Synthesis and Characterization of Wild-Type and Variant γ-Carboxyglutamic Acid-Containing Domains of Factor VII

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ABSTRACT: Synthetic peptides corresponding to portions of the wild-type and variant sequences of the human factor VII γ-carboxyglutamic acid (Gla)-containing domain have been prepared by direct peptide synthesis using the Fmoc-based protection strategy. Peptides were purified by ion-exchange and reversed-phase chromatography and characterized as the correct products. A peptide comprising residues 1–49 (GP 1–49) inhibited the activation of factor X (FX) by soluble tissue factor (sTF) and recombinant activated factor VII (rFVIIa). In the absence of phospholipid, no inhibition by this peptide was observed. GP 1–49 did not inhibit the hydrolysis of a peptidyl substrate by rFVIIa in the presence of either sTF or relipidated TF apoprotein in the presence or absence of phospholipid. A similar peptide (residues 1–38, GP 1–38) that did not contain the aromatic stack region was also inhibitory. Two variant peptides, one identical to GP 1–49 but lacking the N-terminal alanine residue (GP 2–49) and one identical to GP 1–38 but with an arginine to alanine substitution at position 9 (GP 1–38 R9A), showed substantially reduced inhibitory activity. Kinetic analysis of the inhibition of Xa generation by GP 1–49 revealed a noncompetitive mode of inhibition, probably via a substrate-depletion mechanism. GP 1–49 does not inhibit by preventing FX binding to phospholipid surfaces. This indicates that the N-terminal residues of the FVII Gla domain are important for the structural integrity of the peptide, and implicates the Gla domain *per se* in a direct interaction with phospholipid-bound FX.

Factor VII (FVII)[1] is the zymogen for a serine protease which, in complex with its receptor/cofactor TF, initiates the blood coagulation cascade. The physiological substrates for the FVIIa/TF complex are the zymogens FIX and FX (Rapaport & Rao, 1993).

FVII, the single-chain zymogen of the two-chain-activated serine protease factor VIIa, was isolated from human plasma by Bajaj in 1981 (Bajaj et al., 1981). cDNAs for FVII were cloned by Hagen and others (Hagen et al., 1986), and the full gene sequence was reported a year later (O'Hara et al., 1987). FVII, which shows sequence identity to other vitamin K-dependent coagulation enzymes including FIX and FX, consists of an N-terminal γ-carboxyglutamic acid-containing (Gla) domain (residues 1–38), an aromatic stack region (residues 39–49), two epidermal growth factor (EGF)-like domains, a linking peptide where the activating cleavage occurs, and a C-terminal serine protease catalytic domain (Furie & Furie, 1988). There is evidence that both the first EGF-like domain and the protease domain of FVII participate in direct binding to the cofactor tissue factor (Kumar et al., 1991; Clarke et al., 1992; Toomey et al., 1991; O'Brien et al., 1991). The Gla domain of FVII has been implicated in TF binding through an indirect mechanism (Sakai et al., 1990), and has also been shown to be important for the recognition of macromolecular substrates by FVIIa (Ruf et al., 1991), but its precise role remains unclear.

The Gla domain of prothrombin, which shows extensive sequence identity with the other vitamin K-dependent serine protease Gla domains, has been shown to have important roles in both the membrane binding capability of prothrombin (Pollock et al., 1988) and the interaction of prothrombin fragment 1 with FX (Harlos et al., 1987).

The FVII Gla domain has been isolated (Wildgoose et al., 1992; Sakai et al., 1990) and shown to inhibit FX activation by tissue factor/factor VIIa. The mechanism of its interaction has not been determined. In this paper, we report an investigation into both the structural requirements for activity of the FVII Gla domain and the mechanism of its inhibition of factor X activation. We have directly synthesized three peptides with the wild-type sequence corresponding to residues 1–49, 1–38, and 2–49 of FVII (GP 1–49, GP 1–38, and GP 2–49) and one peptide corresponding to residues 1–38 of FVII with an arginine to alanine substitution at position 9 (GP 1–38 R9A). This residue imparts a conserved positive charge in a region which has been shown to be important for the interactions of the Gla domain in FIX, a related coagulation protease (Cheung et al., 1992). We have examined the inhibitory activity of these peptides and investigated the mechanism of inhibition by peptide 1–49. We have also examined the effect of GP 1–49 on the interaction of FX with phospholipid surfaces. The results obtained support a direct interaction of GP 1–49, and by inference a direct interaction of the FVII Gla domain, with phospholipid-bound FX.

EXPERIMENTAL PROCEDURES

Materials. Fmoc-γ-carboxyglutamic acid, di-γ,γ′-*tert*-butyl ester (Fmoc-Gla), was obtained from Nova Biochem (Nottingham, U.K.). All other amino acid derivatives, solvents, and reagents for peptide synthesis were from Applied Biosystems (Warrington, U.K.). Recombinant activated human factor VII was from Novo Nordisk, Denmark. Human factor X, factor Xa, factor IX, and factor IXa were from Enzyme Research Laboratories (Swansea, U.K.). Truncated recombinant human TF mutants 1–243 and 1–220 were the

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[1] Abbreviations: TBS-A, 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 0.1% bovine serum albumin; Gla, γ-carboxyglutamic acid; TF, tissue factor; sTF, soluble tissue factor; FVII, factor VII; FVIIa, activated factor VII; FX, factor X; FXa, activated factor X; pmc, pentamethylchroman; FIX, factor IX; FIXa, activated factor IX; FVIII, factor VIII; bt-FX, biotinylated FX.

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generous gift of Dr. Deborah Higgins, Genentech Inc. (South San Francisco, CA). TF1–243 has procoagulant activity identical to the full-length molecule and has been described elsewhere (Paborsky et al., 1991). The use of soluble TF variants has also been described elsewhere (Morrissey et al., 1993). Concentrations were determined by the absorbance at 280 nm. Peptide substrates S2222 and S2288 and Chromogenix Coatest FVIII:C/4 FVIII assay kit reagents were from Kabi, Sweden. Normal plasma was collected from 10 normal donors, pooled, and snap-frozen. Rabbit brain cephalin, prepared by the method of Bell and Alton (1954) and standardized for use as a platelet substitute for the measurement of activated partial thromboplastin time (APTT), was obtained from Sigma (Poole, Dorset, U.K.), and stock solutions were prepared according to the manufacturer's instructions. Sulfo-NHS-biotin and streptavidin–horseradish peroxidase conjugate were from Pierce (Chester, Cheshire, U.K.). All other reagents not otherwise mentioned in the text were reagent grade or better and obtained from Sigma.

Peptide Synthesis. Peptides were synthesized by the solid-phase approach using an automated peptide synthesizer (Model 431A, Applied Biosystems). Amino acid composition was determined by analysis of samples hydrolyzed by exposure to vapor-phase 6 M HCl for 24 h at 110 °C. The average mass of ions ($M + H^+$) derived from the peptides by secondary ionization mass spectrometry was measured in a VG ZAB SE mass spectrometer. The N-terminal sequences of the peptides were determined in an automated sequencer (Model 473A, Applied Biosystems) and confirmed in particular the substitution of alanine at position 9 in the variant 38-mer.

Peptide 1–49 was synthesized using HOBt/DCC activation chemistry; no special modification was used for the coupling of Fmoc-Gla. Peptide 2–49 was obtained from this synthesis by removing a portion of peptide–resin from the reaction vessel just prior to the addition of residue 1. Peptides 1–38 and 1–38 R9A were cosynthesized using HOBt/HBTU activation chemistry. At the synthetic cycle where residue 9 was to be coupled to the growing peptide chain, the acylating reagent was a 4:1 mixture of Fmoc-Arg(pmc)-OH and Fmoc-Ala-OH. A subpopulation of molecules was thus created with the substitution of alanine at position 9 for the arginine found in the wild type. Cleavage of the peptides from the resin and side-chain deprotection were carried out for 3 h at room temperature in the mixture trifluoroacetic acid/water/ethanedithiol/thioanisole/phenol, 40:2:1:2:3, v/v/v/v/w. The extended time was necessary to fully cleave the pentamethylchroman groups protecting the arginine side chains. Crude peptide was precipitated from the supernatant with ether and washed twice with ether. Initial purification of the peptides was carried out by ion-exchange on DEAE-Sepharose CL-4B (Pharmacia Ltd., Milton Keynes, U.K.) using a gradient of ammonium acetate from 0.05 to 1.0 M at pH 7.5. Further purification was achieved by high-performance liquid chromatography on a C8 reversed-phase column using a gradient of acetonitrile in 0.1% v/v trifluoroacetic acid.

Tissue Factor Relipidation. Typically 5 μL of 10 μM TF1–243 was incubated at 37 °C with 25 μL of 99:1 rabbit brain cephalin/0.25% deoxycholate, 25 μL of TBS-A, and 2.5 μL of 100 mM $CdCl_2$ for 30 min. This solution was then diluted as required.

Tissue Factor/Factor VIIa Amidolytic Assay. Tissue factor/factor VIIa amidolytic activity was determined as follows: FVIIa (final concentration 2 nM) was incubated at 37 °C with sTF (final concentration 20 nM) or relipidated TF apoprotein (final concentration 1.74 nM) in the presence of varying concentrations of Gla peptide and 800 μM S2288. All reagents were diluted in TBS to which 0.1% w/v bovine serum albumin had been added (TBS-A). $CaCl_2$ was added to a final concentration of 5 mM to start the reaction and the reaction stopped after 65 min by the addition of glacial acetic acid. Absorbance was measured at 405 nm. TF apoprotein was relipidated as described above.

Activation of Factor X by Tissue Factor/Factor VIIa. Factor X activation by tissue factor/factor VIIa was determined as follows: FVIIa (final concentration 34 pM) was incubated with relipidated TF apoprotein (final concentration 1.74 nM), FX (final concentration 56 nM), and phospholipids (rabbit brain cephalin, final dilution, 1:300 of stock solution) in the presence of varying concentrations of Gla peptide. $CaCl_2$ was added to start the reaction and the mixture incubated at 37 °C for 10 min. S2222 was added and the mixture incubated for a further 2 min before being stopped with glacial acetic acid. Absorbance was measured at 405 nm. Similarly, FVIIa (final concentration 0.85 nM) was incubated at 37 °C with sTF (final concentration 17 nM), FX (final concentration 56 nM), and phospholipids (rabbit brain cephalin, final dilution, 1:300 of stock solution). $CaCl_2$ was added to start the reaction and the mixture incubated at 37 °C for 60 min. S2222 was added and the mixture incubated for a further 2 min before being stopped with glacial acetic acid. Absorbance was measured at 405 nm.

Activation of Bovine Factor X by FVIII/FIXa. FVIII/FIXa activation was assayed using reagents from the Chromogenix Coatest FVIII:C/4 kit. Solutions were prepared according to the manufacturer's instructions. The FIXa/FX solution contains 6 nkat/mL bovine FX and bovine FIXa. The concentrations and activities of FIXa and phospholipid are proprietary information and have not been released by the manufacturer. The assay was performed as follows: 25 μL of a 1:80 dilution of normal pool plasma in the proprietary dilution buffer was incubated for 5 min at 37 °C with 50 μL of 5:1 FIXa/FX/phospholipid. Twenty-five microliters of Gla peptide and 25 μL of 25 mM $CaCl_2$ were added, and the mixture was incubated for a further 5 min; 25 μL of 2 mg/mL S2222 containing 33.5 μg/mL thrombin inhibitor I2581 was added. The reaction was terminated 5 min later by the addition of 25 μL of acetic acid. The absorbance was measured at 405 nm.

Factor Xa Amidolytic Assay. Various dilutions of FXa were incubated with 6.25 mM $CaCl_2$ and 1 mM S2222 in the presence or absence of 1.58 μM GP 1–49. The reaction was stopped with glacial acetic acid and the absorbance measured at 405 nm.

Biotinylation of FX. FX was biotinylated essentially as described previously (O'Brien et al., 1993) for FVIIa; 75 μL of FX (0.8 mg/mL) was incubated with 25 μL of sulfo-NHS-biotin (4 mg/mL in imidazole-buffered saline, pH 8.0) for 60 min. The biotinylated FX (bt-FX) was purified by gel filtration on a Sephadex G-25 fast desalting column in TBS/10% glycerol and stored at −20 °C until required.

Interaction of FX with Phospholipid Surfaces. The interaction of FX with phospholipids was studied using a modification of the FX and FIX binding assay described by Bloom (1989). Phospholipids were dissolved to a concentration of 0.4 mg/mL in 60:40 CH_3Cl/MeOH; 100 μL of a dilution in methanol (final concentration 2 μg/mL) was added to each well of a microtiter plate. The wells were duplicated with wells containing solvent only. The plates were allowed to air-dry overnight in the dark. Nonspecific protein binding sites were blocked by incubation with 250 μL of TBSA for

Table I: Mass Spectrometric Analysis of Synthetic Peptides

peptide[a]	calcd mass[b] (MH^+)	obsd mass (MH^+)
GP 1–49	6241.5	6242.6
GP 1–49 (33–49)[c]	2076.2	2075.3
GP 1–49 (23–49)[c]	3445.6	3444.9
GP 2–49	6170.4	6170.4
GP 1–38	4929.1	4928.6
GP 1–38 R9A	4844.0	4842.2

[a] All disulfide bridges are present. [b] Isotopic averaged mass. [c] Partial synthesis product.

2 h at room temperature, followed by incubation with TBSA containing 25 mM $CaCl_2$ for 30 min. The wells were washed 5 times with 250 μL of TBS containing 25 mM $CaCl_2$ and 0.05% Tween 20 (TBS-TC); 100 μL of a mixture containing bt-FX (final concentration 150 nM) and various concentrations of GP 1–49 in TBSA containing 25 mM $CaCl_2$ was added to the wells and allowed to incubate at room temperature for 30 min. After five rapid washes with 250 μL of TBS–TC, 100 μL of streptavidin–horseradish peroxidase conjugate (0.5 μg/mL) was added and allowed to incubate for 10 min. After five rapid washes, 200 μL of *o*-phenylenediamine dihydrochloride (0.2 mg/mL in phosphate/citrate buffer, pH 5.0) was added and color allowed to develop. Color development was halted by the addition of 50 μL of 3M sulfuric acid and the absorbance measured at 492 nm.

RESULTS

Peptide Synthesis. Crude peptide products were insoluble in water and acidic media and were initially purified by ion-exchange chromatography in aqueous ammonium acetate at pH 7.5. Peptide 1–49 was eluted by 0.55 M ammonium acetate, and peptide 2–49 by 0.59 M. The two 38-mers prepared by cosynthesis (1–38 and 1–38 R9A) separated in this system, being eluted by 0.40 and 0.43 M ammonium acetate, respectively. The four peptides 1–49, 2–49, 1–38, and 1–38 R9A were then separately purified to homogeneity by HPLC, the four peptides being eluted by 37%, 29%, 26%, and 25% acetonitrile, respectively. Structures of the pure products were confirmed by a combination of amino acid analysis, mass spectrometry, and peptide sequencing. The mass spectrometry data are summarized in Table I. This analysis confirmed that no degradation of the molecule had taken place during deprotection and that all the protecting groups had been removed. In addition, internal sequences covering the whole of the 49-mer were obtained by removing portions of peptide resin at intervals during its synthesis and sequencing these intermediate products (data not shown). The presence of alanine at position 9 in the variant peptide 1–38 R9A was also directly confirmed by sequencing (data not shown). Peptide 1–49 showed no reaction to dithionitrobenzoate (Ellman's reagent) after HPLC purification, indicating that the disulfide bond linking residues 17 and 22 had formed spontaneously (Ellman, 1959). This was confirmed by two sequencing studies after vapor-phase reaction with 4-vinylpyridine (Amons, 1987) with and without the presence of the reductant tri-*n*-tertiary butylphosphine. With reduction, cycles 17 and 22 of sequencing yielded the (pyridylethyl)-cysteine product of reaction of 4-vinylpyridine with cysteine. The peaks are indicated in the lower panel of Figure 1. Without reduction, no pyridylethylation product was observed at these cycles, demonstrating that no free thiols were present and indicating that a disulfide bridge linked those two residues (Figure 1).

Factor X Activation Assays. Incubation of each peptide at a variety of concentrations in a FX activation assay using

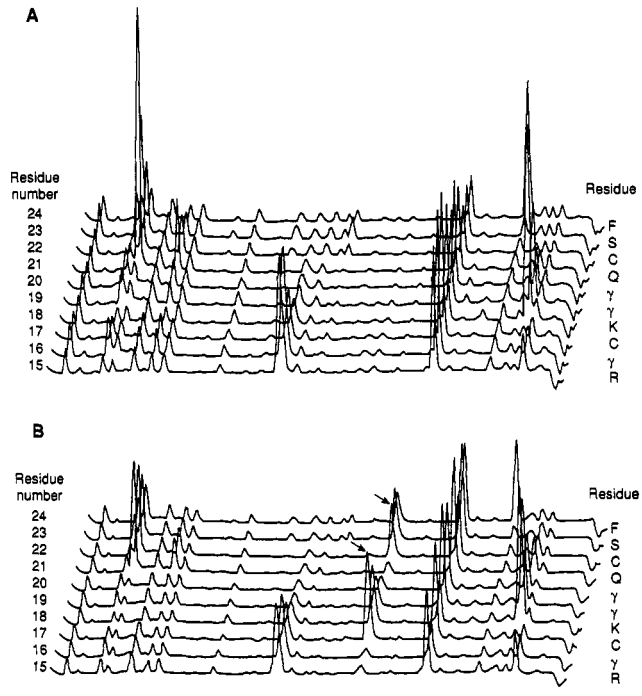

FIGURE 1: Chromatograms from cycles 17–26 (residues 15–24) of (A) unreduced pyridylethylated GP 1–49 and (B) reduced and pyridylethylated GP 1–49. The (pyridylethyl)cysteine residues observed at cycles 19 and 24 in the reduced experiment are indicated by arrows.

sTF or relipidated TF showed inhibition of TF/FVIIa-catalyzed FXa generation (Figure 2A,B). Inhibition of sTF/FVIIa-catalyzed FXa generation occurred with K_i's of 365 nM for GP 1–49, 689 nM for GP 1–38, 5.625 μM for GP 1–38 R9A, and 10.95 μM for GP 2–49. Similarly, inhibition of relipidated TF/FVIIa-catalyzed FXa generation occurred with K_i's of 988 nM for GP 1–49, 5.63 μM for GP 1–38, 23.1 μM for GP 1–38 R9A, and 34.1 μM for GP 2–49. These results are summarized in Table II.

The activation of FX by sTF/FVIIa in the absence of phospholipid was markedly slower than in the presence of phospholipid. This reaction was not inhibited by GP 1–49 at concentrations up to 20 μM. FXa generation by FVIII/FIXa was also inhibited by GP 1–49 at a similar concentration to relipidated TF/FVIIa (Figure 2C).

Amidolytic Assays. GP 1–49 showed no inhibition of TF/FVIIa amidolytic activity at inhibitor concentrations up to 25 μM and similarly showed no inhibition of FXa amidolytic activity at inhibitor concentrations up to 31 μM (data not shown). It is thus apparent that the inhibitory mechanism of these peptides involves prevention of macromolecular substrate recognition and substrate/enzyme complex assembly rather than alteration of the catalytic nature of the enzyme or prevention of TF/FVIIa complex formation, and that the Gla peptide inhibited FX activation rather than FXa activity.

Lineweaver–Burk Plots. Two concentrations of GP 1–49 were incubated with varying concentrations of FX in an activation assay, similar to that described above using relipidated TF apoprotein, to examine the substrate dependency of the inhibition. The Lineweaver–Burk plot obtained (Figure 3) shows an intercept on the *x* axis, corresponding to a K_m for FX in this assay of 3.25 nM. The intercept on the *x* axis indicates a change in the apparent k_{cat} for the complex rather than a change in the K_m, indicating a noncompetitive mode of inhibition.

Further Analysis. Plots of inhibitor concentration *vs* inhibition/activity ratio [[I] *vs* $i/(1-i)$] were constructed for

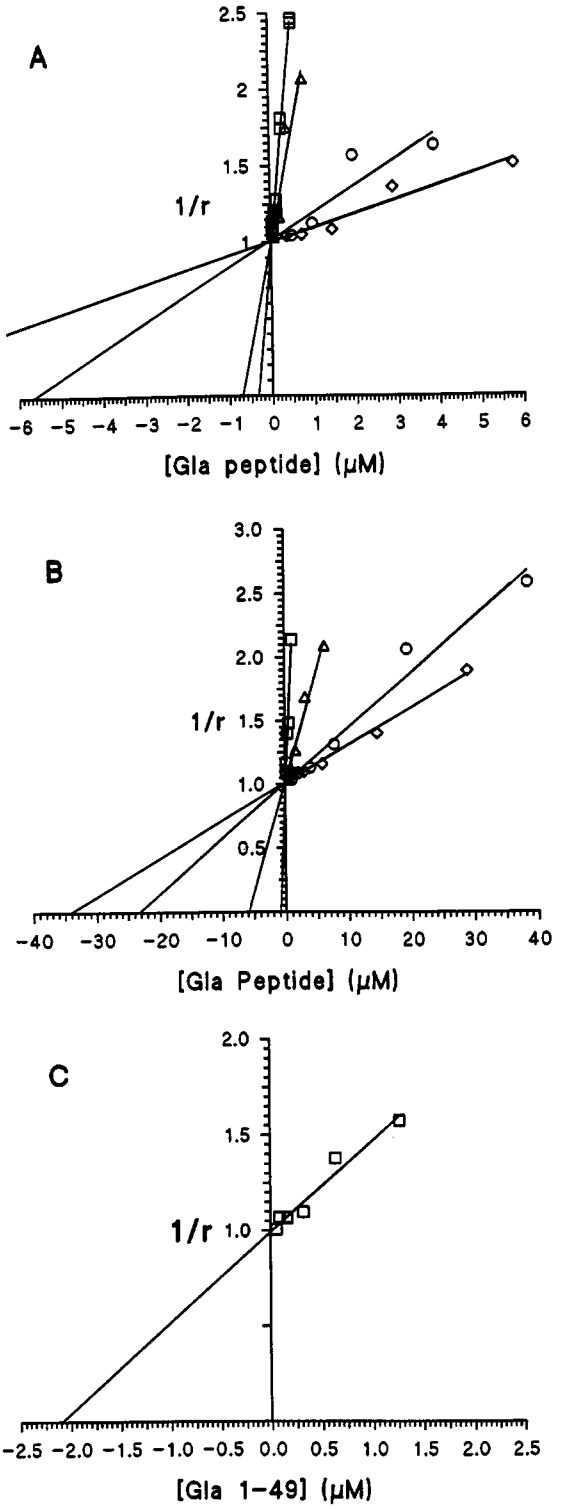

FIGURE 2: K_i determinations of (□) GP 1–49, (△) GP 1–38, (○) GP 1–38 R9A, and (◇) GP 2–49. r is the residual activity. K_i is determined as 1/gradient. Panel A: sTF/FVIIa-catalyzed FX activation. Panel B: relipidated TF_{243}/FVIIa-catalyzed FX activation. Panel C: FVIII/bovine FIXa-catalyzed bovine FX activation.

GP 1–49 (Figure 4). This plot can demonstrate the mechanism of inhibition as described by Reiner (1969). Substrate-oriented inhibition can occur by one of three methods. In the first, the substrate binding site is competitively inhibited. This gives a straight line passing through the origin in an inhibitory analysis. In the second, the inhibitor combines with the substrate to give a nonproductive binding complex. A bilinear curve is seen with the gradient at low i less than the gradient of the asymptote. The extrapolation of the asymptote to the

Table II: Inhibition of Various FX Activating Systems by FVII Gla Domain Variants

peptide	FVIII/FIXa K_i (μM)	TF_{220}/FVIIa K_i (μM)	TF_{220}/FVIIa act. ratio[a]	TF_{243}/FVIIa K_i (μM)	TF_{243}/FVIIa act. ratio[a]
GP 1–49	2.12	0.365	100	0.988	100
GP 1–38	n/d[b]	0.689	53	5.63	17.5
GP 1–38 R9A	n/d	5.625	6.5	23.1	4.3
GP 2–49	n/d	10.95	3.3	34.1	2.9

[a] Activity ratio = $[K_i(\text{GP 1–49})/K_i(\text{variant})] \times 100$. [b] n/d, not determined.

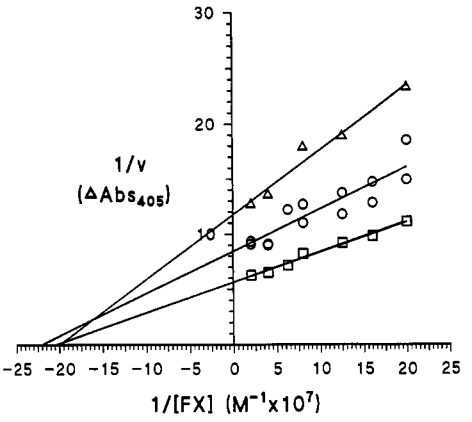

FIGURE 3: Lineweaver–Burk plot of FX activation by relipidated TF_{243}/FVIIa in the presence of (△) 1.056 μM or (○) 528 nM GP 1–49 or (□) no inhibitor.

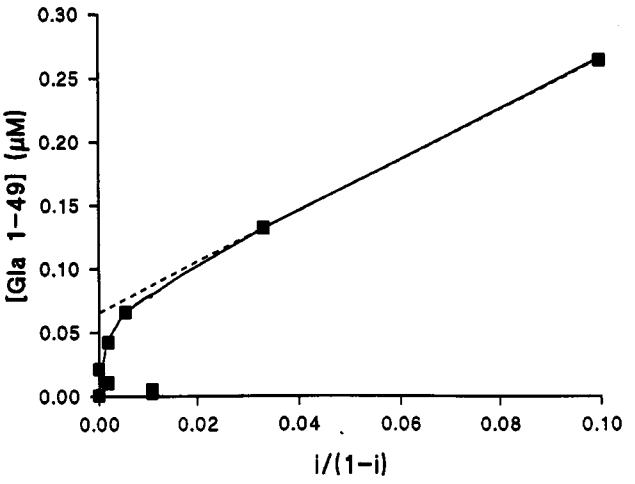

FIGURE 4: Inhibition ratio plot for GP 1–49 with TF_{243}/FVIIa.

y axis gives a negative intercept. The third mechanism also gives a biphasic curve. The substrate binds to the inhibitor and is prevented from binding to the enzyme, effectively being removed from the reaction mixture. In this case, the gradient of the asymptote is less than the initial gradient, and the y intercept of the extrapolation of the asymptote is positive and equal to the concentration of the substrate.

It can be observed from Figure 4 that the result of an inhibitory ratio analysis of GP 1–49 gives a graph corresponding to the latter of these mechanisms. The intercept of the asymptote occurs at a value equal to the total concentration of factor X in the reaction mixture. This analysis confirms that the substrate is being removed from the reaction mixture and supports the postulate that GP 1–49 directly interacts with FX.

Interaction of FX with Phospholipid Surfaces. Incubation of FX with various concentrations of GP 1–49 showed

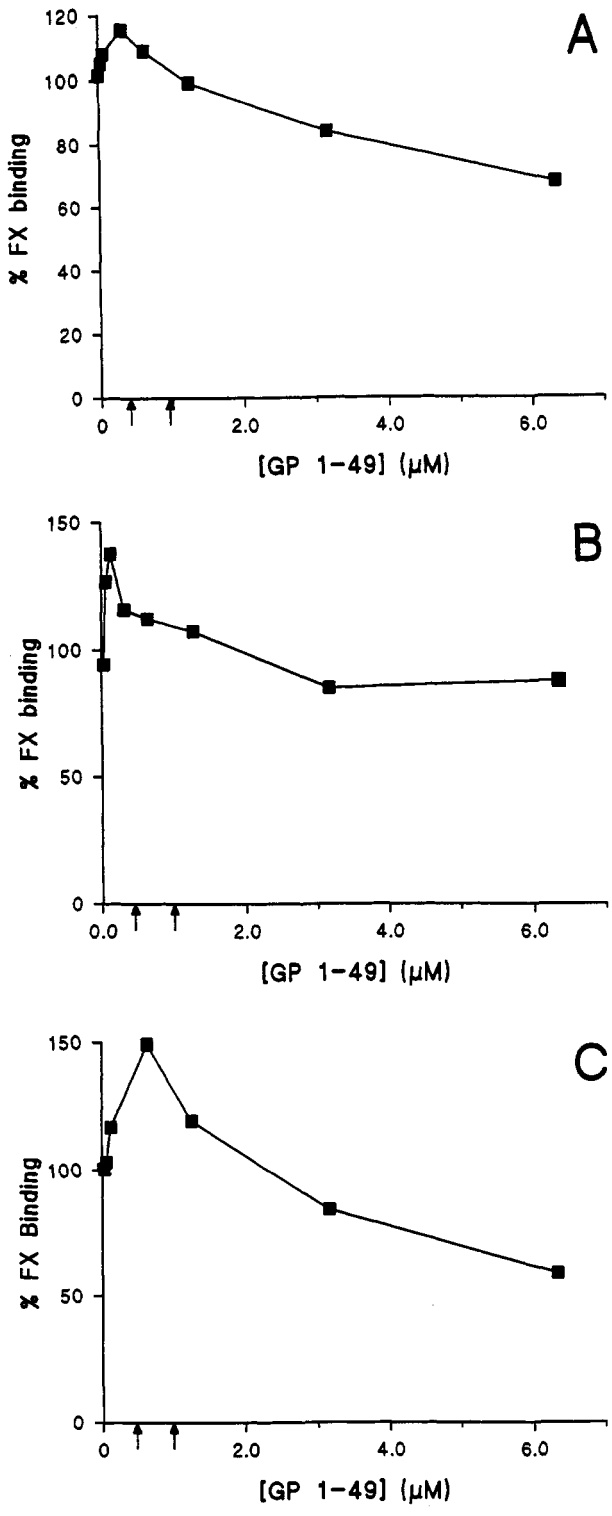

FIGURE 5: FX binding to phospholipid-coated microtitre well plates in the presence of varying concentrations of GP 1–49. (A) Phosphatidylserine; (B) phosphatidylcholine; (C) 30:70 w/w phosphatidylserine/phosphatidylcholine. The K_i's determined for GP 1–49 inhibition of TF/FVIIa-mediated FX activation are indicated by arrows on the x axis.

inhibition of FX binding to phospholipid surfaces with K_i's greater than 10 μM. No inhibition was observed at concentrations of GP 1–49 corresponding to the K_i of GP 1–49 inhibition of TF/FVIIa-mediated FX activation (Figure 5). A significant increase in FX binding was noticed around this concentration. The concentration of FX in these experiments was just saturating, so any increase in FX binding is due to an interaction between GP 1–49 and FX, allowing binding to sites to which FX alone cannot bind.

DISCUSSION

We have synthesized and purified to homogeneity four peptides corresponding to the wild-type and variant sequences of the FVII Gla domain. This is the first chemical production by peptide synthesis of any coagulation protease Gla domain. The peptides have been characterized and their integrity ascertained by mass spectrometry, amino acid analysis, and N-terminal sequencing. We have assayed the inhibitory activity of each of these peptides and have determined both the mechanism of inhibition and the molecular interactions for the most potent, corresponding to residues 1–49 of FVII.

It is apparent from the difference in potency between peptides GP 1–49 and GP 2–49 that residue 1 is an essential structural component, required for the full function of the Gla domain. Removal of the N-terminal alanine reduces the inhibitory activity of the peptide by several orders of magnitude. Examination of the crystal structure of the bovine prothrombin Gla domain (Soriano-Garcia et al., 1992) reveals hydrogen bonding between the N-terminal amino group of Ala-1 and Gla residues 17, 21, and 27. Maintenance of this hydrogen bonding by peptide 2–49 would require a disruption in the conformation of the ω-loop-like structure formed by the N-terminal 12 residues of the peptide. The homologous region (residues 3–11) has been shown to be important in protein–protein interactions in FIX (Cheung et al., 1992). Busby et al. have reported a FVII mutant with an additional N-terminal serine residue at position 0 with a lower coagulant activity than wild-type FVII (Busby et al., 1988). The reduction in activity observed may be due to the presence of the additional serine residue or the incomplete γ-carboxylation of this variant.

We have built a model of the calcium-bound form of the FVII Gla domain using the coordinates of the prothrombin Gla domain (kindly supplied by Dr. A. Tulinsky, Michigan State University) (Figure 6). The N-terminal alanine residue is indicated. Deletion of this residue would cause a distortion in the ω-loop, required to maintain the hydrogen bonding between the new N-terminus (Asn-2) and the Gla side chains. This distortion may also sterically preclude the effective coordination of the Ca^{2+} ions by the Gla side chains. Welsch and Nelsestuen have demonstrated the importance of the N-terminal hydrogen bonds in prothrombin Gla by chemical modification (Welsch & Nelsestuen, 1988). Acetylation of the N-terminus or removal of the N-terminal residue, probably disrupting the structure of the N-terminal loop, abolished the membrane binding function of the molecule.

By comparison to the crystal structure of bovine prothrombin which is essentially homologous, arginine-9 would be expected to form part of a solvent-exposed, positively charged surface region. Mutation of this residue to alanine would not be expected to disrupt the loop backbone structure. This mutation may, however, disrupt the putative intermolecular interactions mediated by the ω-like loop, reducing the inhibitory activity of the peptide. It can be concluded from our data that this arginine residue has an essential function, though whether for the structural integrity of the Gla domain fold or, more probably, for its interaction with other macromolecules remains to be determined. The presence of the highly conserved α-helical aromatic stack region (residues 39–49) of the FVIIa light chain also enhances the inhibitory activity of the Gla domain. A study using chimeras of FIX/FX has indicated that neither the aromatic stack region nor the first EGF-like

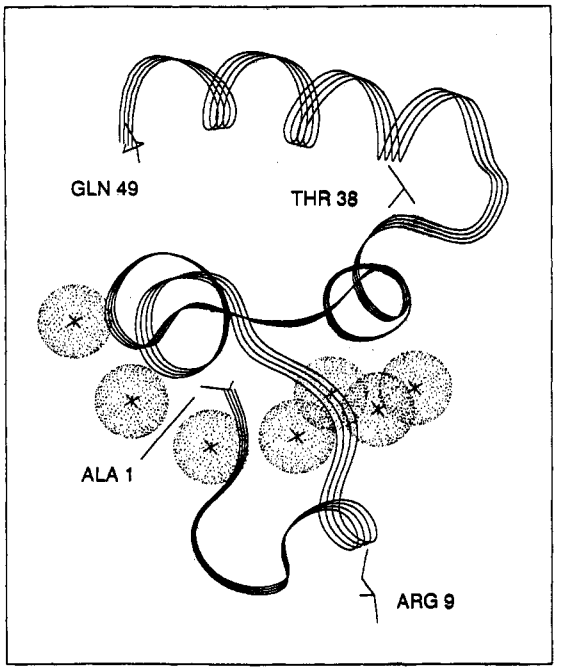

FIGURE 6: Ribbon backbone of the FVII Gla domain modeled on the crystal structure of prothrombin fragment 1 (coordinates kindly provided by Dr. A. Tulinsky). van der Waals surfaces of the Gla-chelated calcium ions are shown. Residues 1, 9, 38, and 49 are indicated.

domain has a significant role to play in determining the specificity of FIX interactions with endothelial cells (Cheung et al., 1991). Whether the enhancement in inhibitory activity is mediated via an enhancement of the packing of the α-helices formed by residues 25–31 and 14–17 and hence stabilizing the formation of the ω-like N-terminal loop, or whether it has a function *per se* in the interactions of the Gla domain remains to be determined.

In an earlier report concerning the FVIIa Gla domain (residues 1–38), produced as a byproduct of recombinant FVIIa production (Wildgoose et al., 1992), the inhibition of tissue factor/FVIIa-catalyzed FX activation was observed. Differences in the inhibitory effect of the Gla were also noted, depending on whether cell-surface tissue factor or relipidated TF was used. The values obtained (0.5 μM using relipidated TF and 2.7 μM using cell-surface TF) are similar to those obtained in this study (0.689 μM using sTF, 5.63 μM using relipidated TF). Minor variations between the values reported for different TF variants by ourselves and others are probably due to differences in the assay methods. Wildgoose et al. also implicated the FVII Gla domain in the induction of subtle structural changes in the factor VII protease domain necessary for full TF binding. Ruf and coworkers have examined both full-length and *des*-1–38-FVIIa and concluded that the Gla domain is not essential for the expression of FVIIa catalytic activity but is essential for extended substrate recognition, and is involved in the binding of FVIIa to membrane-associated TF (Ruf et al., 1991).

By using a range of assays and synthetic peptides representing the whole FVII Gla domain, we have extended these studies.

We have confirmed that the inhibition of FX activation is not due to inhibition of the TF/FVIIa interaction by demonstrating that GP 1–49 does not inhibit TF-dependent FVIIa amidolytic activity. We have also shown that FX activation by FVIII/FIXa can be inhibited by GP 1–49. From these data, it can be seen that the Gla peptides can inhibit FX activation either by a direct interaction with FX or by interactions with both FVIIa/TF and FVIII/FIXa to a similar extent.

It was observed that the inhibitory mechanism is noncompetitive. The inhibitory ratio analysis provides support for a direct Gla peptide/FX interaction, the Gla peptide forming a heterodimer with FX, preventing FX from acting as a substrate for FVIIa and hence giving rise to a noncompetitive mechanism of inhibition. It is plausibly by this mechanism that the Gla peptide inhibits the FVIIIa/FIXa-catalyzed activation of FX. Inhibition via sequestration of phospholipid binding sites would give rise to a competitive mode of inhibition. The binding of FX to phospholipid-coated microtiter plates is not inhibited by GP 1–49 at concentrations around the K_i of inhibition of TF/FVIIa-mediated FX activation. This precludes inhibition occurring via sequestration of phospholipid binding sites, and hence provides support for a model of inhibition involving the formation of a complex of GP 1–49 with phospholipid-associated FX. The inhibitor–substrate complex formed is not a substrate for TF/FVIIa. Dimerization of the coagulation proteases via their Gla domains has previously been postulated by Harlos and co-workers (Harlos et al., 1987). Although these authors have demonstrated the formation of prothrombin fragment 1 homodimers and prothrombin fragment 1/FX heterodimers using chemical cross-linking, they were unable to demonstrate the interaction between FX and FVIIa. Other studies have implicated the Gla domains of other vitamin K-dependent coagulation enzymes in the high-affinity binding to cell surfaces, essential for the interaction of FX and FIX (Rawala-Sheikh et al., 1992; Derian et al., 1989; Astermark & Stenflo, 1991).

The catalytic activation of FX by TF/FVIIa shows no absolute requirement for phospholipid. However, the increase in catalytic activity in the presence of phospholipid suggests that part of the interaction between FVIIa and FX in the formation of the enzyme–substrate complex is stabilized by an interaction with phospholipid. The phospholipid dependence of Gla peptide-mediated inhibition implies that complex formation by the Gla peptide with FX occurs via a similar interaction.

We conclude from this analysis that the Gla peptide sequesters FX in such a manner as to prevent it interacting with FVIIa, thus inhibiting FX activation. The phospholipid-dependent nature of this inhibition, paralleling the difference in TF/FVIIa-catalyzed FX activity in the presence and absence of phospholipid, provides strong evidence that the Gla domain of FVII binds preferentially to FX bound to phospholipid. We have shown that, at concentrations <5 μM, GP 1–49 does not inhibit FX binding to phospholipid surfaces. It has been previously postulated that binding of FX to phospholipid exposes a FVIIa binding epitope, promoting rapid FX activation (Krishnaswamy et al., 1992). We conclude that the isolated FVIIa Gla domain binds to FX in its phospholipid-bound conformation, to prevent substrate recognition by the enzyme. This supports a model whereby the binding of FVIIa to phospholipid-bound FX is, at least in part, mediated via the FVIIa Gla domain. This binding function of the Gla domain does not preclude its participation in maintaining the conformational integrity of FVII required for tissue factor binding.

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