variants. Under identical conditions in these screening assays, all gla variants exhibited greater activity in the prothrombinase complex assembled on thrombin-activated platelets than on synthetic vesicles (Figure 5). In addition,

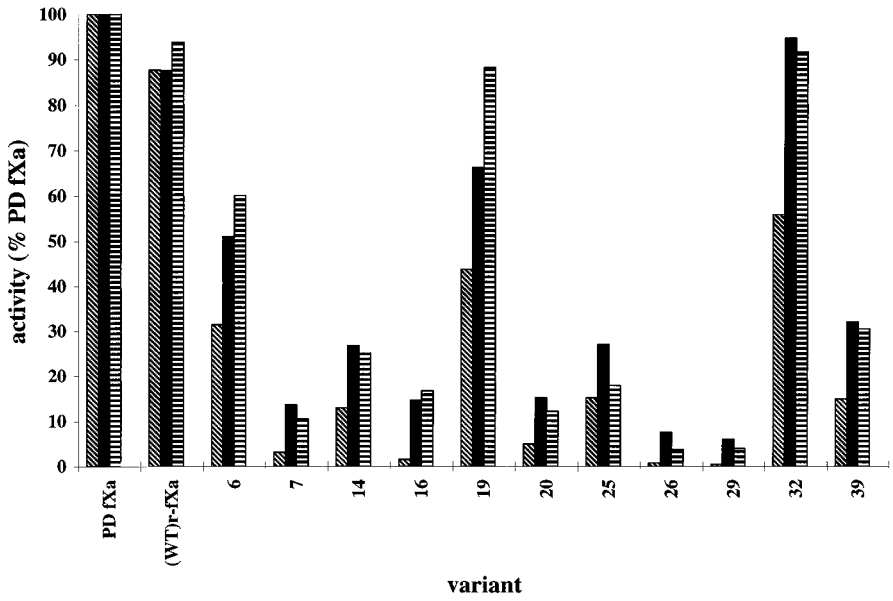

FIGURE 5: Comparison of recombinant fXa variants in prothrombinase assays. The histogram depicts prothrombinase activities (expressed as a percentage of the activity of PD-fXa) of gla variants of human fXa in which a single aspartic acid is substituted for the gla residue at the indicated residue. Column 1 represents the activities of the variant (at 0.05 nM) in the prothrombinase complex assembled on 30:70 (wt %) PSPC vesicles. Column 2 represents the prothrombinase activities of variants (0.05 nM) in the prothrombinase complex assembled on thrombin-activated platelets. Column 3 represents the results of the same experiment presented in column 2 except that 5 nM exogenous fVa was added to the reaction.

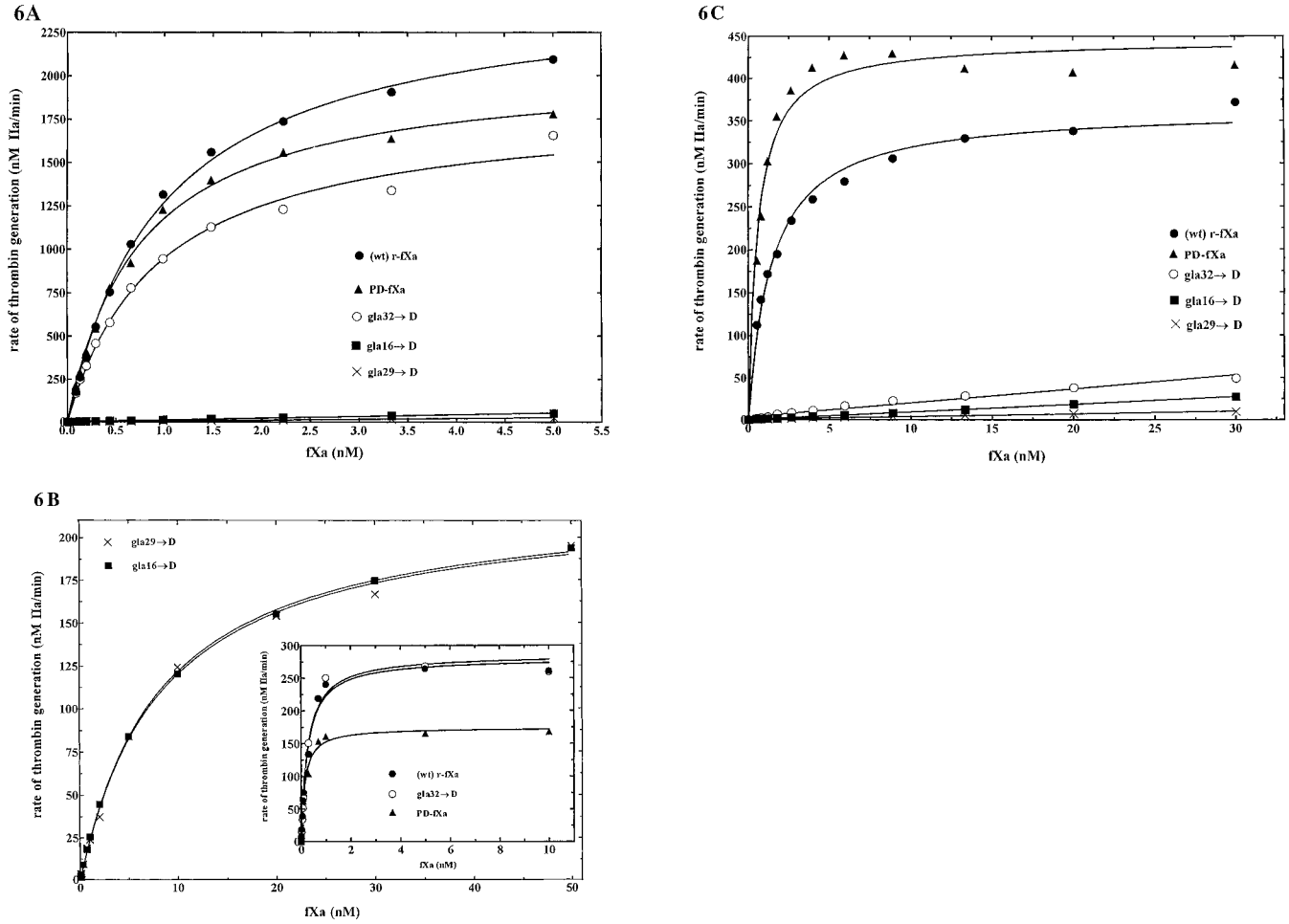

FIGURE 6: Kinetic determination of the incorporation of recombinant fXa variants into the prothrombinase complex. Varied concentrations of fXa were incubated with either 200 μM PSPC vesicles (30:70) (panel A), 1×10^8 platelets/mL (panel B), or 400 nM PS/PC/PE/Sphingomyelin vesicles (11:41:30:17) (panel C) in the presence of 5 nM fVa to form the prothrombinase complex. PS/PC/PE/sphingomyelin vesicle concentrations were chosen to provide the same maximal activity with the wild-type fXa as was observed with thrombin-activated platelets. PS/PC vesicle concentration was optimized based on maximal activity. Panel A: Recombinant (WT) fXa, PD fXa, and the gla32 → D variant fXa have K_D(app)s for binding in the prothrombinase complex assembled on 30:70 PSPC vesicles of 0.977 ± 0.046 nM (r^2 = 0.99), 0.744 ± 0.037 nM (r^2 = 0.99), and 0.930 ± 0.090 nM (r^2 = 0.99), respectively. The gla16 → D and gla29 → D variants show severe defects in binding into the prothrombinase complex. Panel B: Inset PD fXa, (WT) r-fXa, and the gla32 → D mutant fXa have K_D(app)s of 0.142 ± 0.013 (r^2 = 0.99), 0.264 ± 0.028 nM (r^2 = 0.99), and 0.270 ± 0.038 nM (r^2 = 0.98), respectively, for binding into the prothrombinase complex on platelets. The gla29 → D and gla16 → D mutants exhibit measurable binding in the prothrombinase complex on thrombin-activated platelets; K_D(app)s are 8.50 ± 0.57 nM (r^2 = 0.99) and 8.35 ± 0.22 nM (r^2 = 0.99), respectively (30-fold higher than (WT) r-fXa). Panel C: All three gla variants have severe defects when assembled in the prothrombinase complex on synthetic vesicles that contain phospholipid species in concentrations present on the thrombin-activated platelet surface. K_D(app) of (WT) r-fXa and PD fXa are 1.40 ± 0.12 nM (r^2 = 0.99) and 0.574 ± 0.074 (r^2 = 0.98) respectively, and equivalent to those observed in the experiment using (30:70) PSPC vesicles (panel A).

the gla 32 → D variant had normal activity in the prothrombinase complex assembled on platelets in the screening assay; however, its activity on synthetic vesicles was only 50% of PD fXa. To further characterize these differences, kinetic experiments were performed to study the interaction of fXa with the other components of the prothrombinase complex. Detailed analyses were performed with the gla 16 → D, gla 29 → D, and gla 32 → D variants.

Analyses of kinetics of prothrombin activation were used to calculate the interactions of these selected gla variants with other components of prothrombinase assembled on synthetic 30:70 PS/PC vesicles (wt %), thrombin-activated platelets, or vesicles comprised of phospholipids in concentrations reflecting those found on the outer surface of the membrane of thrombin-activated platelets (41:30:11:17 PC/PE/PS/sphingomyelin) (wt %) (*31*) (Figures 6A−C). The rate saturation curves obtained as a function of added gla 32 → D fXa, (WT) r-fXa, or plasma-derived fXa in the presence of a constant and saturating amount of fVa (5 nM) allowed the calculation of apparent K_Ds for each of these fXa species; these K_Ds were indistinguishable from each other when analyzed on PSPC vesicles (Figure 6A, Table 5). Likewise, the gla 32 → D variant incorporated into prothrombinase on thrombin-activated platelets in a manner identical to the (WT) r-fXa (Figure 6B, inset). In contrast, the gla 16 → D and gla 29 → D variants displayed a marked difference between their activity on PSPC vesicles and thrombin-activated platelets. Activity with these two variants was essentially undetectable when assembled into prothrombinase on PSPC vesicles (Figure 6A); however, when thrombin-activated platelets served as the membrane surface for assembly of the complex, saturation curves could be measured, allowing for the determination of K_D(app)s (Figure 6B). When saturation curves were generated with PSPC

Table 5: Binding of fXa Variants into Prothrombinase Assembled on Synthetic Vesicles or Thrombin-Activated Platelets[a]

	PS/PC vesicles[c]		activated platelets		PS/PC/PE/sphingomyelin vesicles[d]	
variant	K_D(app) (nM)	V_{max} (nM of IIa/min)	K_D(app) (nM)	V_{max} (nM of IIa/min)	K_D(app) (nM)	V_{max} (nM of IIa/min)
PD-fXa	0.744 ± 0.037	2052 ± 34.76	0.142 ± 0.013[b]	174.2 ± 4.05[b]	0.574 ± 0.074	445.9 ± 10.56
(WT)-rfXa	0.977 ± 0.046	2510 ± 43.75	0.264 ± 0.028	280.8 ± 7.29	1.40 ± 0.12	363.5 ± 7.62
gla 32 → D	0.930 ± 0.090	1829 ± 65.00	0.270 ± 0.038	286.0 ± 11.2	ND	ND
gla 16 → D	ND	ND	8.35 ± 0.22	223.4 ± 1.89	ND	ND
gla 29 → D	ND	ND	8.50 ± 0.57	222.2 ± 4.82	ND	ND

[a] ND: not able to be determined (see Figure 6). [b] Performed on a different donor (see text). [c] Performed at a vesicle concentration resulting in maximal activity. [d] Performed at a vesicle concentration that gave maximal rates similar those observed with 1×10^8 activated platelets/mL; all $r^2 > 0.980$.

vesicles using 5 nM of the gla 16 → D and gla 29 → D variant fXas (a concentration similar to the K_D determined with the experiments using thrombin-activated platelets), less than 5% of the maximal activity of normal fXa was observed (Figure 6A), whereas on thrombin-activated platelets this concentration of variant fXa had nearly 50% the maximal activity of normal fXa (Figure 6B). The increased affinity of the gla 16 → D and gla 29 → D variants for prothrombinase assembled on thrombin-activated platelets vs PSPC vesicles was not due solely to the difference in phospholipid content since both variants were severely defective in their ability to incorporate into prothrombinase when complex synthetic vesicles comprised of phospholipids in concentrations present on the thrombin-activated platelet surface were used in identical experimental protocols (Figure 6C). This observation is underscored by the markedly reduced affinity of the gla 32 → D variant for prothrombinase assembled on these complex "plateletlike" vesicles (Figure 6C), even though this variant assembled into prothrombinase on thrombin-activated platelets and PSPC vesicles with an affinity identical to that of (WT) r-fXa (Figure 6A and 6B inset).

In addition, we have characterized two mutants of gla 14 (gla 14 → D and gla 14 → K) (data not shown). Binding affinities of these two gla 14 mutants for the prothrombinase complex assembled on thrombin-activated platelets were identical ($K_D = 2-3$ nM) and 5−10-fold higher than that observed for plasma-derived and wild-type r-fXa. These data suggest that the effect observed for this particular residue (gla 14) is due to the loss of the gla at position 14 in fXa and *not* to a specific effect of the substituted amino acid.

DISCUSSION

This report details the expression, purification, and characterization of recombinant wild-type fXa and a series of variants with mutations in the gla domain. A detailed analysis of (WT) r-fXa shows that it is indistinguishable from plasma-derived fXa. Wild-type r-fXa has normal activity in a clotting assay and in the prothrombinase complex assembled on platelets and synthetic phospholipid vesicles. Gla analysis demonstrates full carboxylation on the basis of a comparison with plasma-derived fX purified in an identical fashion. Comparison of a series of gla variants of fXa as components of the prothrombinase complex assembled on thrombin-activated platelets or vesicles of varying lipid concentrations demonstrates that fXa incorporates into prothrombinase differently on thrombin-activated platelets as opposed to synthetic vesicles. Thrombin-activated platelets are more accommodating in supporting the binding and function of the gla variants studied.

The intent of this study was to carry out a systematic characterization of the role of the individual gla residues of fXa in the assembly of the macromolecular enzyme complex prothrombinase on a physiologically important membrane surface. Thus a series of mutant fXas were produced in which aspartate was substituted for individual gla residues. The choice of glu → asp in residues that normally undergo carboxylation is intentionally conservative (i.e. changes in charge and size are minimal except as related to the presence or absence of the second carboxyl group). Although unlikely given the conservative nature of the gla → asp substitution, a potential shortcoming of this strategy is that the choice of a single amino acid (asp) for substitution for gla may compromise interpretation if effects are due to the specific effect of the asp residue and not due to the loss of the specific gla residue or its γ-carboxyl group (i.e. asp results in gain-of-function). None of the mutants in this study, however, result in gain-of-function. Published studies of recombinant fX are sparse because of the difficulties that attend the preparation of fully processed r-fX (e.g. incomplete post-translational modifications such as γ-carboxylation and proteolytic processing) (*14*, *15*). Isolation of wild-type r-fXa with normal specific activity for our studies required modification of the expression construct and the development of a novel purification strategy as outlined in the methods. An important step in the purification is elution of recombinant protein from hydroxylapatite in order to isolate fully carboxylated material. This technique yielded the surprising finding of only two species of immunopurified r-fX; the first contained material that had undergone *no* γ-carboxylation, and the second was *fully* carboxylated when compared to plasma-derived fX purified in an identical fashion. This observation suggests that, for wild-type r-fX expressed in HEK cells, carboxylation by the γ-glutamyl carboxylase proceeds to completion once initiated; otherwise material with intermediate gla content would have been detected. Plasma-derived fX elutes as a single peak from hydroxylapatite, indicating that there is no under-carboxylated material circulating in normal plasma. These data are consistent with previous studies using peptides derived from fIX that show the γ-glutamyl carboxylase to be processive (*32*). The studies reported here were carried out on the fully carboxylated material. Although detailed carbohydrate studies were not performed, functionally important glycosylation differences between the hepatocyte and this expression system are not apparent in these assays as (WT) r-fXa has activity identical to that of PD-fXa. None of the mutations studied here affect known glycosylation sites, and all recombinants were produced in an identical expression system.

The gla domain of the vitamin K-dependent coagulation proteins is highly conserved and confers on these proteins the property of calcium binding which results in a conformational change necessary for phospholipid binding (*1*). Previous studies of a series of gla → asp recombinant mutants of protein C and prothrombin define alterations in phospholipid binding and the gla domain-conformational change that occur in the presence of calcium (*8*, *33*, *34*). Both studies show loss of activity with marked defects in phospholipid binding (*33*) and the calcium-induced conformational change (*8*, *34*) with substitutions at residues 16, 26, and 29; naturally occurring point mutations in human fIX at these positions (residues 17, 27, and 30, respectively) result in severe hemophilia B (*35*). Our study likewise shows these residues to be critical for the function of fXa, as fXa variants with substitutions for glas 16, 26, and 29 had no activity in a clotting assay or in a prothrombinase assay using synthetic phospholipid vesicles and less than 15% activity in a prothrombinase assay using thrombin-activated platelets (Table 4; Figures 5 and 6). Thus, the weight of evidence begins to suggest that carboxylation of these three residues is critically required for activity of the vitamin K-dependent coagulation proteins.

Synthetic phospholipid vesicles are widely used as a consistent source of anionic phospholipid to support prothrombinase, intrinsic tenase, and extrinsic tenase complex assembly in purified component assays. In vivo, however, these enzyme complexes are assembled on activated platelets and perturbed endothelial and subendothelial cells. We hypothesized that differences might be observed between synthetic vesicles and activated platelets because, in the prothrombinase complex, platelet-fXa binding may be regulated by proteins such as EPR-1 as well as membrane phospholipid (*36*). We postulated that such differences would be detectable using gla variants of fXa. Characterization of these fXa variants reveals that their activities in the clotting assay more closely parallel those in the screening prothrombinase assay on thrombin-activated platelets with the exception of (1) gla → asp variants 16, 26, and 29, all of which have *no* activity in clotting or in the prothrombinase complex assembled on PSPC vesicles but detectable activity on thrombin-activated platelets, and (2) the gla 32 → asp variant, which has 25% activity in clotting but *normal* activity with thrombin-activated platelets. Differences observed between the clotting and prothrombinase assays may reflect the fact that the prothrombinase complex is assembled using *activated* fV whereas thrombin generation in the clotting assay is limited by the activation of fV in the system. Since fV can be activated by fXa, mutations in fXa may affect its ability to activate fV (*37*).

Further characterization of the gla 29 → asp and the gla 16 → asp variants provides strong evidence for a difference between the two phospholipid surfaces (activated platelets vs synthetic PSPC vesicles). Both variants exhibit normal K_Ms for prothrombin and severely diminished activity in prothrombinase assembled on synthetic PSPC vesicles (Figure 6A). In contrast, assembly into the prothrombinase complex on thrombin-activated platelets (Figure 6B) demonstrates that a component of the platelet surface can compensate for the altered prothrombinase binding of these variants observed when the complex is assembled on synthetic PSPC vesicles. This is evidenced by the ability to achieve normal activity in the platelet prothrombinase assay with both the gla29→asp and gla16→asp variants when the concentration of fXa is increased approximately 50-fold (Figure 6B). In the prothrombinase assay using synthetic vesicles, however, less than 5% maximal activity was observed with similar increases in the concentration of the gla29 → asp and gla16 → asp variants (Figure 6A,C). Further support for the difference between thrombin-activated platelets and synthetic vesicles is afforded by the gla32 → asp variant, a variant that has activity in the prothrombinase complex on platelets that is normal (inset to Figure 6B) but severely diminished in the prothrombinase complex assembled on vesicles that contain the phospholipid components of the thrombin-activated platelet surface (Figure 6C). Taken together, our results demonstrate that the fVa-dependent binding site for fXa on the thrombin-activated platelet surface is not mimicked by a synthetic phospholipid surface.

One possibility is that these results are due to differences in phospholipid composition between PSPC vesicles and thrombin-activated platelets. Indeed, experiments employing phospholipase degradation or specific labeling of surface-exposed phospholipid headgroups on activated platelets demonstrate that little or no phosphatidylserine is present on the surface of platelets activated with thrombin alone (*38*, *39*). Platelet activation with collagen and thrombin *together*, with the ionophore A23187, or a sulfhydryl oxidizer (diamide) results in increased exposure of PS (*31*, *38*, *39*), and prothrombinase activity is directly correlated with the concentration of PS on the platelet surface (*38*). Other studies show that aminophospholipid translocase activity (important for localizing PS and PE to the inner leaflet of the platelet membrane) is *enhanced* by thrombin stimulation alone (*40*) and that the activity of scramblase (which exposes PS to the outer leaflet) is not detectable in thrombin-activated platelets (*41*). Given these observations, one would expect that PSPC vesicles (which contain a much higher concentration of PS than thrombin-activated platelets) would result in higher prothrombinase activity. The failure of these three fXa variants to assemble into prothrombinase on *complex* synthetic vesicles containing phospholipid headgroups (other than PS and PC alone) at the concentrations present on the outer surface of thrombin-activated platelets (*31*) (Figure 6C) in a manner similar to that observed with thrombin-activated platelets (Figure 6B) argues that the phospholipid components of the outer surface of thrombin-activated platelets alone do not explain the increased activity seen with thrombin-activated platelets. Thus our data lend further support to the notion that prothrombinase activity seen with thrombin-activated platelets is not due to exposure of the procoagulant phospholipid, phosphatidylserine.

A second possibility is that a protein component of the thrombin-activated platelet membrane accounts for the differences observed in prothrombinase complex assembly on PSPC vesicles and activated platelets. For example Bouchard et al. have shown that EPR-1, a fXa binding protein originally identified on monocytes and endothelial cells, is also expressed on the membrane surface of activated human platelets (*36*) and mediates, at least in part, fXa binding into the prothrombinase complex. In a study of fIXa binding to thrombin-activated platelets and synthetic PSPC vesicles, London et al. demonstrated a 25-fold tighter affinity of fIXa

for the platelet surface compared to PSPC vesicles (*42*). Previous ligand blotting studies by the same group also demonstrated an activation-dependent fIXa binding protein(s) on platelets (*43*). These studies and the data presented in this report together provide strong evidence for the existence of other component(s) (besides the total anionic phospholipid content and the protein cofactors, fVIIIa and fVa) of the binding sites on activated platelets for the coagulation serine proteases fIXa and fXa. This component may be a localization of specific phospholipid headgroups in the fXa binding site, a protein component(s), or both.

In summary, we have developed a successful strategy for the expression and purification of recombinant fXa that will allow for the use of site-directed mutagenesis to explore the sites of interaction between this activated serine protease and its cofactors, substrates, and inhibitors. Using a series of recombinants bearing mutations that result in the substitution of aspartate for each of the 11 amino-terminal glutamates that normally undergo γ-carboxylation, we show that three gla residues (at positions 16, 26, and 29) are critical for the activity of human fXa. Kinetic analyses of gla $\rightarrow$ asp variants of fXa at residues 16, 29, and 32 taken together provide strong support for a qualitative difference in the fXa binding site in the prothrombinase complex assembled on thrombin-activated platelets compared to that on synthetic PSPC vesicles.

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