The hydrophobic ω-loop within the prothrombin γ-carboxyglutamic acid-rich (Gla) domain is important in membrane binding. The role of this region in membrane binding was investigated using a synthetic peptide, PT-(1–46)F4W, which includes the N-terminal 46 residues of human prothrombin with Phe-4 replaced by Trp providing a fluorescent probe. PT-(1–46)F4W and PT-(1–46) bind calcium ions and phospholipid membranes, and inhibit the prothrombinase complex. PT-(1–46)F4W, but not PT-(1–46), exhibits a blue shift (5 nm) and red-edge excitation shift (28 nm) in the presence of phosphatidylserine (PS)-containing vesicles, suggesting Trp-4 is located within the motionally restricted membrane interfacial region. PS-containing vesicles protect PT-(1–46)F4W, but not PT-(1–46), fluorescence from potassium iodide-induced quenching. Stern-Volmer analysis of the quenching of PT-(1–46)F4W in the presence and absence of 80% phosphatidylcholine/20% PS vesicles suggested that Trp-4 is positioned within the membrane and protected from aqueous quenching agents whereas Trp-41 remains solvent-accessible in the presence of PS-containing vesicles. Fluorescence quenching of membrane-bound PT-(1–46)F4W is optimal with 7- and 10-doxyl-labeled lipids, indicating that Trp-4 is inserted 5 to 7 Å into the bilayer. This report demonstrates that the ω-loop region of prothrombin specifically interacts with PS-containing membranes within the interfacial membrane region.

The membrane surface (2), however, the mechanism by which the vitamin K-dependent proteins bind phospholipid membranes remains unclear.

The precursor forms of the vitamin K-dependent coagulant proteins (prothrombin, factor VII, factor IX, and factor X) and the anticoagulant proteins (protein C, protein S, and protein Z) are post-translational modified by γ-glutamyl carboxylase (3–6). This enzyme converts 10–13 glutamic acid residues to γ-carboxyglutamic acid (Gla)1 residues, within the N-terminal 40–50 amino acids of its substrates, a region known as the Gla domain (2). Gla has a malonate-like side chain and binds metal ions (2, 7, 8). Calcium binding by the Gla residues within the Gla domain of the vitamin K-dependent proteins leads to stabilization of the membrane binding conformer (9–12). The nature and location of the membrane contact site(s) within the Gla domain remains unsettled (9–15).

Studies of prothrombin fragment 1, which consists of the Gla domain, the aromatic amino acid stack domain, and the first kringle domain of prothrombin, revealed that chemical modification of the free N terminus abolished membrane binding (12, 16, 17). A comparison of the three-dimensional structures of the metal-free and calcium-bound conformers of the prothrombin, factor IX, and factor X Gla domains demonstrate that following calcium binding most of the Gla residues become internalized, resulting in the solvent exposure of three hydrophobic amino acids within a calcium-induced N-terminal loop (ω-loop) from residue 1 to residue 11 (18–20). These residues have been implicated in the binding of vitamin K-dependent proteins to phospholipid membranes (18, 19, 21–24). Site-directed mutagenesis of the highly conserved amino acids (Leu-5 and Leu-8) of protein C reduced its binding affinity for phospholipid vesicles (21, 22). Substitution of the homologous residues in factor IX, Leu-6 and Phe-9, with a photo-activatable cross-linking amino acid leads to cross-linking to the phospholipid membrane, thus identifying that this region is involved in membrane binding (20). However, the importance of the ω-loop hydrophobic amino acids in the phospholipid binding of Gla domain-containing proteins has recently been challenged (15, 25).

PT-(1–46), a synthetic peptide with the sequence of the Gla

The abbreviations used are: Gla, γ-carboxyglutamic acid; Gla domain, γ-carboxyglutamic acid-rich domain; PS, phosphatidylserine; PC, phosphatidylcholine; dansyl, 5-dimethylamino-1-naphthalenesulfonyl; dansyl-PE, phosphatidylethanolamine-N-(5-dimethylamino-1-naphthalenesulfonfonyl); 5-doxyl phosphatidylcholine, 1-palmitoyl-2-stearoyl(5-doxyl)sn-glycero-3-phosphocholine; 7-doxyl-PC, 1-palmitoyl-2-stearoyl(7-doxyl)sn-glycero-3-phosphocholine; 10-doxyl-PC, 1-palmitoyl-2-stearoyl(10-doxyl)-sn-glycero-3-phosphocholine; 12-doxyl-PC, 1-palmitoyl-2-stearoyl(12-doxyl)-sn-glycero-3-phosphocholine; 16-doxyl-PC, 1-palmitoyl-2-stearoyl(16-doxyl)-sn-glycero-3-phosphocholine; lysoPS, phosphatidylserine; TBS, Tris-buffered saline; MALDI, matrix-assisted laser desorption ionization/time of flight.
domain and aromatic amino acid stack domain of human prothrombin, was previously synthesized and characterized (26). The current study investigates the role of the $\omega$-loop region in phospholipid binding using a synthetic peptide, PT-(1–46)F4W, in which Phe-4 is replaced with Trp. This modification provides a fluorescent probe within this region. PT-(1–46)F4W possesses all the anticipated properties of the prothrombin Gla domain, including the ability to undergo calcium-induced conformational changes, interact with anionic phospholipid vesicles and inhibit activation of prothrombin by the prothrombinase complex. We demonstrate that Trp-4 interacts within the interfacial region of anionic phospholipid vesicles, consistent with the general finding that tryptophan residues involved in membrane binding are predominantly positioned at the membrane interface (27–29). These data indicate that the $\omega$-loop region of the prothrombin Gla domain participates in a specific interaction with anionic phospholipid membranes and likely inserts into the interfacial membrane region.

**EXPERIMENTAL PROCEDURES**

**Phospholipids—**Egg phosphatidylcholine, brain phosphatidylserine, 1-palmitoyl-2-stearoyl-sn-glycero-3-phosphoethanolamine, phosphatidylserine, phosphatidylethanolamine, phosphatidylglycerol, L-a-phosphatidylserine, L-a-phosphatidylethanolamine, L-a-phosphatidylglycerol, phosphatidylcholine, doxyl-stearoylphosphatidylcholine, doxyl-phosphatidylcholine, doxyl-phosphatidylserine, doxyl-phosphatidylethanolamine, doxyl-phosphatidylglycerol, and phosphatidylcholine performed on an SLM 8000C fluorescence spectrophotometer. Small unilamellar vesicles (80% PC/20% PS) were sonicated with a bath sonicator to disrupt any aggregates formed during preparation and/or subsequent storage.

**Fluorescence Spectroscopy—**The solvent for phosphatidylcholine vesicles was evaporated (30). The vesicles were prepared using a standard sonication procedure (30).

**Prothrombinase Inhibition Assay—**Prothrombinase inhibition assays were performed as described previously for PT-(1–46) (26). CaCl2 was added in the final concentration indicated to 1 μM PT-(1–46)F4W in TBS, pH 7.4 (20 mM Tris, 150 mM NaCl) previously treated with Chelex 100 (Bio-Rad). The sample was excited at 280 nm and the emission was monitored at 340 nm. The reversibility of the calcium-induced quenching was determined by adding EDTA at the completion of the calcium titration.

**Calcium-induced Quenching of Intrinsic Fluorescence—**Fluorescence quenching experiments were performed as described previously for PT-(1–46) (26). CaCl2 was added in the final concentration indicated to 1 μM PT-(1–46)F4W in TBS, pH 7.4 (20 mM Tris, 150 mM NaCl) previously treated with Chelex 100 (Bio-Rad). The sample was excited at 280 nm and the emission was monitored at 340 nm. The reversibility of the calcium-induced quenching was determined by adding EDTA at the completion of the calcium titration.

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**Phospholipid Membrane Binding Using 90° Light Scattering—**The binding of PT-(1–46) and PT-(1–46)F4W to phospholipid vesicles was evaluated by 90° light scatter according to the method of Nelsestuen and Lim (32) on an SLM 8000C fluorescence spectrophotometer. Small unilamellar vesicles (80% PC/20% PS) were sonicated with a bath sonicator to disrupt any aggregates formed during preparation and/or subsequent storage.

**Synthesis of PT-(1–46) and PT-(1–46)F4W—**The phosphorothioate peptides were synthesized as described previously (10, 26). Briefly, the reaction mixture was dialyzed against 50 mM ammonium acetate, pH 7.4. Following the addition of protein, the vesicle suspension was centrifuged at 160,000 $g$ for 2 h to remove unbound peptide. The supernatant was removed and the phospholipid vesicles were washed twice with methylene chloride and dried as before. The phospholipid vesicle complex to phospholipid vesicles alone and is plotted versus peptide concentration. A representative experiment is shown in detail by Blosein et al. (26). The scatter intensity of phospholipid plus protein was corrected for increases in scattering due to the peptides themselves. Additionally, the scatter intensity of phospholipid alone was corrected for the decrease in intensity due to dilution with each addition of protein. $M_\mu M$ is the molecular weight ratio of the peptide/phospholipid vesicle complex to phospholipid vesicles alone and is plotted versus peptide concentration. A representative experiment is shown and dissociation constants are reported as the means ± S.E.

**Blue Shift and Red-edge Excitation Shift Analysis—**For blue shift experiments performed in the presence or absence of phospholipid vesicles, the excitation wavelength was 280 nm and the emission scan was monitored from 300 to 400 nm. For the red-edge excitation shift studies, the fluorescence emission spectra of the sample were collected at excitation wavelengths from 270 nm to 310 nm. Peptides were diluted to 1 μM in TBS, pH 7.4, containing 2 mM CaCl2. Emission spectra of PT-(1–46) or PT-(1–46)F4W in the absence or presence of 100 μM 80% PC/20% PS or 100 μM 100% PC small unilamellar vesicles were collected. The emission due to the vesicles alone was subtracted from each spectrum. Additionally, the fluorescence of a reference fluorophore (l-Trp) was used to correct the change in peptide fluorescence that results from the addition of 80% PC/20% PS vesicles and 100% PC vesicles. These artifacts are due to the light scattering effects caused by the vesicles, which may influence the fluorescence signal (33). To simplify the results of our red-edge excitation experiments, the maximum emission wavelength of each corrected spectrum was graphed versus the excitation wavelength.

**Iodide-induced Quenching of Intrinsic Fluorescence—**For iodide quenching experiments, aliquots of a freshly prepared potassium iodide (KI) stock solution were added to samples containing 1 μM PT-(1–46) or PT-(1–46)F4W in TBS, pH 7.4 containing 2 mM CaCl2 to achieve the indicated KI concentration. Potassium iodide (KI), which does not quench fluorescence, was added to each sample to maintain a constant salt concentration. Following the addition of the quenching agent, emission spectra were collected between 300 and 400 nm or the emission intensity at 350 nm was measured. Experiments were performed in the presence of 100 μM 80% PC/20% PS or 100% PC vesicles in the absence of phospholipid vesicles. The fluorescence data were analyzed using the Stern-Volmer equation for collisional quenching (Equation 1) or a modified version of the equation, which describes a system of two independent fluorophores with different $k_{sv}$ values (Equation 2).

$$F/F_0 = [f/(F_0/K_{sv}(Q + 1))]^{-1}$$

(Eq. 1)

$$F/F_0 = [f/(F_0/K_{sv}(Q + 1))]^{-1} + [f/(F_0/K_{sv}(Q + 1))]$$

(Eq. 2)
centration, \(f_a\) and \(f_b\) are the fractional contributions of the fluorophores a and b to the total intensity and \(K_{aSVa}\) and \(K_{bSVb}\) are the quenching coefficients of the accessible fractions. The values of \(f_a, f_b, K_{aSVa}\), and \(K_{bSVb}\) can be determined by a linear least squares fit of \(F/\alpha\) versus \(Q\). All experiments were corrected for the inner filter effects due to absorption of \(I_0\) that is formed at high concentrations of \(KI\) (34).

**Doxyl-lipid Quenching of Intrinsic Fluorescence—**To study quenching by doxyl-lipids, 15% (molar percent) of doxyl-labeled PC was incorporated into small unilamellar vesicles that contained 20% PS. The doxyl labeled vesicles were added to 1 \(\mu\)M PT-(1–46)F4W or PT-(1–46) in TBS, pH 7.4, and 2 mM CaCl\(_2\). The fluorescence intensities of the emission maxima were measured in the presence and absence of doxyl-labeled small unilamellar vesicles. The amount of quenching due to vesicles containing the doxyl moiety at various positions along the acyl chain was measured. \(F/\alpha\) values were calculated, where \(F_0\) is the fluorescence in the absence of doxyl-containing vesicles and \(F_\alpha\) is the fluorescence in the presence of doxyl-containing vesicles.

**RESULTS**

A peptide based on the N-terminal 46 residues of human prothrombin, PT-(1–46), was previously synthesized and characterized (26). We have synthesized an analog of PT-(1–46), PT-(1–46)F4W, with Phe-4 replaced by Trp (Table I). PT-(1–46)F4W has an intrinsic fluorescence probe within the hydrophobic \(\omega\)-loop (residues 1–11) allowing us to probe the interaction of this region of the peptide with phospholipid vesicles. The amino acid sequence of the peptide was confirmed by automated Edman degradation and amino acid analysis (data not shown). The molecular mass of the peptide, determined by MALDI mass spectrometry in the negative ion mode, was 5308 daltons, which corresponds with the theoretical molecular mass of the decarboxylated peptide (5308.2 daltons).

PT-(1–46)F4W was characterized as described for PT-(1–46) (26). Initially, we assessed the ability of the peptide to undergo a calcium-dependent conformational alteration using intrinsic fluorescence spectroscopy (Fig. 1A). The peptide was excited at 280 nm, and the emission was monitored at 340 nm. PT-(1–46)F4W contains two tryptophan residues and two tyrosine residues; however, the intrinsic fluorescence is predominantly attributed to Trp-4 and Trp-41. The addition of 80% PC/20% PS phospholipid vesicles to the PT-(1–46)F4W results in a 20–30% decrease in the fluorescence of the peptide and reference sample (data not shown). Based on these results, the emission spectra were corrected for light scattering effects (33). These results indicate that the environment of Trp-4 is unaltered by the addition of PS-containing phospholipid vesicles to the Gla domain peptides in the presence of calcium ions, whereas Trp-4 is in a less polar, more hydrophobic environment in the presence of these vesicles, suggesting that Trp-4 has partitioned into the phospholipid membrane. These results are consistent with the x-ray crystal structure of calcium-bound bovine prothrombin fragment 1, containing the Gla domain, aromatic amino acid side chain, and kringle 1 domain of prothrombin (18). In this structure Trp-4 is disulfide bond in the Gla domain and is buried in the interior of the protein. The position of Trp-4 relative to the membrane surface strongly governs its fluorescence properties (37). The modest blue shift observed for Trp-4 suggests that it is within the interfacial region. In addition to the observed blue shift, the 80% PC/20% PS vesicles caused an increase in the intrinsic fluorescence of both peptides (Fig. 2A and B). This fluorescence enhancement is most likely due to an increased lifetime of the tryptophan fluorescence following binding to the phospholipid vesicles as described previously.

To further examine the interaction of PT-(1–46)F4W with membranes, we used wavelength-selective fluorescence spectroscopy or red-edge excitation shift spectroscopy, which provides an additional approach for monitoring the environment and dynamics of fluorophores in complex biological systems (27, 34, 38). The fluorescence emission of the tryptophan residues in PT-(1–46)F4W and PT-(1–46) as a function of excitation wavelength are shown in Fig. 3. No shift in the fluorescence maximum was seen for PT-(1–46) or PT-(1–46)F4W in

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**TABLE I**

| Peptide     | Sequence                              |
|-------------|----------------------------------------|
| PT-(1–46)   | NH\(_2\)-ANTFLY7YRKGNRLYRyCV7Y7CSY7YAF7yALy7sSTATDVFYWAKYTA-COOH |
| PT-(1–46)F4W| NH\(_2\)-ANTFLY7YRKGNRLYRyCV7Y7CSY7YAF7yALy7sSTATDVFYWAKYTA-COOH |

**Interaction of Human Prothrombin Gla Domain with Membranes**

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the presence of 100% PC phospholipid vesicles, which do not support prothrombin binding or function. Upon the addition of 80% PC/20% PS vesicles, the emission maximum for PT-(1–46) was not shifted, whereas the emission maximum for PT-(1–46)F4W was shifted from 350 to 378 nm. The 28 nm shift in emission maximum with increasing excitation wavelength for PT-(1–46)F4W indicates that Trp-4 of PT-(1–46)F4W is localized in a motionally restricted environment in the presence of 80% PC/20% PS vesicles. Red-edge excitation shifts primarily result from a decreased rate of solvent relaxation for those solvent molecules around the excited state fluorophore. This is due to motional restrictions imposed on these solvent molecules by their environment (27, 34, 38). The interfacial membrane region has unique motional (39, 40) and dielectric properties (41). Water molecules at the membrane interface are expected to be motionally restricted (27). The significant red-edge effect demonstrated for PT-(1–46)F4W argues that Trp-4 interacts with the heterogeneous motionally restricted interfacial region of the phospholipid membrane (27, 34, 42).

To further probe this interaction, we employed the aqueous phase quenching agent, potassium iodide (KI), which does not readily penetrate into phospholipid membranes (43), to assess the ability of PS-containing membranes to effectively shield tryptophan quenching of PT-(1–46)F4W and PT-(1–46). In the absence of phospholipid vesicles, iodide quenched PT-(1–46)F4W and PT-(1–46) intrinsic fluorescence 80% at the highest concentration of quenching agent used (Figs. 4, A and B). In
the presence of 80% PC/20% PS phospholipid vesicles, we observed 45% quenching of PT-(1–46)F4W fluorescence intensity. At the same KI concentration (0.2 M), PT-(1–46)F4W fluorescence was not protected from quenching in the presence of 100% PC vesicles (Fig. 4A). The presence of vesicles of either composition did not protect PT-(1–46) fluorescence from quenching by 0.2 M KI (Fig. 4B). These data suggest that PS-containing vesicles effectively shield one population of tryptophan residues in PT-(1–46)F4W and that Trp-4 is protected from KI quenching due to a specific interaction with PS-containing vesicles. To assure that the anionic nature of KI did not influence the experimental results through electrostatic interaction of the quenching agent with charged moieties on the peptide or the vesicles, these experiments were repeated with a cationic aqueous quenching agent, CoCl$_2$, added as Co$^{2+}$ (44). Similar results were obtained (data not shown).

A quantitative examination of PT-(1–46)F4W fluorescence quenching due to KI was performed using the Stern-Volmer equation. Initial data collected in the absence of phospholipid vesicles were analyzed using the standard Stern-Volmer equation (Equation 1). The KI quenching of the intrinsic fluorescence of PT-(1–46)F4W in the absence of phospholipid vesicles generated a straight line with a $K_{SV}$ of 5.5 ± 0.5 m$^{-1}$ (Fig. 5A). However, in the presence of 80% PC/20% PS vesicles, the Stern-Volmer plot showed a negative deviation from linearity, indicative of two independent fluorophore populations possessing different accessibility coefficients for the aqueous quenching agent (Fig. 5B). These results were analyzed using a modified version of the Stern-Volmer equation (Equation 2). One class of fluorophores was readily accessible to potassium iodide and possessed a $K_{SV}$ of 9.0 ± 2.0 m$^{-1}$, whereas the other class of fluorophores was effectively shielded from potassium iodide quenching and had a $K_{SV}$ of $1.7 \times 10^{-8} \pm 2.9 \times 10^{-8}$ m$^{-1}$. The fractional accessibilities were 0.63 ± 0.18 and 0.37 ± 0.18, respectively. A decrease in $K_{SV}$ reflects a decrease in solvent exposure of the tryptophan or a decrease in tryptophan lifetime (45, 46). However, lifetime is often associated with fluorescence intensity upon binding lipid vesicles (46). We demonstrated an increase in intensity (Fig. 2A) reflecting an increased lifetime. Thus, the observed decrease in $K_{SV}$ is most likely due to tryptophan shielding from the quenching agent. The data presented in Figs. 4 and 5 demonstrate that in the presence of PS-containing vesicles ~50% of the tryptophan residues are effectively shielded from iodide and hence associated with the anionic phospholipid vesicle. The $K_{SV}$ values of 9.0 ± 2.0 m$^{-1}$ and $1.7 \times 10^{-8} \pm 2.9 \times 10^{-8}$ m$^{-1}$ are consistent with Trp-41 being accessible and Trp-4 being protected from potassium iodide quenching in the presence of PS-containing vesicles.

The previous fluorescence quenching experiments demonstrate that Trp-4 of PT-(1–46)F4W specifically interacts with PS-containing membranes, effectively removing this region of the peptide from the bulk aqueous environment. However, these studies cannot determine the penetration depth of this tryptophan into the membrane. To estimate this we employed spin-labeled PC that carries a nitroxide (doxyl) group attached to the methylene carbon at position 5, 7, 10, 12, or 16 of the fatty acyl chain (for review, see Ref. 47). Tryptophan quenching by the doxyl-moiety is primarily a static event, and thus pro-
vides an accurate probe for estimating the penetration depth of this residue into the lipid bilayer (48). Quenching is dependent upon direct distance between the spin label and the fluorophore; the greatest quenching efficiency is observed when the doxyl-moiety is located closest to the tryptophan residue (less than 5 Å) (34, 47, 49). As demonstrated in Fig. 6A, tryptophan fluorescence is quenched most significantly when the doxyl moiety is located at the 7- or 10-position of the acyl chain. We identified that the amount of quenching varied with the doxyl moiety position; 22%, 33%, 37%, 28%, and 25% quenching were estimated for the 5-, 7-, 10-, 12-, and 16-doxyl-lipids, respectively. The reported percentages are the average of independent quenching experiments repeated at least twice for vesicles containing the doxyl label at each position. Tryptophan fluorescence is incrementally quenched as the concentration of PS-containing vesicles possessing 10-doxyl-PC increases as illustrated in Fig. 6B and C. Maximum quenching values were seen in the presence of 75 μM doxyl-containing vesicles. In control experiments in which the doxyl-containing vesicles were added to PT-(1–46), which lacks the α-loop fluorescent probe, Trp-4, only 10% quenching was observed independent of the doxyl position (data shown for 10-doxyl-PC-containing vesicles in Fig. 6C). This minimal amount of quenching was determined to be nonspecific since the location and thus penetration depth of the nitroxide moiety did not alter the amount of quenching observed. The maximum fluorescence quenching for PT-(1–46)F4W is observed with vesicles containing 7-doxyl and 10-doxyl. These doxyl labels are estimated to be positioned 5 and 7 Å into the bilayer, respectively (48, 50–52). This places Trp-4 5 to 7 Å into the outer leaflet of the membrane, based on a bilayer thickness of 30 Å (48, 50, 52) (Fig. 7). The indole ring...
hydrophobic residues within the ω-loop of the prothrombin Gla domain selectively interact with PS-containing vesicles, providing a membrane anchor. The modest blue shift, significant red-edge excitation effect, shielding of one class of fluorophores from potassium iodide quenching and doxyl quenching of PT-(1–46)F4W fluorescence by PS-containing vesicles positions Trp-4 within the bilayer and suggests that Trp-4 of PT-(1–46)F4W penetrates into the interfacial region of the membrane as shown in our model (Fig. 7). These results are consistent with previous studies examining the ω-loop region of the Gla domains of protein C and factor IX. Site-directed mutagenesis of the highly conserved amino acids (Leu-5 and Leu-8) of protein C reduces the affinity of binding to phospholipid vesicles (21, 22). Substitution of the corresponding residues in factor IX, Leu-6, and Phe-9, with a photo-activatable cross-linking amino acid allows cross-linking to the phospholipid membrane, thus identifying that this region is involved in membrane binding (20). This mechanism of membrane binding has been proposed for other protein-membrane interactions (49).

The non-vitamin K-dependent protein cofactors for the tenase and prothrombinase enzymatic complexes, factor VIII and factor V, may employ a similar mechanism for phospholipid binding. The crystal structures of the C2 domains of these proteins share a conserved β-barrel framework with three protruding loops that contain a group of solvent exposed hydrophobic residues (53, 54). The proposal that these hydrophobic groups participate in phospholipid binding has been tested by mutating two tryptophan residues in one of these loops of factor V to Ala and determining that this mutant protein had impaired ability to interact with phospholipids (55).

Although structures of the Gla domains in the presence of calcium ions, determined to date for prothrombin, factor VII, and factor IX, indicate that the ω-loop containing several hydrophobic residues is a conserved feature of these domains (18–20, 24), a recent analysis of accumulated biochemical data on vitamin K-dependent protein-phospholipid interaction resulted in an alternative proposal for a membrane contact site (25). Mutation of hydrophobic residues within the ω-loop resulted in only a 4-fold decrease in affinity of protein C for phospholipid vesicles (22), and removal of the three terminal amino acids of bovine prothrombin, which should disrupt the structure of the ω-loop, reduced membrane affinity only 5-fold (17) representing a loss of free energy of protein-membrane binding of only 10–15%. Similarly, analyses based on hydrophobic exposure on vesicle surfaces, effects of surface pressure on phospholipid monolayers, calcium binding properties of phospholipid bound and unbound prothrombin, and effects of ionic strength, coupled with comparison of the amino acid sequences of known vitamin K-dependent proteins and their membrane binding properties, led to an alternate proposal for the phospholipid binding site in the Gla domain (15, 25). Residues 11, 33, and 34 (bovine prothrombin numbering system), which are clustered on the surface of the protein, were identified as a potential site (15, 25).

Our findings demonstrate that Trp-4 penetrates the interfacial phospholipid membrane region (Fig. 7) and that the ω-loop of the Gla domain serves as a site of interaction for vitamin K-dependent protein-membrane interaction. These interpretations are not necessarily in conflict with this alternative model or with other proposed sites, for example those identified by focusing on the electrostatic component of the protein-phospholipid interaction based on electrostatic considerations (56, 57). Rather, we prefer a model in which the binding of the Gla domain of vitamin K-dependent proteins to phospholipid membranes is facilitated by interaction of multiple sites in the Gla domain with different regions of the phospholipid moieties.
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This model could serve to explain some of the apparently contradictory data regarding the hydrophobic or ionic nature of the interaction and the modest influence of amino acid mutations upon the free energy of binding. In this construct we propose that the hydrophobic contribution to the binding energy arises from the interaction of the ω-loop within the interfacial region of the membrane bilayer, whereas other sites on the Gla domain interact with the head groups of the phospholipids. Mutation at one interaction site in the Gla domain would be expected to have only a small to modest effect on binding energy, depending on its contribution to the whole. This model can account for the specificity of the interaction with regard to phospholipid head group requirement. In addition to the long recognized importance of PS, a role for phosphatidyethanolamine has more recently been identified in hemostasis (58–62). The specificity of the Gla domain interaction with these phospholipids is likely based on the presence of a specific binding site or sites for these head groups. Indeed, in the absence of PS (e.g. 100% PC vesicles), as was anticipated, we found no evidence for insertion of Trp-4 of PT-(1–46)F4W into the bilayer of these phospholipid vesicles, suggesting that other electrostatic interactions may be required to induce peptide vesicle insertion (63). Evidence for insertion of Trp-4 of PT-(1–46)F4W into the bilayer of these phospholipid vesicles, suggesting that other electrostatic interactions may be required to induce peptide vesicle insertion (63).

In conclusion, we demonstrate that hydrophobic residues in the ω-loop of the prothrombin Gla domain penetrate the interfacial region of anionic phospholipid membrane bilayers. Further studies are required to understand if other sites of the prothrombin Gla domain are involved in binding anionic phospholipid membranes and/or if unique regions of this domain recognize other phospholipid compositions.

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