The role of the Gla domain of human prothrombin in interaction with the prothrombinase complex was studied using a peptide with the sequence of the first 46 residues of human prothrombin, PT-(1–46). Intrinsic fluorescence measurements showed that PT-(1–46) undergoes a conformational alteration upon binding calcium; this conclusion is supported by one-dimensional $^1$H NMR spectroscopy, which identifies a change in the chemical environment of tryptophan 41. PT-(1–46) binds phospholipid membranes in a calcium-dependent manner with a $K_c$ of 0.5 μM and inhibits thrombin generation by the prothrombinase complex with a $K_c$ of 0.8 μM. In the absence of phospholipid membranes, PT-(1–46) inhibits thrombin generation by factor Xa in the presence but not absence of factor Va, suggesting that PT-(1–46) inhibits prothrombin-factor Va binding. The addition of factor Va to PT-(1–46) labeled with the fluorophore sulfosuccinimidyl-7-amino-4-methylcoumarin-3-acetic acid (PT-(1–46)AMCA) caused a concentration-dependent quenching of AMCA fluorescence, providing direct evidence of a PT-(1–46)-factor Va interaction. The $K_c$ for this interaction was 1.3 μM. These results indicate that the N-terminal Gla domain of human prothrombin is a functional unit that has a binding site for factor Va. The prothrombin Gla domain is important for interaction of the substrate with the prothrombinase complex.

Prothrombin, a vitamin K-dependent protein, is the zymogen form of thrombin, the final enzyme generated during blood coagulation. Prothrombin is activated to thrombin by the enzyme factor Xa in the presence of phospholipid membranes and factor Va. Although factor Xa is able to convert prothrombin to thrombin in solution, the presence of phospholipid membranes and factor Va results in a 100,000-fold acceleration in thrombin generation allowing this enzymatic reaction to proceed at a physiologically relevant rate (1). Phospholipid membranes decrease the apparent $K_c$ of factor Xa for prothrombin approximately 100-fold, and factor Va increases the $k_{cat}$ of factor Xa for prothrombin approximately 3000-fold (1).

Prothrombin is composed of five domains; an N-terminal γ-carboxyglutamic acid (Gla)$^3$ domain, an aromatic amino acid stack domain, two kringle domains, and a C-terminal serine protease domain. The N-terminal Gla domain contains 10 Gla residues that are responsible for the calcium binding properties of prothrombin. The Gla domain defines the binding of vitamin K-dependent proteins to phospholipid membranes (for review, see Refs. 2 and 3).

Upon binding calcium ions, prothrombin undergoes two sequential conformational alterations, one can be detected spectroscopically (4), and both can be detected with conformation-specific antibodies (5). The second conformational change, which is supported only by calcium or strontium ions, is necessary to allow prothrombin to bind phospholipid membranes (6). The crystal structure of bovine prothrombin fragment I, which comprises the Gla, aromatic amino acid stack, and first kringle domains, has been solved both in the absence (7) and presence of calcium ions (8). The latter structure demonstrates that five calcium ions are chelated by six Gla residues (Gla$^8$, Gla$^{15}$, Gla$^{17}$, Gla$^{26}$, Gla$^{27}$, Gla$^{30}$), rendering 4 of the calcium residues inaccessible to solvent. Two additional calcium ions are bound to Gla residues 15, 20, and 21, and remain exposed to solvent. The dequaternization of calcium ions in the interior of the protein results in the burying of the N-terminal alanine and contributes to the structural stability of the amino-terminal domain (8). In human prothrombin, site-directed mutagenesis studies demonstrate that Gla$^{16}$, Gla$^{26}$, and Gla$^{29}$ (equivalent to Gla$^{15}$, Gla$^{27}$, and Gla$^{30}$ in bovine prothrombin) are critical for phospholipid binding (9).

The Gla domain of the vitamin K-dependent proteins serves functions in addition to mediating phospholipid binding. A mutation in the Gla domain of factor IX (Gly$^{12} \rightarrow$ Arg) results in defective activation of factor X by the complete tenase complex in the presence but not absence of factor VIIIa (10). A mutation in tissue factor, the cofactor of factor VIIa, results in defective activation of factor X but not Gla-domainless factor X by the factor VIIa-tissue factor complex (11). Furthermore, computational protein-protein docking analysis of the factor VIIa-tissue factor-factor Xa complex suggests interactive sites between the Gla domain of factor Xa and tissue factor-factor VIIa (12). Although the N-terminal 12 residues of the Gla domain are important for phospholipid binding (13), the C-terminal region of the Gla domain may be involved in protein-protein interactions. A chimeric protein C molecule in which the Gla domain of the protein was replaced with the corresponding region of prothrombin renders activated protein C activity independent of its cofactor, protein S. This suggests that the Gla domain of protein C is required for protein S

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1. The abbreviations used are: Gla, γ-carboxyglutamic acid; AMCA, sulfosuccinimidyl-7-amino-4-methylcoumarin-3-acetic acid; HPLC, high pressure liquid chromatography; MALDI-TOF, matrix-assisted laser desorption time of flight; ppm, parts per million.
cofactor activity. This protein S-independent activity has been localized to residues 23–46 of the Gla domain of protein C (14, 15). Domains within prothrombin that have been shown to bind to factor Va include the kringle 2 domain (16) and the kringle 1 domain (17). However, the Gla domain of prothrombin may be important for factor Va binding when these proteins are assembled on membranes. Such a hypothesis is consistent with factor Va and prothrombin interacting through several contact sites located in different domains. This paradigm has been demonstrated in the factor VIIa-tissue factor complex, in which the crystal structure identifies multiple interactive sites between the two proteins, including a hydrophobic interaction between the C-terminal helix of the Gla domain of factor VIIa and the second fibronectin domain of tissue factor (18).

To gain insight into the structure/function relationships of the Gla domain of prothrombin, we have synthesized and characterized the properties of a peptide comprising the Gla and aromatic amino acid stack domains of human prothrombin. The results demonstrate that this peptide binds calcium, binds to phospholipid membranes, and inhibits the prothrombinase complex. Using this synthetic Gla domain, we demonstrate a direct interaction between the Gla domain of human prothrombin and factor Va.

**Experimental Procedures**

**Materials**—Human prothrombin, human factor IXa, human factor Xa, and human factor Va were purchased from Hematologic Technologies. Chromogenic substrate S-2238 was purchased from Diapharma Group Inc. Phosphatidyserine and phosphatidylcholine were obtained from Avanti Polar Lipids. Sulfo succinimidyl-7-amine-4-methylcoumarin-3-acetic acid (AMCA) was purchased from Pierce. Rabbit IgG was purchased from Sigma. Rabbit IgG F(ab)2 fragments were prepared as described previously (19) with the following modifications. The cleavage reaction was performed in trifluoroacetic acid/1,2-ethanediol/thioanisole/water/phenol (10:2.5:5:5:5, v/v) for 5 h at 25 °C. A cleavage reaction was performed in trifluoroacetic acid/1,2-ethanediol/thioanisole/water/phenol (10:2.5:5:5:5, v/v) for 5 h at 25 °C. A cleavage reaction was performed in trifluoroacetic acid/1,2-ethanediol/thioanisole/water/phenol (10:2.5:5:5:5, v/v) for 5 h at 25 °C.

**Synthesis of PT-(1–46)**—PT-(1–46) was synthesized as described previously for factor IX-(1–47) (19) with the following modifications. The cleavage reaction was performed in trifluoroacetic acid/1,2-ethanediol/thioanisole/water/phenol (10:2.5:5:5:5, v/v) for 5 h at 25 °C. A cleavage reaction was performed in trifluoroacetic acid/1,2-ethanediol/thioanisole/water/phenol (10:2.5:5:5:5, v/v) for 5 h at 25 °C. A cleavage reaction was performed in trifluoroacetic acid/1,2-ethanediol/thioanisole/water/phenol (10:2.5:5:5:5, v/v) for 5 h at 25 °C.

**Measurement of Phospholipid Binding**—The binding of PT-(1–46) to phospholipid vesicles was evaluated by 90° light scatter according to the method of Nelsestuen and Lim (24) on an SLM 8000C fluorescence spectrophotometer. The peptide was dissolved in 150 mM NaCl, 50 mM Tris-HCl, pH 7.4, 2 mM CaCl₂. Aliquots of the peptide were added to 3 ml of buffer containing 9.4 μM small unilamellar phospholipid vesicles (PC:PS, 60:40) to achieve the final peptide concentration shown. The samples were excited at a wavelength of 420 nm and emission was monitored at a wavelength of 320 nm with a slit width of 16 nm. Dissociation constants were calculated according to the equations of Lim et al. (24):

\[
\frac{(I_fO_f) - 2}{M2/M1} = \frac{[PT-(1–46):PL_1]}{[PL_1/n]} \tag{1}
\]

\[
\frac{M2}{M1} \left( \frac{Msat}{M1} - 1 \right) = \frac{[PT-(1–46):PL_1]}{[PL_1/n]} \tag{2}
\]

where \(I_fO_f\) is the scatter intensity of phospholipid plus protein corrected for increases in scatter intensity due to the protein itself. Io is the scatter intensity of phospholipid alone corrected for the decrease in intensity due to dilution with each addition of protein, and M2/M1 is the signal due to light scattering of PT-(1–46) bound to phospholipid when all the binding sites for PT-(1–46) are occupied. Dissociation constants were calculated using a simple bimolecular model analogous to that used by Gilbert et al. (25):

\[
K_d = \left( \frac{[PT-(1–46)] + [PL_1/n]}{[PT-(1–46):PL_1]} \right) \left( \frac{[PL_1/n]}{2[PL_1/n]} \right) \tag{3}
\]

The initial value of M2/M1 is adjusted to zero by subtraction of 1. M2/M1 is similarly normalized. PT-(1–46), is the total concentration (μM) of peptide. The equilibrium binding constant, \(K_d\), was determined by fitting the light scattering data to the above equation using non-linear regression analysis with SigmaPlot for Windows 4.0 (SPSS).

**Metal-induced Quenching of Intrinsic Fluorescence**—Fluorescence quenching experiments were performed at 25 °C using an SLM 8000C fluorescence spectrophotometer. PT-(1–46) (4.4 μM) was dissolved in 20 mM Tris-HCl, pH 7.4, previously treated with Chelex 100. The sample was heated to approximately 50 °C at a neutral pH of 7.0 in the presence of Chelex 100 to ensure that all trace metal ions were removed. Spectra were collected on a Bruker AMX-500 spectrometer with a proton frequency of 500.14 MHz. One-dimensional spectra were acquired at 25 °C with 16,000 real data points, 2,048 summed scans, and a spectral width of 12,045.1 Hz. Following data acquisition, each spectrum was processed by applying a squared sine bell window function, shifted by 30°. The carrier frequency was set on the water resonance, which was suppressed using presaturation.

**Kinetic Analysis**—The prothrombinase assay was a modification of that of Rosing et al. (1). The assay was performed in Titertek (ICN Biomedical Inc., Aurora) microtiter plates at 25 °C. The reaction mixture (40 μl) contained 0.1 mM factor Xa, 5 mM factor Va, 1.25 μM phospholipid vesicles (PC:PS, 60:40), and varying concentrations of PT-(1–46) in 150 mM NaCl, 20 mM HEPES, pH 7.4, 2 mM CaCl₂, and 0.1% bovine serum albumin. The reaction was initiated by the addition of 20 μl of prothrombin (final concentration, 300 nM) and stopped 60 s later by adding 20 μl of buffer containing 150 mM NaCl, 20 mM HEPES, pH 7.4, 20 mM EDTA, and 0.1% bovine serum albumin. Thrombin activity was measured at 25 °C using the chromogenic substrate S-2238 (0.25 mg/ml) on a Molecular Devices enzyme-linked immunosorbent...
assay plate reader.

For assays performed in the absence of phospholipid membranes, the concentration of factor Xa was 1.7 nM when factor Va (1.56 nM) was present and factor Xa was 5 nM when factor Va was omitted. The concentration of prothrombin was 300 nM for both conditions. The reaction was stopped after 60 min. For assays performed in the absence of factor Va but in the presence of phospholipid membranes, the reaction time was 5 min. Reactant concentrations were 1 mM factor Xa, 1.25 μM phospholipid vesicles, and 300 nM prothrombin.

The K_i for PT-(1–46) inhibition of prothrombin activation by prothrombinase was determined graphically using the method of Dixon (27). Reactions were performed with 0.1 mM factor Xa, 5 mM factor Va, 1.25 μM phospholipid vesicles at three different concentrations of prothrombin, namely, 50, 75, and 100 nM in the presence of varying concentrations of PT-(1–46) as indicated. A K_i for PT-(1–46) inhibition of factor Xa (1 mM) activation of prothrombin in the absence of factor Va was calculated in a similar fashion except the concentrations of prothrombin were 100, 400, and 800 nM and phospholipid vesicles were 1.25 μM. A K_i for PT-(1–46) inhibition of factor Xa (5 mM) activation of prothrombin in the absence of phospholipid membranes was also determined. Reactant concentrations were 5 mM factor Xa, 25 mM factor Va, and 150, 300, or 450 nM prothrombin.

Labeling of PT-(1–46) with AMCA—To label PT-(1–46) with AMCA-Sulfo-N-hydroxysuccinimide, 100 μg of peptide was dissolved in 200 μl of 150 mM NaCl, 50 mM sodium borate, pH 8.5, and 1 mM CaCl_2 AMCA (10 μl, 0.67 mg/ml in water) was added, and the mixture was incubated at 25 °C for 60 min shielded from light. The reaction was stopped with 100 μl of 0.2 mM Tris-HCl, pH 7.4, and the resulting product was dialyzed against Tris-buffered saline, pH 7.4, to remove any free AMCA. PT-(1–46)AMCA (150 μg) in 150 mM NaCl, 50 mM Tris-HCl, pH 7.4, was added to a gel filtration column (D-Salt Extracellulose Desalting Column from Pierce, molecular weight cutoff 5000) and eluted with 150 mM NaCl, 50 mM Tris-HCl, pH 7.4. Fractions (0.5 ml) were collected, and the absorbance at 340 nm was measured. After labeling and dialysis, PT-(1–46)AMCA was subjected to analytical HPLC on a C18 column (Vydac, 2.2 × 25 cm) employing a linear gradient of 35–45% Buffer B (buffer A: 0.1% trifluoroacetic acid, water; Buffer B: 0.1% trifluoroacetic acid in acetonitrile) over 30 min. Peaks were detected with a fluorescence detector (Beckman) using an excitation wavelength of 350 nm and an emission wavelength of 450 nm. Collected peaks were analyzed by MALDI-TOF mass spectrometry on a Voyager linear MALDI-TOF spectrometer.

Measurement of Factor Va Binding to PT-(1–46)AMCA—Binding of factor Va to PT-(1–46)AMCA was monitored by quenching of AMCA fluorescence upon addition of factor Va using a Photon Technology International fluorescence spectrophotometer (Barnegat, NJ). The excitation wavelength was 350 nm with a slit width of 1 nm; emission scans were monitored from 400 to 500 nm with a slit width of 8 nm. Aliquots of factor Va in Tris-buffered saline, pH 7.4, 2 mM CaCl_2, 0.001% Tween 80 were added to 0.1 μM PT-(1–46)AMCA in the same buffer. Correction was made for changes in fluorescence signal due to the addition of buffer alone. Factor Va was added in aliquots of 0.25–0.5 μM up to a concentration of 3 μM. At the end of the titration, excess unlabeled PT-(1–46) was added to assess the reversibility of the fluorescence change. As a negative control, rabbit IgG was added to 0.1 μM PT-(1–46) up to a concentration of 3 μM. The binding affinity of factor Va for PT-(1–46) was calculated from fluorescence quenching data using a bimolecular equilibrium model as described previously (28).

RESULTS

A peptide consisting of the γ-carboxylated N-terminal 46 residues of human prothrombin, PT-(1–46), was synthesized by solid phase peptide synthesis using N-(9-fluorenyl)methoxycarbonyl/N-methylpyrrolidone chemistry (Fig. 1). Following synthesis, the peptide was air-oxidized at pH 8.0 at room temperature for 24 h and purified using reverse phase HPLC. Automated Edman degradation revealed that the synthetic peptide had the correct sequence; amino acid analysis also confirmed the expected amino acid composition of the peptide. The peptide had the expected decarboxylated molecular mass of 5268 daltons (decarboxylation occurs during ionization) determined by MALDI-TOF mass spectrometry in linear mode-negative ionization.

An Ellman assay for free sulphydrols of the oxidized, purified peptide revealed that the molar ratio of cysteine to half-cystine was less than 10%. The purified peptide was subjected to SDS-gel electrophoresis in the presence and absence of 10% β-mercaptoethanol. A single band migrating with a molecular weight of about 6000 was visible in the oxidized form of the peptide, confirming that dimerization or multimerization had not occurred during disulfide bond formation (Fig. 2).

To determine whether PT-(1–46) undergoes a conformational alteration upon binding calcium ions, we measured the effect of increasing CaCl_2 concentration on PT-(1–46) intrinsic fluorescence. When irradiated at a wavelength of 280 nm, PT-(1–46) has an emission maximum at 340 nm. The single tryptophan residue at position 41 accounts for this intrinsic fluorescence. The intrinsic fluorescence of PT-(1–46) was quenched with increasing CaCl_2 concentration, reaching a maximal quenching of 55% (Fig. 3). Half-maximal quenching was observed at 0.2 mM CaCl_2. This effect was reversed by addition of EDTA. These results indicate that PT-(1–46) undergoes a change in its tertiary structure upon binding calcium ions, similar to that observed with factor IX-(1–47) (19), prothrombin fragment 1 (29), and prothrombin (4).

The calcium-induced structural perturbation of PT-(1–46) was assessed using one-dimensional 1H NMR spectroscopy (Fig. 4). The peptide was initially rendered metal-free using Chelex 100. The proton resonances associated with the metal-free conformation of PT-(1–46) suggest that the peptide is partially structured, with the spectral dispersion associated with the amide protons shifted by greater that 0.15 parts per million (ppm) from their random coil values (30). However, the γ-protons associated with the malonate-like side chains of the 10 Gla residues were spectrally degenerate within a limited frequency envelope in this metal-free conformer (data not shown). To assess the ability of the peptide to bind calcium ions, a series of one-dimensional spectra were collected at increasing molar
equivalents of CaCl₂ to peptide. Following the addition of CaCl₂, the proton frequencies showed increased spectral dispersion in all regions of the spectrum, including the amide proton region (Fig. 4). Many of the amide protons were shifted downfield (0.25 ppm), the γ-protons became spectrally dispersed, and many of the α-protons were shifted upfield from their random coil values indicative of increased helical structure at concentrations of calcium ions in excess of 40 equivalents (30, 31). The presence of two resonances for the indole proton (Nₑ₁) of tryptophan 41 (9.9 and 10.1 ppm) in the one-dimensional spectra illustrates that the metal-free and calcium-induced structures are in slow exchange on the NMR time scale. The metal-free resonance remains sharp, suggesting that the correlation time (τₓ) of this conformer is shorter. The calcium-induced resonance is shifted upfield and broadened, identifying the presence of a new structure that places the tryptophan moiety in an altered chemical environment. At elevated calcium ion concentrations, this resonance broadens due to self-aggregation of the peptide.

We determined that PT-(1–46) binds to phospholipid membranes in a calcium-dependent manner analogous to prothrombin. Binding was measured by the relative 90° light scattering technique. When increasing amounts of PT-(1–46) were added to phospholipid vesicles (PC:PS, 60:40) in the presence of 2 mM CaCl₂, saturable binding was observed (Fig. 5). A dissociation constant, Kᵣ, of 0.5 ± 0.2 μM with an n value of 50 phospholipid monomers per binding site was calculated by fitting the data to a bimolecular equilibrium model. The binding was reversible following the addition of excess EDTA.

When PT-(1–46) was incubated with prothrombinase in the presence of the substrate prothrombin, thrombin generation was inhibited. Thrombin generation was inhibited 70% at a concentration of 40 μM PT-(1–46). Higher concentrations of peptide could not be tested due to aggregation (Fig. 6A). PT-(1–46) inhibition of prothrombin activation by prothrombinase fits a model of competitive inhibition. A Kᵢ of 0.8 μM was measured (Fig. 6B). To further elucidate the mechanism by which PT-(1–46) inhibits prothrombin activation, the ability of PT-(1–46) to inhibit prothrombin cleavage by factor Xa in the presence of phospholipid membranes and absence of factor Va was measured. The Kᵢ for inhibition of prothrombin activation under these conditions was 2.0 ± 0.5 μM, similar to the Kᵢ of inhibition for the prothrombinase complex (Fig. 7).

To test our hypothesis that the Gla domain of prothrombin, in addition to binding to phospholipid membranes, binds directly to factor Va, we assessed the ability of PT-(1–46) to
inhibit prothrombin activation by factor Xa and factor Va in the absence of phospholipid. As indicated in Fig. 8, PT-(1–46) inhibits at least 70% of the thrombin generation in the absence of phospholipid membranes and in the presence of factor Va. A $K_i$ of 14.9 ± 1 μM was determined for inhibition of this reaction by PT-(1–46). In the absence of both phospholipid membranes and factor Va, no inhibition was observed (Fig. 8). PT-(1–46) is able to inhibit the generation of thrombin in the absence of phospholipid membranes, and this inhibition is dependent on the presence of factor Va. These results suggest a prothrombin-factor Va interaction mediated, in part, by the Gla domain of prothrombin.

To provide a probe for measurement of direct interaction between PT-(1–46) and factor Va, PT-(1–46) was labeled with the fluorophore AMCA (excitation maximum 350 nm, emission maximum at 450 nm). This fluorophore selectively labels free primary amines, including lysine residues and the N terminus of a polypeptide. To prevent labeling of the N terminus of PT-(1–46) and loss of function (32), calcium ions were included in the labeling buffer to protect the N-terminal alanine (33). Analysis of the labeled peptide by reverse phase HPLC and mass spectrometry revealed that the preponderance of labeled peptide had a molecular mass of 5491 daltons corresponding to derivatization with a single AMCA moiety. The fluorescence emission spectrum of PT-(1–46) after excitation at 350 nm is shown in Fig. 9. Addition of 3 μM factor Va to PT-(1–46)AMCA results in a fluorescence quenching of 30%. This quenching is completely reversed by addition of 20-fold excess of unlabeled PT-(1–46). IgG at 3 μM has no effect on PT-(1–46)AMCA fluorescence.

Direct binding between the Gla domain of prothrombin and factor Va was demonstrated in experiments in which factor Va was added to PT-(1–46)AMCA. Addition of factor Va resulted in a concentration-dependent quenching of emission at 450 nm after excitation at 350 nm (Fig. 9A). Data were corrected for sample dilution and fitted to a bimolecular model of factor Va binding to PT-(1–46)AMCA (Fig. 9B). A binding constant, $K_d$, of 1.3 ± 0.5 μM, was determined.

**DISCUSSION**

During blood coagulation, prothrombin is converted to thrombin by factor Xa in complex with factor Va and phospholipid membranes. This enzyme complex is known as prothrombinase. Phospholipid membranes are key components for the
conversion of prothrombin to thrombin to occur at a physiologically relevant rate. The membrane binding portion of prothrombin resides in the N-terminal domain, which contains γ-carboxyglutamic acid and is referred to as the Gla domain (2). The Gla domain may also mediate protein–protein interactions within the prothrombinase complex as has been demonstrated in other vitamin K-dependent coagulation proteins (10, 11, 14, 15, 18). To study Gla domain-mediated protein–protein and protein–membrane interactions within the prothrombinase complex, a 46-residue peptide comprising the Gla domain and aromatic amino acid stack domains of human prothrombin was chemically synthesized. This peptide was anticipated to be the minimal size required to confer the membrane binding properties of N-terminal fragments of several vitamin K-dependent proteins generated by either proteolysis or chemical synthesis indicate that both the Gla and aromatic amino acid stack domains are required to achieve binding similar to that of the native protein (19, 33, 35). Although these domains are encoded by separate exons, they work together as a functional unit. Our results with PT-(1–46) support this notion. We show that PT-(1–46) binds to phospholipid membranes in a calcium-dependent fashion with a dissociation constant, $K_d$, of 0.5 ± 0.2 μM, similar to that of prothrombin. It has been hypothesized that the second disulfide loop (Cys46-Cys61) of the first kringle domain of bovine prothrombin is involved in membrane binding by forming a scaffold with residues Cys46-Arg55 of the Gla domain on which the remainder of the Gla domain anchors (8, 37). The results of our study and those of others (33) refute the importance of the second disulfide loop, and hence, the first kringle domain, for membrane binding. Our study supports the concept that only the Gla domain of prothrombin is required for protein-membrane binding, in a manner analogous to that of other vitamin K-dependent proteins. Nevertheless, prothrombin is unique among the vitamin K-dependent proteins in that it has two kringle domains instead of two EGF domains. The EGF domains of other blood-clotting proteins bind calcium and may play a role in stabilizing the structure of the Gla domain, as has been shown for factor X (38). It remains unclear whether the kringle domains bind calcium and play a similar role in prothrombin. However, site-directed mutagenesis studies of human prothrombin in which one or both kringle domains are deleted demonstrate that the kringle domains are not required for phospholipid binding (39). Furthermore, the crystal structure of prothrombin fragment 1 in the presence of calcium does not reveal many close contacts between the Gla and kringle 1 domains, although van der Waals interactions between Arg55 and Gla19/Gla20 were identified (8). An additional site-directed mutagenesis study of the Gla domain of human prothrombin in agreement that the interactions between the prothrombin Gla domain, and kringle 1 domains are not important for phospholipid binding. Mutations of residues Gla14 and Gla19 and one-dimensional $^1$H NMR analysis. The peptide binds reversibly to phospholipid vesicles containing acidic phospholipid. Furthermore, the peptide inhibits activation of prothrombin by prothrombinase. Thus PT-(1–46) is a useful tool for assessing Gla domain-mediated protein-phospholipid and protein–protein interactions.

PT-(1–46) binds calcium ions and undergoes a calcium ion-induced conformational reorientation as measured by quenching of intrinsic fluorescence and one-dimensional $^1$H NMR spectroscopy. Intrinsic fluorescence quenching is initiated by half-maximal quenching observed at 0.2 mM CaCl$_2$; this value is similar to that previously observed for prothrombin fragment 1 (4, 29, 36). The one-dimensional $^1$H NMR spectra of PT-(1–46) acquired in the current study demonstrate a calcium-induced conformational reorientation of tryptophan 41. These results are supported by a comparison of the crystal structure of the calcium-bound and apo forms of bovine prothrombin fragment 1, which demonstrate reorientation of tryptophan 42 (equivalent to tryptophan 41 in human prothrombin) relative to the disulfide loop at residues Cys18-Cys33 in the presence of calcium ions (8). The results of another study with N-terminal fragments of prothrombin (a mixture of residues 1–44 and 1–41) obtained with chymotryptic digestion of prothrombin found results similar to ours (33).

One of the major functions of the Gla domain of the vitamin K-dependent proteins is to support binding of these proteins to phospholipid membranes. The phospholipid binding properties of N-terminal fragments of several vitamin K-dependent blood coagulation proteins generated by either proteolysis or chemical synthesis indicate that both the Gla and aromatic amino acid stack domains are required to achieve binding similar to that of the native protein (19, 33, 35). Although these domains are encoded by separate exons, they work together as a functional unit. Our results with PT-(1–46) support this notion. We show that PT-(1–46) binds to phospholipid membranes in a calcium-dependent fashion with a dissociation constant, $K_d$, of 0.5 ± 0.2 μM, similar to that of prothrombin. It has been hypothesized that the second disulfide loop (Cys46-Cys61) of the first kringle domain of bovine prothrombin is involved in membrane binding by forming a scaffold with residues Cys46-Arg55 of the Gla domain on which the remainder of the Gla domain anchors (8, 37). The results of our study and those of others (33) refute the importance of the second disulfide loop, and hence, the first kringle domain, for membrane binding. Our study supports the concept that only the Gla domain of prothrombin is required for protein-membrane binding, in a manner analogous to that of other vitamin K-dependent proteins. Nevertheless, prothrombin is unique among the vitamin K-dependent proteins in that it has two kringle domains instead of two EGF domains. The EGF domains of other blood-clotting proteins bind calcium and may play a role in stabilizing the structure of the Gla domain, as has been shown for factor X (38). It remains unclear whether the kringle domains bind calcium and play a similar role in prothrombin. However, site-directed mutagenesis studies of human prothrombin in which one or both kringle domains are deleted demonstrate that the kringle domains are not required for phospholipid binding (39). Furthermore, the crystal structure of prothrombin fragment 1 in the presence of calcium does not reveal many close contacts between the Gla and kringle 1 domains, although van der Waals interactions between Arg55 and Gla19/Gla20 were identified (8). An additional site-directed mutagenesis study of the Gla domain of human prothrombin in agreement that the interactions between the prothrombin Gla domain, and kringle 1 domains are not important for phospholipid binding. Mutations of residues Gla14 and Gla19

**Fig. 9.** Binding of factor Va to PT-(1–46)AMCA. A, factor Va (3 μM) was added to 0.1 μM PT-(1–46)AMCA in 150 mM NaCl, 50 mM Tris-HCl, pH 7.4, 2 mM CaCl$_2$, 0.001% Tween 80. Excitation was at 350 nm; the emission spectrum was obtained from 400 to 500 nm. PT-(1–46)AMCA emission prior to factor Va addition (solid line), PT-(1–46)AMCA emission after the addition of 3 μM factor Va (○), PT-(1–46)AMCA emission after the addition of 3 μM IgG (▲), PT-(1–46)AMCA emission after the addition of 2 μM of unlabeled PT-(1–46) to 3 μM factor Va and 0.1 μM PT-(1–46)AMCA (●). B, aliquots of factor Va were added to 0.1 μM PT-(1–46)AMCA in 150 mM NaCl, 50 mM Tris-HCl, pH 7.4, 2 mM CaCl$_2$, 0.001% Tween 80. Data are plotted as a percentage of quenching of emission at 450 nm after excitation at 350 nm. Non-linear regression curve fitting was employed as described under “Experimental Procedures.”
PT-(1–46) inhibits prothrombin activation by factor Xa-factor Va but not by factor Xa alone, suggesting that the prothrombin GlA domain participates in the interaction of prothrombin with factor Va. The Kᵢ for PT-(1–46) inhibition of prothrombin activation by factor Xa-factor Va is 14.9 μM. This Kᵢ of inhibition is greater than the Kᵢ for binding of PT-(1–46)AMCA for factor Va, 1.3 μM. It is known that kringle 2 and possibly kringle 1 also constitute binding sites on prothrombin for factor Va (16, 17). Because domains other than the GlA domain contribute to the binding energy for interaction of prothrombin with factor Va, it is not surprising that the Kᵢ for inhibition of prothrombin activation by factor Xa-factor Va is higher than the Kᵢ for PT-(1–46) binding to factor Va. Although PT-(1–46) does not inhibit activation of prothrombin by factor Xa alone, we cannot rule out the possibility that the presence of factor Va induces interaction between the prothrombin GlA domain and factor Xa. Disruption of such an interaction would also be expected to contribute to the Kᵢ.

Analytical ultracentrifugation studies of prothrombin and factor Va interactions in solution reveal that prethrombin 1 and prothrombin bind equally (10) and kringle 1 peptides inhibit prothrombin turnover by factor Xa in solution in the presence of factor Va. Prothrombin fragment 1 and kringle 1 peptides inhibit prothrombin turnover by factor Va when these proteins bind in solution. Given that kringle 1 (17) and kringle 2 (16) have also been shown to bind to factor Va, a model of prothrombin-factor Va interaction likely involves multiple contact sites in these proteins. This paradigm is analogous to that of the factor VIIa-tissue factor complex in which the crystal structure reveals multiple contact sites, including an interaction between the C-terminal helix of the GlA domain of factor VIIa and the second fibronectin domain of tissue factor (18). A similar model has also been proposed for the ternary complex of factor Xa-factor VIIa-tissue factor (12). Further study is required to identify the factor Va contact sites in the prothrombin GlA domain and to determine whether this protein-protein interaction occurs when factor Va and prothrombin assemble on phospholipid membranes.

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