Identification of the Phospholipid Binding Site in the Vitamin K-dependent Blood Coagulation Protein Factor IX*

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Steven J. Freedman‡§*, Mark D. Blostein§†‡, James D. Balejai, Margaret Jacobs§, Barbara C. Furie§§, and Bruce Furiei§§

From the ‡Center for Hemostasis and Thrombosis Research, Division of Hematology-Oncology, New England Medical Center and the Departments of §Medicine and ¶Biochemistry, Tufts University School of Medicine and Sackler School of Graduate Biomedical Sciences, Boston, Massachusetts 02111

The blood coagulation and regulatory proteins that contain γ-carboxyglutamic acid are a part of a unique class of membrane binding proteins that require calcium for their interaction with cell membranes. Following protein biosynthesis, glutamic acids on these proteins are converted to γ-carboxyglutamic acid (Gla) in a reaction that requires vitamin K as a cofactor. The vitamin K-dependent proteins undergo a conformational transition upon metal ion binding, but only calcium ions mediate protein-phospholipid interaction. To identify the site on Factor IX that is required for phospholipid binding, we have determined the three-dimensional structure of the Factor IX Gla domain bound to magnesium ions by NMR spectroscopy. By comparison of this structure to that of the Gla domain bound to calcium ions, we localize the membrane binding site to a highly ordered structure including residues 1-11 of the Gla domain. In the presence of Ca2+, Factor IX Gla domain peptides that contain the photoactivatable amino acid p-benzoyl-l-phenylalanine at positions 6 or 9 cross-link to phospholipid following irradiation, while peptides lacking this amino acid analog or with this analog at position 46 did not cross-link. These results indicate that the NH2 terminus of the Gla domain, specifically including leucine 6 and phenylalanine 9 in the hydrophobic patch, is the contact surface on Factor IX that interacts with the phospholipid bilayer.

The vitamin K-dependent blood coagulation and regulatory proteins interact with phospholipid membranes in the presence of Ca2+. This interaction plays an important biological role in the formation of protein complexes with enhanced catalytic properties (Furie and Furie, 1988). For instance, the catalytic activity ($k_{cat}/K_m$) of Factor IXa for the activation of Factor X is increased almost 100-fold by the presence of phospholipids, calcium ions and Factor VIIla (van Dieijen et al., 1981). The γ-carboxyglutamic acid (Gla)-rich domain of the vitamin K-dependent proteins mediates phospholipid recognition upon the ligandng of calcium ions and the subsequent formation of a phospholipid binding site (for review, see Furie and Furie (1988)). γ-Carboxyglutamic acid, which defines the calcium ion binding sites of this domain, is synthesized posttranslationally from glutamic acid in an enzymatic reaction directed by a prepeptide on the precursor form (Jorgensen et al., 1987) and catalyzed by the vitamin K-dependent carboxylase (Suttie, 1985). In contrast to Ca2+, other divalent metal ions induce a stable conformer of the Gla domain that does not bind phospholipids (Nelsestuen et al., 1976). A two-step sequential conformational transition model for the metal ion-induced structural changes has been proposed based on observations using conformation-specific antibodies (Borowski et al., 1986; Liebman et al., 1987). In the first step, the binding of a variety of divalent metal ions to the Gla domain results in a conformational change with the expression of new antigens common among the metal ion-protein binary complexes. This conformer does not bind phospholipid vesicles. In the second step, saturation with calcium ions induces a specific conformer that binds to phospholipids and expresses an additional neoantigenic determinant. Antibodies and their Fab fragments directed against the Ca2+-stabilized epitope block phospholipid binding (Borowski et al., 1986; Liebman et al., 1987). The differences between the metal-free structures and the structures of Ca2+-bound Gla domains from Factor IX and a homologous protein, prothrombin, have been determined in the absence of phospholipid (Soriano-Garcia et al., 1992; Freedman et al., 1995a, 1995b). However, the location of the phospholipid binding site within the Gla domain remains unknown.

A synthetic peptide, Factor IX (1-47), which contains the Gla domain and the aromatic amino acid stack domain of Factor IX, has been used to study the structural requirements of phospholipid binding by Factor IX (Jacobs et al., 1994). We show that the Factor IX (1-47) peptide, like Factor IX, does not interact with phospholipid membranes in the presence of magnesium ions but does interact with phospholipids in the presence of calcium ions. Here, we have localized the phospholipid binding site by determining the solution structure of the Factor IX Gla domain peptide when fully saturated with Mg2+. Since the Ca2+- and Mg2+-bound Gla domains undergo a common metal-induced fluorescence transition and share a structural epitope (Borowski et al., 1986; Liebman et al., 1987), the differences between the Ca2+- and Mg2+-bound structures identify the NH2-terminal 11 amino acids as critical for phospholipid binding. The covalent cross-linking of a Factor IX Gla domain analog containing a photoactivatable amino acid, p-benzoyl-l-phenylalanine, at position 6 or position 9 to phospholipid proves that residues 6 and 9 are within or in close proximity of the membrane.

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The atomic coordinates (code 1MGX) have been deposited in the Protein Data Bank, Brookhaven National Laboratory, Upton, NY. Distance and torsion angle restraints and molecular topology file are available as R1MGXMR.

‡‡ These authors made equal contributions to this work and should both be considered primary authors.

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EXPERIMENTAL PROCEDURES

The synthesis, folding, and purification of the Factor IX (1–47) peptide have been described previously (Iacobs et al., 1994; Freedman et al., 1995a). Factor IX (1–47)p-benzoyl-L-phenylalanine (Bpa) 6, Factor IX (1–47)/Bpa 9, and Factor IX (1–47)/Bpa 46 were prepared by solid phase peptide synthesis using Fmoc chemistry, as for Factor IX (1–47). However, Fmoc-p-benzoyl-L-phenylalanine was substituted for leucine 6 in Factor IX (1–47)/Bpa 6, for phenylalanine 9 in Factor IX (1–47)/Bpa 9, and for valine 46 in Factor IX (1–47)/Bpa 46. After oxidation to form the disulfide bond and purification by high performance liquid chromatography, these peptides migrated as a single homogeneous band upon SDS-gel electrophoresis. Synthetic Factor IX (1–47) peptides were labeled, where indicated, with 125I using the lactoperoxidase method. The 125I-labeled peptides were purified by gel filtration on Sephadex G-50 column equilibrated in 50 mM Tris, pH 7.4, 50 mM NaCl. CaCl2, MgCl2, or 1.5 mM peptide, 3 M urea, 2.5 M acetate, pH 5.4. We observed concentration-dependent aggregation of the Factor IX (1–47) peptide with 125I-labeled Factor IX (1–47) was purified by the same method except that anti-Factor IX-Mg(II) antibodies bound to Sepharose and were eluted with CaCl2. The three-dimensional structures of Factor IX (1–47)-Ca2+- and Factor IX (1–47)-Mg2+-bound peptides were formally compared using QUANTA (Molecular Simulations). These structures were overlaid to minimize the backbone rmsd. Mean-square deviation values between residues 15 and 47, using the Molecular Similarity utility, the molecular volumes of both structures were defined, and the volume common to both structures identified. All residues in Factor IX (1–47)-Ca2+ within and outside of this common volume of matched atoms were color-coded. The three-dimensional structures of Factor IX (1–47)-Ca2+ and Factor IX (1–47)-Mg2+ were formally compared using QUANTA (Molecular Simulations). These structures were overlaid to minimize the backbone root-mean-square deviation values between residues 15 and 47. Using the Molecular Similarity utility, the molecular volumes of both structures were defined, and the volume common to both structures identified. All residues in Factor IX (1–47)-Ca2+ within and outside of this common volume of matched atoms were color-coded.

Characteristic spin system patterns were observed in the Factor IX (1–47)-Mg2+-bound peptide that coincided with the spin system patterns for the 20 naturally occurring amino acids as described by Wüthrich (1986). γ-Carboxyglutamic acid residues had the spin system pattern as described previously (Freedman et al., 1995a, 1995b). The main starting point for spectral assignment was the identification of the spin system characteristic for the single tryptophan side chain of Factor IX (1–47). Correlations from aromatic side chains from the ring protons to the β protons (and backbone) were achieved primarily from the D2O NOESY spectrum. For some residues, the cross-peak patterns for the Ca2+-bound Factor IX (1–47) peptide and metal-free Factor IX (1–47) peptide were used as a guide in assigning the proton resonances. In TOCSY spectra, correlation from the amide protons generally extended to the β protons. Side-chain identification beyond the β protons were mainly completed from the aliphatic region of TOCSY (and DQF-COSY) spectra. Here correlations were generally observed from the α and β protons out to the end of the chain. The intraresidue information from these spectra in conjunction with the NOESY spectra, which displayed all sequential connectivities (δNN–δN, δN–δi, δi–δi+1, Wüthrich (1986)), made nearly complete proton resonance assignments possible. The proton resonance assignments are presented in Table I.

NMR spectroscopy was performed on three different samples of Factor IX (1–47) peptide in the presence of magnesium ions that, under certain conditions, resulted in minor amounts of peptide precipitation. We have previously reported Mg2+-induced linebroadening in one-dimensional NMR spectra of the Factor IX (1–47) peptide (Freedman et al., 1995a). Despite these minor impediments to the acquisition of high quality spectra, two-dimensional 1H NMR studies were performed on the Mg2+-saturated Factor IX (1–47) peptide.

Spectra acquisition and processing were similar to that performed previously and Ca2+-bound peptides (Freedman et al., 1995a, 1995b). Briefly, two-dimensional NOESY spectra were recorded with mixing times of 150, 250, and 300 ms at 25°C, and 250 ms at 35°C. A total of 2048 real data points were acquired in t2, 384–512 points in t1, a spectral width of approximately 7000 Hz, 128–160 summed scans, and a relaxation delay of 1.3 s between scans. As an alternative to presaturation for H2O suppression, the jump-and-return technique was also used to maximize amide intensity (Plateau and Guéron, 1982). Total correlation spectroscopy (TOCSY) data were recorded using a mixing time of 55 ms, 80 summed scans, and an MLEV-17 mixing sequence (Bax and Davis, 1985). Double-quantum filtered correlation spectroscopy (DQF-COSY) data were recorded with 2048 t2, 512 t1 points, 64 summed scans, and 1024 increments (Piantini et al., 1982). As described previously, the NMR data sets were multiplied by sine bell window functions and zero-filled to 2000 by 1000 (real) matrices (Freedman et al., 1995a, 1995b). Sequence-specific resonance assignments were made using the methodology described for the metal-free Factor IX (1–47) and Ca2+-bound peptides (Freedman et al., 1995a, 1995b). Briefly, the NOE contacts were classified into five categories: (1) "Intrar residue" for NOEs within a residue; (2) "Sequential" for contacts between the backbone and side-chain protons of residue i with the backbone amide proton of residue i + 1; (3) "Short range" for all other contacts between residue i and i + 1; (4) "Medium range" for NOEs between protons on residues separated by 3 amino acid positions or less in the sequence; and (5) "Long range" for contacts between residues that are separated by 4 amino acid positions or more in sequence.

1 The abbreviations used are: Fmoc, N-(9-fluorenylmethoxycarbonyl); dansyl, 5-dimethylaminonaphthalene-1-sulfonyl; NOE, nuclear Overhauser effect; NOESY, NOE spectroscopy; DQF-COSY, double-quantum filtered correlation spectroscopy; TOCSY, total correlation spectroscopy; Bpa, p-benzoyl-L-phenylalanine.

RESULTS

Fluorescence energy transfer studies have shown that the Factor IX (1–47) peptide behaves like full-length Factor IX in that it binds phospholipid vesicles in the presence of Ca2+, but
The short, medium, and long range nuclear Overhauser effect (NOE) contacts for the carboxy-terminal 36 residues were nearly identical to those of the Ca\(^{2+}\)-bound peptide (Fig. 2). Only a few cross-peaks were weak or obscured by spectral overlap. A comparison of the chemical shift values between the Ca\(^{2+}\)- and Mg\(^{2+}\)-bound structures showed that they were similar for residues 14–47 (Fig. 3), except for residues 21–23 (see below). In contrast, there was little similarity in the short, medium, and long range NOE contacts within the amino-terminal 13 residues between the spectra collected on the two metal ion-bound structures. All NOE contacts confined to this peptide region in Factor IX (1–47)-Mg\(^{2+}\) were instead similar to those found in the apoFactor IX (1–47) spectra (Fig. 2) (Freedman et al., 1995b). In addition, the chemical shift values for all residues in this region were centered around random coil values (Wishart et al., 1991). For residues 21–23, the \(\alpha\) proton chemical shift differences between the Ca\(^{2+}\)- and Mg\(^{2+}\)-bound peptides are large, as indicated in Fig. 3. These may be accounted for by contacts between Tyr-1 and residues 21–23 in the Ca\(^{2+}\)-stabilized Factor IX (1–47) peptide. However, the metal ion-dependent phospholipid binding characteristics of full-length Factor IX are reflected in the Factor IX (1–47) peptide. The differences between the three-dimensional structures of the Ca\(^{2+}\)-stabilized Factor IX (1–47) peptide and the Mg\(^{2+}\)-stabilized Factor IX (1–47) peptide should also define a region critical for phospholipid binding. For these reasons, we determined the solution structure of the Mg\(^{2+}\)-stabilized Factor IX (1–47) peptide by using NMR spectroscopy.

Table I

| Residue | NH | \(\delta\)H | \(\gamma\)H | Others |
|---------|----|------------|------------|--------|
| Tyr-1   | 4.15 | 3.05, 3.06 | H\(\delta\), 7.08; H\(\varepsilon\), 6.81 |
| Asn-2   | 4.66 | 2.65, 2.78 | H\(\delta\), 6.73, 6.90 |
| Ser-3   | 8.55 | 4.32 | 3.91, 3.96 |
| Gly-4   | 8.48 | 3.93, 3.95 |
| Lys-5   | 8.04 | 4.33 | 1.71, 1.84, 1.38, 1.38 | H\(\delta\), 1.65, 1.65; H\(\varepsilon\), 2.97, 2.97 |
| Leu-6   | 8.32 | 4.27 | 1.64, 1.65 | 1.59 | H\(\delta\), 0.85, 0.89 |
| Gla-7   | 8.68 | 4.13 | 2.12, 2.23 | 3.23 |
| Gla-8   | 8.38 | 4.13 | 2.12, 2.12 | 3.13 |
| Phe-9   | 8.09 | 4.48 | 3.11, 3.17 |
| Val-10  | 7.69 | 3.67 | 2.02 | 0.75, 0.92 |
| Gin-11  | 8.15 | 3.99 | 1.98, 2.06, 2.39, 2.39 |
| Gly-12  | 8.22 | 3.81, 3.89 |
| Asn-13  | 8.31 | 4.57 | 2.62, 2.88 |
| Leu-14  | 8.32 | 3.50 | 1.25, 1.42 | 1.18 |
| Thr-15  | 8.31 | 4.00 | 2.13, 2.22 | 3.37 |
| Arg-16  | 7.84 | 3.87 | 1.68, 1.77 | 1.51, 1.51 | H\(\delta\), 3.18, 3.18; H\(\varepsilon\), 7.60 |
| Gla-17  | 8.45 | 4.10 | 2.39, 2.45 | 3.47 |
| Cys-18  | 8.15 | 5.29 | 2.86, 3.08 |
| Met-19  | 7.36 | 5.12 | 2.16, 2.58 | 2.46, 2.81 | H\(\delta\), 2.17 |
| Gla-20  | 8.27 | 4.26 | 2.22, 2.35 | 3.35 |
| Gla-21  | 6.91 | 3.48 | 2.20, 2.22 | 3.27 |
| Lys-22  | 8.17 | 4.41 | 1.77, 1.86 | 1.48, 1.48 | H\(\delta\), 1.68, 1.68; H\(\varepsilon\), 2.91, 2.91 |
| Cys-23  | 9.05 | 5.50 | 3.27, 3.33 |
| Ser-24  | 8.96 | 5.06 | 4.18, 4.39 |
| Phe-25  | 8.54 | 3.02 | 1.72, 2.55 |
| Gla-26  | 8.55 | 4.33 | 1.82, 2.21 | 3.55 |
| Gla-27  | 7.46 | 3.91 | 2.15, 2.42 | 3.24 |
| Ala-28  | 7.67 | 3.67 | 1.41 |
| Arg-29  | 8.84 | 4.27 | 1.63, 1.65 | 1.27, 1.27 | H\(\delta\), 2.86, 3.33; H\(\varepsilon\), 8.69; H\(\eta\), 6.71, 6.95 |
| Gla-30  | 8.00 | 3.89 | 2.22, 2.22 | 3.68 |
| Val-31  | 7.29 | 3.62 | 1.82 | 0.27, 0.67 |
| Phe-32  | 7.74 | 4.08 | 2.87, 2.87 |
| Gla-33  | 9.44 | 3.78 | 2.17, 2.58 | 3.19 |
| Asn-34  | 6.84 | 4.89 | 2.25, 2.99 |
| Thr-35  | 3.67 | 4.20 | 1.30 |
| Gla-36  | 8.77 | 4.40 | 2.04, 2.18 | 3.50 |
| Arg-37  | 8.77 | 3.82 | 1.42, 1.45 | 1.29, 1.34 | H\(\delta\), 2.95, 3.20; H\(\varepsilon\), 6.78 |
| Thr-38  | 7.44 | 3.46 | 4.09 | 0.43 |
| Thr-39  | 8.56 | 4.03 | 4.42 | 1.31 |
| Gla-40  | 8.33 | 4.13 | 2.22, 2.40 | 3.66 |
| Phe-41  | 7.74 | 4.36 | 3.20, 3.22 |
| Trp-42  | 9.44 | 4.03 | 3.54, 3.78 |
| Lys-43  | 7.89 | 4.08 | 1.74, 1.94 | 1.57, 1.57 |
| Gin-44  | 7.08 | 4.39 | 2.03, 2.05 | 2.23, 2.29 |
| Tyr-45  | 7.57 | 4.07 | 1.86, 2.02 |
| Val-46  | 7.17 | 3.78 | 1.77 | 0.56 |
| Asp-47  | 7.53 | 4.25 | 2.50, 2.55 |
The presence of 1mM MgCl₂ (ison to binding studies performed with the Factor IX (1–47) peptide in containing 1.5 mM peptide in H₂O, pH 5.8, had a small subclass could not be measured. The NOE data indicate that the major- short, medium, and long range distances. Due to cross-peak were intraresidue and sequential distances, and 330 were geometry methods using 710 distance restraints, of which 380

46. For this region, an average backbone root-mean-square transition with the geometric average. A comparison with the anal- stics of less than 6 Hz occur. As a consequence, torsion angle

Fig. 4. The root-mean-square deviation upon superimposition of the average Mg²⁺-bound structure with the average Ca²⁺-bound structure shows that they 12–46 of the Mg²⁺-bound structure that could not be identified in either the apo or Ca²⁺-bound peptide spectra reported pre- viously (Freedman et al., 1995a, 1995b) (Fig. 2). These 22 NOE contacts represent intermolecular contacts since additional spectra collected using the solvent system previously used to determine the Factor IX (1–47)-Ca²⁺ structure (Freedman et al., 1995b): 3 M urea and 2.5 M guanidine HCl, eliminated the novel NOE contacts. These NOE contacts were also eliminated at lower concentrations of peptide (0.5 mM) in the absence of guanidine/urea.

Mg²⁺-bound peptide structures were generated by distance geometry methods using 710 distance restraints, of which 380 were intraresidue and sequential distances, and 330 were short, medium, and long range distances. Due to cross-peak broadening, coupling constants below approximately 7.5 Hz could not be measured. The NOE data indicate that the majority of residues are found in helices where α-NH coupling con- stants of less than 6 Hz occur. As a consequence, torsion angle information was not available for structure calculations. The Mg²⁺-bound peptide had defined structure from residues 12–46. For this region, an average backbone root-mean-square deviation value of 0.9 ± 0.1 Å was determined by superimposition with the geometric average. A comparison with the anal- ogous region of the Ca²⁺-bound structure shows that they contain the same secondary structural elements, predomi- nantly helices, and the same tertiary structure (Fig. 4). The lowest energy Ca²⁺-bound structure (residues 12–46) is shown superimposed with a series of the Mg²⁺-bound structures in Fig. 4. The root-mean-square deviation upon superimposition of the average Mg²⁺-bound structure with the average Ca²⁺- bound structure is 1.2 Å for residues 12–46. In this region, both the Mg²⁺ and Ca²⁺-bound forms are compact and approxi- mately globular. In contrast, the amino-terminal 11 residues lacked defined structure in Factor IX (1–47)-Mg²⁺, with the exception of a short loop from residue 6 to 9. The same loop was found in the apoFactor IX (1–47) structure (Freedman et al., 1995a). The two-step conformational transition of Factor IX can now be defined in structural terms; the apoFactor IX lacks formal structure in residues 1–36, with the exception of resi-

dues 6–9 and 18–23. Upon occupancy of specific metal bindings sites that interact with many divalent cations, including Mg²⁺, the Gla domain assumes formal structure except that residues 1–11 remain flexible and motile. With occupancy of another set of metal bindings, sites that can be occupied only by Ca²⁺, the entire polypeptide backbone of the Gla domain is defined, in- cluding the NH₂-terminal loop (Fig. 5).

To compare the two binary complexes quantitatively, the structural model of Factor IX-Mg²⁺ was overlaid on the structural model of Factor IX (1–47)-Ca²⁺. The molecular volume of each structure was determined, and the volumes that are shared by equivalent atoms within both structures were de- fined (Fig. 6A). Using only the structural model of Factor IX (1–47)-Ca²⁺, residues contained within the common volume are identified in white; these are the atoms whose positions are preserved in the two structures (Fig. 6B). Residues outside this volume represent the region from residue 1 to residue 11. Thus, the yellow atoms represent structural differences between the two metal-protein complexes, differences that correlate with phospholipid binding function, i.e., residues 1–11 represent the putative phospholipid binding site.

To prove that residues 1–11 of Factor IX are in close prox- imity of the phospholipid membrane in the Factor IX-phos- pholipid-Ca²⁺ ternary complex, we used a photoactivatable cross-linker to covalently couple the Factor IX (1–47) peptide to phospholipid in the presence of Ca²⁺. Bpa is a photolabile amino acid analog composed of a side-chain benzophenone (Dorman and Prestwich, 1994) (Fig. 7A). Upon irradiation at
The absolute proton chemical shift differences for α protons between Factor IX (1–47) peptide structures. Top graph, the chemical shift differences between apoFactor IX (1–47) and Factor IX (1–47)-Ca$^{2+}$. Bottom graph, the chemical shift differences between Factor IX (1–47)-Ca$^{2+}$ and Factor IX (1–47)-Mg$^{2+}$. The chemical shift values reflect the structural environment of the protons. The bars below the abscissa indicate that the chemical shift differences are small for α protons in the designated peptide region (apoMg$^{2+}$ residues 1–13: αH, 0.1 ± 0.1 ppm; Ca$^{2+}$/Mg$^{2+}$ residues 14–47: αH, 0.1 ± 0.2 ppm). The peptide regions designated by the bar are predicted to be structurally similar since the backbone α and amide protons of amino acids undergo an average chemical shift change of approximately 0.4 ppm from the random coil value when they are incorporated in different secondary structures (Wishart et al., 1991). The arrows indicate large chemical shift differences for the α protons in a region where all other α protons show small chemical shift differences. Similar findings were observed for the backbone amide protons (apoMg$^{2+}$ residues 1–13: NH, 0.1 ± 0.1 ppm; Ca$^{2+}$/Mg$^{2+}$ residues 14–47: NH, 0.2 ± 0.2 ppm).

350 nm, the benzophenone decomposes and forms a covalent bond with carbon-hydrogen bonds within 3 Å. By substituting Bpa for specific hydrophobic residues in Factor IX (1–47), we evaluated the ability of Bpa incorporated into specific regions of the peptide to mediate cross-linking to phospholipid. In order to avoid disruption of the tertiary structure of the Gla domain, we designed peptides that placed Bpa in positions of hydrophobic residues whose side chains are oriented toward solvent (Freedman et al., 1995b). Leucine 6, phenylalanine 9, and valine 46 meet these criteria. Three additional peptides were prepared by solid phase synthesis: Factor IX (1–47)/Bpa 6, Factor IX (1–47)/Bpa 9, and Factor IX (1–47)/Bpa 46 (Fig. 7B). These peptides were homogeneous by high performance liquid chromatography and by SDS-gel electrophoresis.

To demonstrate that these Bpa-containing peptides exhibited the membrane binding properties characteristic of Factor IX (1–47), we used fluorescence energy transfer to study peptide-membrane interaction. Using fluorescence energy transfer with phospholipid vesicles composed of phosphatidylserine: phosphatidylcholine:dansyl-phosphatidylethanolamine (40:50: 10), we observed binding of the Bpa-containing peptides to phospholipid membranes in the presence of Ca$^{2+}$ (Fig. 8). The $K_d$ for Factor IX (1–47)/Bpa 6 binding to phospholipid was 1.0 μM; Factor IX (1–47)/Bpa 9 was 1.5 μM; Factor IX (1–47)/Bpa 46 was 6.0 μM. These values are equivalent within experimental error to the $K_d$ of 2.4 μM measured for Factor IX (1–47). The interaction was Ca$^{2+}$-dependent; the addition of EDTA inhibited binding. The results indicate that, in the absence of conditions that induce covalent cross-linking, the Factor IX/Bpa-containing peptides interact non-covalently with phospholipid membranes with characteristics indistinguishable from Factor IX (1–47).

Using gel filtration to assess covalent binding of $^{125}$I-labeled Bpa-containing peptides to phospholipid, we investigated the nature of the peptide-phospholipid complex following photoactivation and after dissociation of non-covalently bound peptide to phospholipid by the removal of Ca$^{2+}$ with EDTA. The extent of covalent cross-linking using a photoactivatable reagent is related to the conditions of the reaction, the geometry of the labile group and the target, and the chemical reactivity of the photolabile compound and the target bonds. Therefore, the direct comparison of the relative amount of cross-linking with each peptide to phospholipid vesicles is critical for analysis. Factor IX (1–47)/Bpa 6 in the presence of phospholipid and Ca$^{2+}$ was irradiated at 350 nm for 20 min. EDTA was then added to remove non-covalently bound peptide from the vesicles. The mixture was then analyzed by gel filtration in the

**Fig. 4.** Superimposition of the Factor IX (1–47)-Mg$^{2+}$ structures with the Factor IX (1–47)-Ca$^{2+}$ structure. Seven Factor IX (1–47)-Mg$^{2+}$ structures (gray), arbitrarily chosen from the 15 calculated, are superimposed. For comparison, the lowest energy Factor IX (1–47)-Ca$^{2+}$ structure (black) is overlaid. Only the backbone atoms are displayed. The amino-terminal 11 residues of the Factor IX (1–47)-Mg$^{2+}$ peptide are not structurally defined, in contrast to the well defined loop for this region in the Factor IX (1–47)-Ca$^{2+}$ structure. Possibly due to intermolecular association, the Mg$^{2+}$-induced linewidth broadening also blunts the magnitude and resolution of the NOE cross-peaks, reducing the number and accuracy of NOE distance measurements. Therefore, the resolution of the Mg$^{2+}$-bound structure is not as high as that of the Ca$^{2+}$-bound structure. During data analysis, we identified some NOE cross-peaks that were due to intermolecular interactions. These NOEs were not present when data were collected at lower peptide concentration or when the peptide was dissolved in aqueous solution including guanidine and urea. These additional NOE cross-peaks were not employed in defining the Factor IX (1–47)-Mg$^{2+}$ model.
The vitamin K-dependent proteins are a unique class of membrane-binding proteins, interacting with membrane surfaces through the Gla domain in the presence of calcium ions (Furie and Furie, 1988). This domain exhibits multiple metal binding sites for calcium ions. Protein-membrane interaction is reversible. The removal of calcium ions leads to rapid separation of the protein from the membrane surface (Nelsenstuen et al., 1976). Furthermore, this class of proteins has a requirement for acidic phospholipid, such as phosphatidylserine, as a component of the membrane surface. Speculations on the molecular details of the nature of the protein-membrane interface over the past 20 years have resulted in conflicting models. Some groups have proposed that the vitamin K-dependent blood clotting proteins bind to acidic lipid membranes through calcium ions that bridge γ-carboxyglutamic acid residues on the protein to the phosphate head groups on the lipid membrane (Lim et al., 1977; Mann et al., 1982; Schwabke et al., 1989). This paradigm requires that the side chains of the γ-carboxyglutamic acid residues extend into the solvent and are available on the surface to link to the membrane through calcium ion bridges. A salient feature of this model is that γ-carboxyglutamic acid participates as a contact residue in the protein-membrane interface. However, three significant experimental observations have argued against this interpretation. 1) We had proposed from NMR studies of a peptide fragment of prothrombin that the high affinity metal binding sites were internal and formed by intramolecular γ-carboxyglutamic acid residues, both bound to a common metal ion (Furie et al., 1979); 2) Rhee et al. (1982) have shown that the hydration of metal ions bound by γ-carboxyglutamic acids in prothrombin is not altered by the addition of phospholipid membranes, thus demonstrating the absence of metal-phosphate interaction in the ternary complex; 3) many groups have observed that excess concentrations of calcium ions do not disrupt protein-membrane interaction. Borowski et al. (1986) put forth an alternative model in which the metal ion binding sites are created by the γ-carboxyglutamic acid residue side chains oriented toward the interior of the protein, thus with internal bound Ca²⁺, this Ca²⁺-stabilized conformer expresses a phospholipid binding site that does not include γ-carboxyglutamic acid at the protein-membrane interface. From the prothrombin fragment 1 crystal structure (Soriano-Garcia, 1992), a definitive structure of the protein-Ca²⁺ complex has emerged. In this structure, the calcium ions are coordinated by oxygens of the carboxylate groups of γ-carboxyglutamic acid. These calcium ions are, for the most part, not exposed to solvent, but rather form an internal array that stabilizes the folding of the NH₂-terminus of the prothrombin. Only a few of the γ-carboxyglutamic acid side chains are available on the surface of the molecule. Our solution structure of the Factor IX Gla domain is consistent with this structure (Freedman et al., 1995b). However, the location of the phospholipid binding site of the vitamin K-dependent proteins remains speculative and has been based on the comparison of the Ca²⁺-bound structures and the metal-free structures (Freedman et al., 1995a, 1995b; Sunnerhagen et al., 1995).

A number of approaches have been taken to localize the phospholipid binding site on the vitamin K-dependent proteins.
Fragment 1, the NH2-terminal third of prothrombin, contains the phospholipid binding site of prothrombin (Gitel et al., 1973). The region between the Gla-aromatic amino acid stack domains and the serine protease domain, including epidermal growth factor and kringle domains, does not appear to play a significant role in mediating phospholipid binding in the vitamin K-dependent proteins (Kotkow et al., 1993). Proteolytic fragments of Factor X and prothrombin that contain the Gla domain and the aromatic amino acid stack domain bind to phospholipid vesicles (Schwalbe et al., 1989; Pollock et al., 1988), and synthetic fragments based on the Gla-aromatic amino acid stack domain of protein C and Factor IX bind to acidic phospholipid vesicles (Jacobs et al., 1994; Castellino, 1994). These results have led us and others to conclude that the Gla-aromatic amino acid stack domains constitute the unit for phospholipid binding. Zhang and Castellino (1994) have used site-specific mutagenesis to demonstrate that mutation of leucine 5 in protein C interferes with lipid binding, and Christianson et al. (1995) showed that the mutation of leucine 8 and to a lesser extent, phenylalanine 4, also affected lipid binding. We have mutated Arg-15 in prothrombin and observed significant decreases in binding affinity when a glycine is substituted at position 15 (Dietcher et al., 1994). Furthermore, mutation of Glu-16, -20, -25, or -26 to aspartic acid abolishes high affinity phospholipid binding in human prothrombin (Ratcliffe et al., 1993). None of these experiments allow interpretation that a specific amino acid is located at the protein-membrane interface since alteration of side chains of amino acids that are located at the protein-membrane interface and mutation of amino acids that lead to a significant change in the tertiary structure of the Gla domain structure can both disrupt membrane binding. More recently, we have compared the structures of the Factor IX Gla domain in the presence and absence of Ca2+ (Freedman et al., 1995a, 1995b). This analysis concluded that Ca2+-induced folding of polypeptide backbone linking the three well structured regions in the apo form correlate with the induction of phospholipid binding properties. The apo structure of the Factor X proteolytic fragment including the Gla, aromatic amino acid stack, and the first epidermal growth factor domains revealed somewhat more stable structure than the apo form of the Factor IX Gla domain (Sunnerhagen et al., 1995). Comparison of this structure to a homology model based upon the prothrombin fragment 1-Ca2+ crystal structure (Soriano-Garcia et al., 1992) also suggested that Ca2+ induces further folding of the NH2 terminus of the Gla domain. Although a hydrophobic patch near the NH2 terminus has been noted previously (Soriano-Garcia et al., 1992), there has been no direct evidence for the interaction of this region with acidic phospholipid vesicles.

To circumvent the problems of interpretation associated with site-directed mutagenesis and large changes in structure in the presence and absence of calcium ions, we determined the three-dimensional structures of the Gla domain in the presence of calcium ions and in the presence of magnesium ions to identify the phospholipid binding site. Calcium and magnesium ions induce a conformational change that can be monitored by fluorescence quenching and the expression of neoantigens. Calcium ions induce unique neoantigens in addition to those present in the protein-Mg2+ complex (Borowski et al., 1986; Liebman et al., 1987). Furthermore, despite the common metal-induced conformational change, the Ca2+-stabilized conformer to both structures is shown in violet. The visible white residues are amino acids in Factor IX (1–47)-Ca2+ that are distinct from equivalent residues in Factor IX (1–47)-Mg2+ (red). B, only the Factor IX (1–47)-Ca2+ is shown. Amino acid residues completely outside of the common molecular volume (violet) are shown in yellow.
binds to phospholipids but the Mg$^{2+}$-stabilized conformer does not. Thus, we anticipated that the structural comparison of the Factor IX (1–47)-Ca$^{2+}$ and the Factor IX (1–47)-Mg$^{2+}$ complexes would reveal both regions of structure common to these two forms and regions of structure that were unique to each liganded form, allowing identification of the site on Factor IX that is required for phospholipid binding. We have now shown that the region of difference, required for phospholipid binding, includes the NH$_2$ terminus of Factor IX, from residues 1 to 11.

Chemical modification of the NH$_2$ terminus of prothrombin is blocked in the presence of calcium ions but not in the presence of magnesium ions or in the absence of metal ions (Welsch and Nelsestuen, 1988; Schwalbe et al., 1989). Chemical modification of the metal-free forms of the vitamin K-dependent proteins preclude phospholipid binding in the presence of Ca$^{2+}$. These results suggest that the NH$_2$ terminus is buried in the

**Fig. 7.** A, chemical structure of Bpa. B, chemical synthesis of Factor IX (1–47) peptides containing benzoyl-L-phenylalanine. Bpa, a photoreactive homolog of phenylalanine, was incorporated into peptides using solid phase synthesis and Fmoc chemistry. Bpa was substituted for leucine 6 (Factor IX (1–47)/Bpa 6), for phenylalanine 9 (Factor IX (1–47)/Bpa 9), and for valine 46 (Factor IX (1–47)/Bpa 46). Bpa, ● ●.

**Fig. 8.** Phospholipid binding properties of Factor IX (1–47) containing Bpa. Ca$^{2+}$-dependent interaction of Factor IX (1–47)/Bpa 6, Factor IX (1–47)/Bpa 9, Factor IX (1–47)/Bpa 46, and Factor IX (1–47) with phospholipid vesicles studied by fluorescence energy transfer, as per Fig. 1. The change in fluorescence was monitored by irradiating at 280 nm and recording dansyl emission at 520 nm as a function of increasing peptide concentration. $I_0$, fluorescence (arbitrary units) in the absence of peptide; $I$, fluorescence at indicated peptide concentration (μM). A, Factor IX (1–47) (wild type); B, Factor IX (1–47)/Bpa 6 (6BPA); C, Factor IX (1–47)/Bpa 9 (9BPA); D, Factor IX (1–47)/Bpa 46 (46BPA).

**Fig. 9.** Photo-induced cross-linking of Bpa-containing Factor IX (1–47) peptides to phospholipid. $^{125}$I-Labeled Factor IX (1–47)/Bpa 6, $^{125}$I-labeled Factor IX (1–47)/Bpa 9, $^{125}$I-labeled Factor IX (1–47)/Bpa 46, and $^{125}$I-labeled Factor IX (1–47) were incubated with phospholipid vesicles (40% phosphatidylserine, 60% phosphatidylcholine). A, 10 mM CaCl$_2$; B, 10 mM MgCl$_2$; C, 10 mM EDTA; D, 10 mM CaCl$_2$. In A–C, the sample was irradiated at 350 nm for 20 min; the sample in D was not irradiated. Non-covalent Ca$^{2+}$-mediated protein-phospholipid binding was reversed by the addition of EDTA after irradiation. Bound and free peptide were separated by gel filtration. The percentage of total peptide that was cross-linked to phospholipid vesicles is shown on the y axis. Factor IX (1–47)/Bpa 6 (black); Factor IX (1–47)/Bpa 9 (gray); Factor IX (1–47)/Bpa 46 (striped); Factor IX (1–47) (white).
The presence of calcium ions, but is exposed in the absence of metal ions or in the presence of Mg\textsuperscript{2+}. Indeed, the crystal structure of prothrombin fragment 1 and the NMR structure of Factor IX support this model for the Ca\textsuperscript{2+}-bound form (Soriano-Garcia et al., 1992; Freedman et al., 1995b), and our current study supports this model for the Mg\textsuperscript{2+}-bound form.

We have tested the hypothesis that residues 1–11 define the phospholipid contact site by localizing the lipid binding site on the peptide with a photocross-linking reagent. Residues 1–11 include a hydrophobic patch, noted by ourselves (Freedman et al., 1995b) and others (Soriano-Garcia et al., 1992; Colpitts and Castellino, 1994; Sunnerhagen et al., 1995), that might represent a component of the phospholipid binding site that inserts into the phospholipid bilayer. The NH\textsubscript{2}-terminal tyrosine 1 (orange-yellow) interacts with Lys-22. Tyrosine 1 (Y1), leucine 6 (L6), phenylalanine 9 (F9), and valine 10 (V10) are indicated.

\textbf{FIG. 10.} The phospholipid binding site of Factor IX. The atomic coordinates of this binary complex were derived from two-dimensional NMR spectroscopy (Freedman et al., 1995b). The positions of atoms of residues 12–47 (white) are nearly identical in the Factor IX (1–47)-Mg\textsuperscript{2+} and Factor IX (1–47)-Ca\textsuperscript{2+} structures. Residues 1–11 (yellow and shades thereof) include the regions required for phospholipid binding. Hydrophobic residues (dark yellow), including leucine 6, phenylalanine 9, and valine 10, define a hydrophobic patch on the exterior of the protein that likely buries inside the phospholipid bilayer. The NH\textsubscript{2}- terminal tyrosine 1 (orange-yellow) interacts with Lys-22. Tyrosine 1 (Y1), leucine 6 (L6), phenylalanine 9 (F9), and valine 10 (V10) are indicated.

\textbf{FIG. 11.} Hypothetical model of the interaction of Factor IX and phospholipid membranes. In this model, the hydrophobic residues (black) that form the hydrophobic patch in the phospholipid binding site of Factor IX are buried in the phospholipid bilayer. Specific residues in the Gla domain interact with the phospholipid head groups and are responsible for the requirement for anionic phospholipids for effective binding.

\begin{center}
\textbf{Phospholipid Binding Site of Factor IX}
\end{center}
may be critical for phospholipid binding or, and more likely, the fold of the NH2-terminal amino acids 1–11, including the interaction of tyrosine 1 with Gla-21 and Lys-22, define the critical phospholipid binding surface. Based upon the cross-linking analysis, we propose that residues 6–10, including leucine 6, phenylalanine 9, and valine 10, form a hydrophobic patch on the exterior of the protein that likely buries inside the lipid bilayer (Fig. 11). This patch represents at least part of the contact site, but electrostatic interactions involving charged amino acids may also be important (Atkins and Ganz, 1992).

Membrane proteins, including families of integral membrane proteins and extrinsic membrane proteins, fall into structural classes that characterize their functional properties. The vitamin K-dependent, γ-carboxyglutamic acid-containing proteins interact reversibly in the presence of calcium ions with acidic phospholipids on membranes following cell activation. The regulation of blood coagulation on cell surfaces involves the formation of membrane-bound protein complexes, including protein cofactors and the enzymes formed following zymogen activation (Furie and Furie, 1988). The structural motif responsible for Factor IX interaction with membrane surfaces is a general feature of the homologous Gla regions in this family of proteins.

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