Membrane Binding Properties of the Factor IX \(\gamma\)-Carboxyglutamic Acid-rich Domain Prepared by Chemical Synthesis*  
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Margaret Jacobs, Steven J. Freedman, Barbara C. Furie, and Bruce Furie†  
From the Center for Hemostasis and Thrombosis Research, Division of Hematology-Oncology, New England Medical Center and the Department of Medicine and Department of Biochemistry, Tufts University School of Medicine, Boston, Massachusetts 02111  

The fully \(\gamma\)-carboxylated peptides based upon the complete and truncated Gla/aromatic amino acid stack domains of human Factor IX were prepared by solid phase peptide synthesis using Fmoc (N-(9-fluorenyl)methoxycarbonyl) chemistry. A 47-residue peptide Factor IX-(1-47) and a 42-residue peptide Factor IX-(1-42), both containing 12 residues of \(\gamma\)-\(\gamma\)-carboxyglutamic acid, were purified by high performance liquid chromatography and oxidized to form the disulfide bond. Quantitative \(\gamma\)-carboxyglutamic acid analysis of Factor IX-(1-47) and Factor IX-(1-42) indicated the presence of 12.1 and 11.2 \(\gamma\)-carboxyglutamic acid residues/mol of peptide, respectively; no glutamic acid was detected. As monitored by fluorescence quenching, calcium ions induced the prototypical conformational transition in Factor IX-(1-47), but not in Factor IX-(1-42), that is observed with Factor IX. Half-maximal quenching of the intrinsic fluorescence of Factor IX-(1-47) was observed at Ca(II) concentrations of about 50 \(\mu\)M. Factor IX-(1-47) bound to the conformation-specific antibodies, anti-Factor IX-Mg(II) and anti-Factor IX-Ca(II)-specific in the presence of metal ions. Factor IX-(1-47) bound to phospholipid membranes, as monitored by energy transfer from intrinsic fluorophores to dansyl (5-dimethylaminonaphthalene-1-sulfonyl)-phosphatidylethanolamine incorporated into a lipid bilayer composed of phosphatidylserine:phosphatidylcholine. In contrast, Factor IX-(1-42) bound poorly to these same membranes. Factor IX-(1-47) did not inhibit Factor Xa activation of Factor IX but did inhibit the activation of Factor X by Factor IXa bound to Factor VIII in the presence of calcium ions and phospholipid. These results show that phospholipid membrane binding is a property of the Gla/aromatic amino acid stack domain and that the Factor IX-(1-47) peptide, prepared by chemical synthesis, preserves the membrane binding properties and the metal-induced conformational transitions observed in native Factor IX. These results indicate that Factor IX-(1-47) but not Factor IX-(1-42) is a suitable model for structural studies of Factor IX-membrane interaction.

Factor IX is a vitamin K-dependent plasma zymogen that plays a central role in blood coagulation (1). The mature protein is composed of a series of domains, including the Gla domain, the aromatic amino acid stack domain, two epidermal growth factor domains, and a serine protease domain (2, 3). The fully carboxylated form of human Factor IX binds calcium ions (4). Factor IX contains two classes of metal-binding sites which are defined by \(\gamma\)-carboxyglutamic acid (5, 6) and a third class which is a component of the EGF domain (7, 8). More recently, an additional site within the protease domain has been suggested (9). The metal binding site within the EGF domain is defined by aspartic acid 64, a residue that is partially (about 30%) \(\beta\)-hydroxylated (10-12). Similar to the other vitamin K-dependent proteins, Factor IX and Factor IXa bind to phospholipid membranes composed of phosphatidylserine (PS):phosphatidylcholine (PC) (13, 14). Factor IX affinity for PS:PC membranes is independent of the PS concentration with PS compositions above 20-30%; a \(K_c\) of about 1-2 \(\mu\)m has been measured (15). This interaction requires both calcium ions and fully carboxylated Factor IX (14).

When Factor IX binds to calcium, the protein undergoes conformational transitions that can be monitored by fluorescence quenching and the expression of neoantigenic determinants. With conformation-specific antibodies, Liebman et al. (14) demonstrated that, like prothrombin (16), Factor IX undergoes two sequential conformational changes. Most divalent metal ions support the first conformational change while only calcium ions support the second conformational change. It is this final conformer that expresses phospholipid binding properties. The location of the phospholipid binding site in Factor IX is not known, but studies on the other vitamin K-dependent proteins provide insight. We have prepared mutants of prothrombin in which the first kringle domain, the second kringle domain, or both kringle domains have been deleted and shown that these mutant forms bind to phospholipid vesicles in the presence of calcium (17). These results indicate that the kringle domains, and by analogy the EGF domains in Factor IX, are not implicated in phospholipid binding. Despite considerable study of the membrane binding properties of the vitamin K-dependent proteins that spans two decades, the molecular details of the nature of protein-membrane interaction of this class of membrane-binding proteins remain elusive. Solution of the crystal structure of prothrombin fragment 1 has defined an internal core of calcium ions liganded by the carboxylate groups of \(\gamma\)-carboxyglutamic acid (18). Most of the carboxylate oxygen atoms of \(\gamma\)-carboxyglutamic acid residues participate in forming this core and are thus unavailable for bridging to phospholipid through calcium ions. This structure confirmed earlier suggestions that the membrane-binding sites of the vitamin K-dependent proteins did not necessarily involve \(\gamma\)-carboxyglutamic acids as contact residues that inter-
acted with the phospholipid membrane (16, 19), in contrast to alternative models that had been proposed earlier (20). To determine the features of the protein-Ca(II)-membrane complex at atomic resolution requires crystallization of the ternary complex or examination of small but functional Gla-containing protein domains in complex with membranes in the presence of Ca(II) by two-dimensional nuclear magnetic resonance spectroscopy. In the current study we have determined whether the Factor IX Gla/aromatic amino acid stack domain in its fully carboxylated form is a suitable model for structural studies of Factor IX-membrane interaction. We show that Factor IX (1-47) has membrane binding properties. This peptide can serve as a suitable analog for the characterization of the structure of the Gla domain of Factor IX and its interaction with membrane surfaces.

**EXPERIMENTAL PROCEDURES**

Chemical Synthesis of Factor IX Residues 1-47 and of Factor IX Residues 1-42—Factor IX (1-47) and Factor IX (1-42) were synthesized using monomethyl polyethyleneamine chemistry on an Applied Biosystems model 430A peptide synthesizer. Amino acids were coupled as 1-hydroxybenzotriazole esters onto 0.55 mmol of p-hydroxymethylphenoxymethyl polystyrene resin. Side chain protecting groups included 2,2,5,7,8-pentamethylchromane-6-sulfonyl (arginine), OtBu (aspartic acid and y-carboxyglutamic acid), trityl (cysteine), t-butoxycarbonyl (lysine), t-butoxycarbonyl (lysine), and 9-fluorenylmethoxycarbonyl (serine, threonine, and tyrosine). Following each coupling step all uncoupled α-NH₂ terminals were acetylated. All ω-y-carboxyglutamatic acid residues were coupled using 1 mmol of protected amino acid per 100 pr of 1 mg/lO ml) was adjusted to pH 8.5 with ammonium hydroxide. The above HPLC gradient was employed. Following oxidation the reaction mixture was lyophilized. The amino acid sequence was verified using a Millenium 6000 ProSequence. The purity and molecular size of the peptides were confirmed by SDS-polyacrylamide gel electrophoresis in the presence and absence of β-mercaptoethanol, according to Schagger and von Jagow (21). The peptides were visualized with Coomassie Blue.

Materials—Phosphatidylcholine, phosphatidylserine, and dansylphosphatidylethanolamine were obtained from Avanti Polar Lipids and DEGR-CMK from Calbiochem. The chromogenic substrate specific for Factor IXa (CBS 31.39) was obtained from Diagnostica Stago. Human Factor Xa was purchased from Enzyme Research Laboratories. Human Factor Xa was purchased from Hematologic Technologies. Human Factor IX and Factor VIII/IX/FVWF were the generous gift of Dr. William Drohan (22). Factor IXa was preincubated with Factor Xa at a weight ratio of 50:1 in the presence of 5 mm CaCl₂, 3.5 h at 37 °C. The reaction was stopped by the addition of 10 mm EDTA.

Analysis for y-Carboxyglutamic Acid—The y-carboxyglutamic acid content of the purified peptide was determined by a modification of the method of Mallinson and Katayama. The peptide (10 nmol) was hydrolyzed in 2.5 m KOH for 16 h at 110 °C. The alkali, filtered to remove glass beads, was derivatized with o-phthalaldehyde and ethanedithio-. The derivatized amino acids were separated by high performance liquid chromatography on a Nucleosil 55 BBN anion exchange column (4.6 x 250 mm; Macherey-Nagel, Germany) under isocratic conditions at a flow rate of 1 ml/min. A 1:1 volume to volume mixture of 0.1 m sodium citrate, pH 5.2; acetoni/trile was used as the mobile phase (23). The column effluent was irradiated at 240 nm using an Applied Biosystems model 540A diode array spectrophotometer. Factor IXa (1-47) and Factor IX (1-42) were preincubated with Factor Xa or Factor Xa previously treated with 100 units of trypsin. The sample was irradiated at 280 nm using an slt width of 4 nm and the emission monitored at 340 nm using a slt width of 16 nm. The fluorescence titrations were performed by the addition of Ca(II) or Mg(II), as described originally by Nelsestuen et al. (24). At the completion of titration, the reversibility of metal-induced quenching was tested by the addition of 1.4 mM EDTA.

Immunochromatographic Analysis with Conformation-Specific Antibodies—The binding of Factor IX (1-47) and Factor IX (1-42) to conformation-specific anti-Factor IX antibodies was studied using a solution phase radioimmunoassay. Factor IX was iodinated with Na³¹I using EnzymoLink (25). The iodinated protein was separated using gel filtration on Sephadex G-25, then by immunooaffinity chromatography with anti-Factor IX: Mg(II) ovelinkingly bound to Sepharose 4-B. Anti-Factor IX: Ca(II)-specific antibodies and anti-Factor IX: Mg(II) antibodies were purified as described previously (26). A competition radioimmunoassay was configured to study the displacement of ³¹I-labeled Factor IX from anti-Factor IX antibodies at increasing concentrations of Factor IX (1-47) or Factor IX (1-42). Varying concentrations of unlabeled competitor (Factor IX (1-47) or Factor IX (1-42)), normal rabbit immunoglobulin (1 mg/ml), and ³¹I-labeled Factor IX were preincubated with either Factor IX: Ca(II)-specific antibody or Factor IX: Mg(II) antibodies. A final assay volume of 300 ml containing 15 m NaCl, 50 mM Tris, pH 7.4, 1 mM benzamidine, 0.1% bovine serum albumin, 0.1% Tween 20, and either 3 mM CaCl₂ or 5 mM MgCl₂ was incubated overnight at 4 °C. Following incubation, 1 ml of rabbit anti-goat immunoglobulin (25 mg in 1 ml of 0.1% Tris, 0.15 m NaCl, 0.1% NaN₃, 2.5% polyethylene glycol 4000, pH 7.4) was added. The precipitate that was formed was removed by centrifugation and assayed for ³¹I using a Packard 5000 Series Auto-Gamma scintillation counter.

The Effect of Factor IX (1-47) on Factor IX Activation by Factor Xa—The effect of Factor IX (1-47) on Factor IX activation by Factor Xa was studied using SDS-gel electrophoresis and autoradiography to monitor the development of the Factor Xa light chain and the intact Factor IX. Factor Xa (0.4 nm) was added to ³¹I-labeled Factor IX (2 nm) in a volume of 100 ml containing 20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM CaCl₂. The reaction, performed in the presence or absence of Factor IX (1-47) (2 μg/ml), was incubated at 37 °C with aliquots removed at various time intervals over 90 min. The reaction was stopped with the addition of Laemmli SDS gel loading buffer containing 10% β-mercaptoethanol and 16 mM EDTA. After the samples were subjected to electrophoresis in SDS gels, the gels were dried and exposed to Kodak X-Omet AR film. Autoradiographs were scanned for densitometric analysis on LKB Ultrascan XL densitometer.
(1 nm), human Factor VIII (4.5 units/ml), human Factor X (250 nm), and phospholipid vesicles composed of PC/PS/PE-dansyl (50:40:10, 25 µm). Factor IX-(1-47), at indicated concentrations between 1 and 25 µm, was added to the reaction mixture. The reaction was allowed to proceed at 25 °C for 18 min, and 25-µl aliquots were removed at 2-min intervals and placed in wells containing 10 µm EDTA to stop the reaction. The amount of Factor Xa generated was determined using the chromogenic substrate CBS 31.39. CBS 31.39 (50 µl; 625 µm final concentration) was added to each sample in a 96-well plate, and the absorbance at 405 nm was determined over 10 min at 37 °C using a Thermomax kinetic enzyme-linked immunosorbent assay reader (Molecular Devices). From these data the rate of activation of Factor X was determined.

**Phospholipid Binding Studies**—The binding of the Factor IX-(1-47) peptide and the Factor IX-(1-42) peptide to phospholipid vesicles was evaluated by energy transfer measurements performed on a SLM 8000C fluorescence spectrophotometer. Fluorophores in the peptide were excited at 280 nm. When the peptide is in close proximity to the dansyl group of the phosphatidylethanolamine, the dansyl emission is proportional to the amount of peptide at 520 nm. Because the efficiency of energy transfer has an inverse relation to the distance between the peptide and the dansyl group, the amount of peptide was determined based upon the Gla aromatic amino acid stack domains of Factor IX. Various calculations were used to prepare Factor IX-(1-42).

**RESULTS**

Factor IX-(1-47) (Fig. 1) was synthesized using Fmoc/N-methylpyrrolidone chemistry. All γ-carboxyglutamic acid residues were coupled using 1 µm of protected amino acid per cartridge after activation. Following coupling of each Gla residue, a ninhydrin assay was performed to determine coupling efficiency prior to proceeding with the synthesis. Cleavage of the peptide from its solid support resin and simultaneous side chain deprotection was performed using trifluoroacetic acid. The crude deprotected peptide was purified by HPLC using a Hi-Pore 318 column monitored at 214 nm. The identity of the peak was confirmed by automated Edman degradation from residues 1 through 47. The purified peptide, containing two free sulfhydryl groups associated with cysteine 18 and cysteine 23, was oxidized by incubating the dilute peptide for 30 h at pH 8.5. The peptide was chromatographed on a Bio-Rex 70 ion exchange column and the column developed with a 10–50% acetic acid gradient. The folded peptide was then repurified by HPLC. The overall yield of purified Factor IX-(1-47) was about 600 mg or 64% of the theoretical yield of crude peptide. Similar methods of synthesis and analysis were used to prepare Factor IX-(1-42).

The purified Factor IX-(1-47) migrated as a single band in SDS gels following electrophoresis in the presence or absence of 10% β-mercaptoethanol (Fig. 2). The reduced form showed slightly reduced electrophoretic mobility. The amino acid analysis of the acid hydrolysate of Factor IX-(1-47) and of Factor IX-(1-42) yielded amino acid compositions within 10% of the theoretical expected results (Table I). Direct γ-carboxyglutamic acid analyses of the alkaline hydrolysates were performed to ensure that no carboxylation accompanied peptide synthesis, decoupling, or deprotection. Both peptides contained within 8% of the expected 12 residues of γ-carboxyglutamic acid; no glutamic acid was observed.

Factor IX undergoes a conformational transition upon interaction with Ca(II) and Mg(II) ions. This transition may be monitored by the quenching of intrinsic fluorescence and the exposure of novel antigenic determinants that are recognized by conformation-specific antibodies directed at the metal ion-stabilized conformer. We asked whether the 47-residue peptide, based upon the Gla/aromatic amino acid stack domains of Factor IX, might similarly exhibit these properties. Factor IX-(1-47), when irradiated at 280 nm, exhibits a fluorescence emission spectrum dominated by the single tryptophan at residue 42. As shown in Fig. 3A, the addition of Ca(II) to a solution containing Factor IX-(1-47) induced quenching of the intrinsic fluorescence of the peptide by about 75%. Half-maximal fluorescence quenching was observed at 50 µM CaCl₂. This effect was completely reversible by the addition of EDTA at a concentration equivalent to the cumulative calcium ion concentration. In contrast, Factor IX-(1-42) showed minimal fluorescence quenching upon the addition of CaCl₂ under similar experimental conditions. As shown in Fig. 3B, the addition of MgCl₂ to Factor IX-(1-47) was associated with a minimal increase in fluorescence followed by fluorescence quenching of about 75%; half-maximal fluorescence quenching was observed at about 0.4 mM MgCl₂. These results indicate that the Factor IX-(1-47) binds to calcium ions and induces the conformational change characteristic of Factor IX. However, Factor IX-(1-42) failed to demonstrate this characteristic conformational change.

To determine the ability of the Factor IX peptides to assume the metal-stabilized conformation characteristic of Factor IX, the interaction of these peptides with conformation-specific an-
Anti-Factor M:Ca(II)-specific antibodies bind to Factor M only unreduced. SDS gels were stained with Coomassie Blue. Lane 1, reduced; lane 2, unreduced. MW, molecular weight markers.

The interaction of the Factor M-(1-47) peptide with phospholipid vesicles was studied by fluorescence energy transfer. Acid hydrolysates of Factor IX-(1-47) or Factor IX-(1-42) were titrated in either Factor M-(1-47), or Factor M-(1-42). The sample was irradiated at 280 nm and the fluorescence emission monitored at 520 nm. The binding of peptide to the lipid vesicles was associated with proximity of intrinsic fluorophores in the peptide with the dansyl group in the membrane, thus facilitating energy transfer. As shown in Fig. 5, the addition of Factor IX-(1-47) led to increased fluorescence emission at 520 nm. This fluorescence was completely reversible upon the addition of 2 mM EDTA. A binding constant, $K_p$, was calculated to be 0.64 μM by fitting these data to a simple bimolecular model (27).

The Factor IX-(1-47) peptide was added in increasing concentration to phospholipid vesicles composed of phosphatidylserine, phosphatidylcholine, and dansyl-phosphatidylethanolamine (40:50:10) in the presence of 1 mM Ca(II). The sample was irradiated at 280 nm and the fluorescence emission monitored at 520 nm. The binding of peptide to the lipid vesicles was associated with proximity of intrinsic fluorophores in the peptide with the dansyl group in the membrane, thus facilitating energy transfer. As shown in Fig. 3, the addition of Factor IX-(1-47) led to increased fluorescence emission at 520 nm. This fluorescence was completely reversible upon the addition of 2 mM EDTA. A binding constant, $K_p$, was calculated to be 0.64 μM by fitting these data to a simple bimolecular model (27). In contrast, Factor IX-(1-42) showed only weak interaction with the phospholipid vesicles; this interaction was not reversible upon the addition of EDTA.

The activation of Factor IX by Factor XIa proceeds in the presence of Ca(II), but phospholipid membranes are not required. To determine whether Factor IX-(1-47) might bind to Factor XIa and thus interfere with the enzymatic activation of the peptide with the dansyl group in the membrane, thus facilitating energy transfer.

### Table 1

| Amino acid analysis of Factor IX peptides containing γ-carboxyglutamic acid |
|------------------|------------------|
| Amino acid       | Factor IX (1-47) | Factor IX (1-42) |
|                  | Actual | Expected | Actual | Expected |
| Alkaline hydrolysate | 12.1  | 12     | 11.2  | 12     |
| γ-Carboxyglutamic acid | 4.0  | 3.0     | 4.0   | 3.0     |
| Glutamic acid     | 13.5  | 13.1    | 13.1  | 13.1    |
| Acid hydrolysate  | 1.9   | 2.0     | 2.0   | 2.0     |
| Aspartic acid     | 1.8   | 1.7     | 1.7   | 1.7     |
| Glutamic acid/glutamine | 3.2  | 3.0     | 3.0   | 3.0     |
| Serine            | 2.8   | 2.9     | 2.9   | 2.9     |
| Glycine           | 1.2   | 1.2     | 1.2   | 1.2     |
| Histidine         | 0.4   | 0.3     | 0.3   | 0.3     |
| Arginine          | 0.0   | 0.0     | 0.0   | 0.0     |
| Threonine         | 1.8   | 1.4     | 1.4   | 1.4     |
| Alanine           | 3.0   | 1.9     | 1.9   | 1.9     |
| Proline           | 1.1   | 0.6     | 0.6   | 0.6     |
| Tyrosine          | 0.1   | 0.1     | 0.1   | 0.1     |
| Valine            | 0.0   | 0.0     | 0.0   | 0.0     |
| Methionine        | 4.0   | 4.0     | 4.0   | 4.0     |
| Leucine           | 3.2   | 1.8     | 1.8   | 1.8     |
| Phenylalanine     | ND    | ND      | ND    | ND      |
| Lysine            | ND    | ND      | ND    | ND      |
| Tryptophan        | ND    | ND      | ND    | ND      |
| Cysteine          | ND    | ND      | ND    | ND      |

* ND, not determined.
Factor IX to Factor IXa, we evaluated the effect of Factor IX-(1-47) on this reaction. Factor IX was incubated with Factor XIa in the presence of 1 mM Ca(II) in the presence or absence of Factor IX-(1-47). The concentration of Factor IX-(1-47) was 2 μM, 1000-fold higher than that of Factor IX. The progress of the reaction was monitored over time by SDS-gel electrophoresis in the presence of reducing agent. The disappearance of Factor IX (Fig. 6) and the appearance of the light chain of Factor IXa (data not shown) were determined by quantitative densitometric analysis of the SDS gels. Under the conditions employed, the conversion of Factor IX to Factor IXa was nearly complete by 40 min. The presence of Factor IX-(1-47), even at high concentration, did not accelerate or decrease the rate of Factor IX activation (Fig. 6) or the rate of Factor IX light chain appearance. These results suggest that Factor IX-(1-47) alone does not bind directly to Factor XIa and interfere with substrate binding.

It has been demonstrated previously that Factor IX-(1-43) inhibits the activation of Factor X by the complex of Factor IXa and Factor VIII in the presence of Ca(II) and phospholipid (28, 29). To determine whether Factor IX-(1-47) is also inhibitory in this reaction, we used the competition tenase assay system. The progress of the conversion of Factor X to Factor Xa was

**FIG. 4. Interaction of conformation-specific antibodies with Factor IX-(1-47) and Factor IX-(1-42).** 

A, anti-Factor IX:Ca(II)-specific antibodies. B, anti-Factor IX:Mg(II) antibodies. C, plasma-derived Factor IX; ■, Factor IX-(1-47); ○, Factor IX-(1-42).

**FIG. 5. Comparison of the phospholipid binding properties of Factor IX-(1-47) and Factor IX-(1-42).** The interaction of peptides Factor IX(1-47) and Factor IX(1-42) with phospholipid vesicles was studied by monitoring energy transfer from the peptide to the dansyl group in the phosphatidylethanolamine incorporated into the lipid vesicles. The sample was irradiated at 280 nm and the fluorescence emission monitored at 520 nm. The change in fluorescence was monitored as a function of increasing peptide concentration. I₀, fluorescence (arbitrary units) in the absence of peptide; I, fluorescence at indicated peptide concentration. ●, Factor IX-(1-47); □, Factor IX-(1-42).

**FIG. 6. Effect of Factor IX-(1-47) on the activation of Factor IX by Factor XIa.** Factor XIa (0.4 nm) was added to [35S]-labeled Factor IX (2 nm) in a volume of 100 μl containing 20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM CaCl₂. The reaction, performed in the presence (○) or absence (□) of Factor IX-(1-47) (2 μM), was incubated at 37 °C, with aliquots removed at the indicated times and the reaction stopped with the addition of Laemmli SDS gel loading buffer containing 10% 2-mercaptoethanol and 10 mM EDTA. After SDS-gel electrophoresis, the gels were dried and exposed to Kodak X-Omat AR film. Autoradiographs were quantitated by densitometry. The disappearance of Factor IX versus time reflects the rate of Factor IX activation.
Factor IX Gla Peptide

![Graph](image)

**Fig. 7. Effect of Factor IX(1-47) on the activation of Factor X by Factor IXa and Factor VIII in the presence of calcium ions and phospholipid vesicles.** Factor Xa generation was measured from the amidolytic activity of Factor Xa. The reaction mixture, in a volume of 250 μl, contained human Factor IXa (1 nm), human Factor VIII (4.5 units/ml), human Factor X (250 nm), and phospholipid vesicles composed of PC/PS/PE-dansyl (50:40:10 25 μM) in a buffer of 20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.1% bovine serum albumin, 5 mM CaCl₂. Factor IX(1-47), at a indicated concentration, was added to the reaction mixture. The reaction was allowed to proceed at 25 °C for 18 min, and 25-μl aliquots were removed at 2-min intervals, and the amount of Factor Xa generated was determined using the chromogenic substrate CBS 31.39. The Factor IX(1-47) concentration is plotted versus the percent inhibition of Factor Xa activation monitored using the chromogenic substrate CBS 31.39. As shown in Fig. 7, Factor IX(1-47) inhibited the proteolytic conversion of Factor X by the tenase complex. Half-maximal rates of Factor Xa generation were observed with concentrations of Factor IX(1-47) of about 25 μM.

**DISCUSSION**

To study the interaction of the Gla domain of Factor IX with phospholipid membranes, we have evaluated the properties and characteristics of synthetic peptides based upon the primary structure of Factor IX. Our goal was to identify the minimal region of Factor IX that retained the complete membrane binding properties that characterize Factor IX. We describe a high yield chemical synthesis of a 47 residue peptide that contains 12 residues of ω-carboxyglutamic acid. Analysis of the purified peptide demonstrated that under the synthetic and purification conditions employed, the ω-carboxyglutamic acid residues were incorporated quantitatively and did not undergo decarboxylation. This chemical synthesis, parallel to the synthesis of ω-carboxyglutamic acid-containing peptides based upon the structure of Factor VII (30) and protein C (31), offers new opportunities for analysis of structure-function relationships in the vitamin K-dependent proteins. This approach provides a strategy to obtain large quantities of a homogeneous Gla-containing peptide of predetermined length and primary structure for purposes of structural and functional characterization. This has significant advantages over the use of proteolytic digestion products of the intact vitamin K-dependent proteins, and, particularly for Gla-containing peptides, expression of specific domains in heterologous recombinant systems.

In the current work, we have established that Factor IX(1-47) exhibits many of the membrane binding properties that characterize Factor IX. This peptide undergoes a conformational change induced by Ca(II) ions as measured by both quenching of intrinsic fluorescence and the exposure of conformation-dependent antigenic determinants. Furthermore, this peptide bound reversibly to phospholipid vesicles composed of acidic phospholipids in the presence of Ca(II). This peptide inhibited the enzymatic activation of Factor X by Factor IXa in a system that requires phospholipid membranes, but did not inhibit the activation of Factor IX by Factor XIa in a reaction that does not require phospholipid membranes. By these criteria, Factor IX(1-47) has intrinsic calcium-dependent membrane-binding properties that parallel those of Factor IX. In contrast, Factor IX(1-42), a peptide truncated within the aromatic amino acid stack domain, lacks these properties. Our results on Factor IX(1-42) confirm previous work in which bovine Factor IX(1-42), prepared by proteolytic digestion of bovine Factor IX, did not bind to phospholipid membranes in the presence of calcium ions (32). These results indicate that both the entire Gla and aromatic amino acid stack domains are required for membrane binding in the case of Factor IX. The peptide and Gla domain of Factor IX (residues -18 to 38) are encoded by Exon II, whereas the aromatic amino acid stack domain (residues 39-46) is encoded by Exon III (2,3). Thus, the phospholipid binding results of Factor IX(1-47) indicate that the functional unit for phospholipid binding involves domains, the Gla domain and the aromatic amino acid stack domain, encoded by two separate exons. Previously published data on protein C(1-38) have demonstrated more modestly reduced phospholipid binding solely by the Gla domain (31) encoded by Exon II of the protein C gene compared with the Gla-aromatic amino acid stack domain. Our results indicate that a functional lipid binding unit must include not only the Gla domain but also the aromatic amino acid stack domain to support calcium-mediated Factor IX-phospholipid binding. Whether the aromatic amino acid stack domain contributes residues for contact with the membrane or whether this region is critical to the proper folding and expression of other regions of the Gla domain that contact the membrane surface remains to be determined.

Previous study of the metal ion-induced conformational transitions in Factor IX that can be monitored by fluorescence quenching have shown that half-maximal quenching occurs at about 50 μM Ca(II) (33). Factor IX(1-47) binds to Ca(II) and Mg(II) ions, which induce the characteristic fluorescence quenching observed in the vitamin K-dependent proteins. Factor IX(1-47) demonstrated half-maximal quenching at 50 μM Ca(II), a somewhat lower concentration than observed for intact Factor IX. This likely represents the absence of conformational constraints on the Gla/aromatic amino acid acid stack domains imposed by the EGF domain. Evidence exists for the interaction of the Gla domain and the first EGF domain or the combined effect of multiple conformational changes in distinct portions of Factor IX in response to calcium ions (34, 35); this interaction may be favored by a structure stabilized by the occupancy of metal binding sites by calcium ions and may contribute thermodynamically to the global energy of calcium binding and stabilization of the polypeptide backbone conformation, including calcium-dependent interaction between the Gla and EGF domains. The absence of these interactions in Factor IX(1-47) may be responsible for the lower Ca(II) concentration for half-maximal fluorescence quenching. By comparison, protein C(1-49) exhibited most of the calcium and membrane properties of intact protein C, but did not demonstrate calcium ion-induced fluorescence quenching (31). These authors suggest that the absence of fluorescence quenching does not preclude a metal-induced conformational transition,
particularly when high levels of calcium ions are required to effect this transition in proteolytically derived peptides lacking part of the aromatic stack. It is important to emphasize that our Factor IX peptide, from residues 1 to 47, undergoes the conformational transition and the amount of calcium ion needed to effect this transition is lower than that required in the intact Factor IX. These may be intrinsic differences between Factor IX and protein C.

Anti-Factor IX:Ca(II)-specific antibodies and anti-Factor IX: Mg(II) antibodies are polyclonal immunofinity-purified conformation-specific antibodies that recognize the conformational transition in Factor IX induced by metal ions (14). The location of the antigenic determinants against which these antibodies are directed has not been known, although studies on prothrombin have implicated residues 1-44 (36). However, the anti-Factor IX:Ca(II)-specific antibodies are known to inhibit binding of Factor IX to phospholipid membranes and thus are directed at or near the phospholipid binding site on Factor IX. In the current experiments, we show that Factor IX(1-47) contains the antigenic determinants against which anti-Factor IX:Ca(II)-specific antibodies and anti-Factor IX: Mg(II) antibodies are directed. The Ca(II)-stabilized epitopes are expressed nearly quantitatively as compared directly with Factor IX. It would appear that Factor IX(1-47) undergoes the Ca(II)-induced conformational change leading to the expression of these Ca(II)-stabilized epitopes and that the conformational motility of this peptide favors a Factor IX-like native structure.

The activation of Factor IX by Factor Xa in the presence of calcium ions occurs independent of phospholipid membranes. Based upon the ability of anti-Factor IX: Ca(II)-specific antibodies to inhibit Factor IX-phospholipid binding and Factor IX activation by Factor Xa, we had previously proposed that the Gla domain of Factor IX might interact directly with Factor Xa, thus facilitating Factor IX cleavage (14). Based upon the current experiments in which Factor IX(1-47) failed to inhibit Factor IX cleavage by Factor Xa, even at a molar excess of Factor IX(1-47) over Factor IX of 1000-fold, this would appear not to be the case. However, we cannot rule out the possibility that the Gla/aminergic amino acid stack domains and the RGD domains may be sufficient to inhibit this reaction. In contrast, Factor IX(1-47) did inhibit the activation of Factor X by the tensase complex, a phospholipid-dependent reaction. Quantitative analysis of this inhibition indicates results parallel to those reported by Astermark et al. (28) on inhibition by bovine Factor IX(1-43). The basis of this inhibition, which was observed by Astermark et al. (28) only when Factor VIII was present but was independent of phospholipid (28), remains uncertain but may be a consequence of an interaction between the Gla domain of Factor IX with Factor VIII (28). Similarly, the inhibition of this reaction by Factor VII(1-49) (30) has been interpreted as resulting from a phospholipid-dependent interaction of the peptide with Factor IX. A model in which the phospholipid-dependent binding of Factor VIIa to Factor X is partially mediated by the Factor VIIa Gla domain is suggested (30). However, these potential interactions of the Gla domains of Factor IX and Factor VII must be taken within the context of the well established role of the Gla domains of the vitamin K-dependent proteins in facilitating the protein enzyme-substrate complexes required for blood coagulation.

Although it had been generally believed that the Gla domain and particularly individual Gla residues play identical roles in each of the vitamin K-dependent proteins, it is now becoming clear that this is not the case. For example, mutagenesis studies of the Gla residues of human prothrombin and human protein C suggest that not all Gla regions in different proteins work identically despite the marked sequence homology (36-40). Using the Factor IX numbering system, prothrombin and protein C have 3 or 4 Gla residues (Gla15, Gla27, Gla36, and possibly Gla38) which define an internal carboxylate core that chelates calcium that is not accessible to solvent; disruption of these residues destroys membrane binding activity and function. We suspect that these residues in Factor IX have an identical function. Gla1 is not essential for prothrombin or protein C, and based upon the naturally occurring but fully active mutant Factor IX Oxford 2 (Gla1 to Ala) (41), Gla1 is not important for Factor IX either. However, disruption of the other Gla residues in prothrombin and protein C have markedly different effects. For example, disruption of Gla15 and Gla20 in prothrombin decreases functional activity but has no effect of the activity of protein C. Conversely, disruption of Gla8 or Gla21 in protein C eliminates activity, but the same mutation in prothrombin only partially inhibits function. Based upon these findings we have proposed (36) that some of the Gla residues may contact directly with the membrane surface, whereas others stabilize interdomain interaction between the Gla domain and the linking region adjacent to the first kringle domain. The differences in phospholipid and metal binding characteristics between Factor IX(1-47) and protein C(1-48) must be appreciated within this context. As discussed, protein C(1-48) does not undergo calcium-induced fluorescence quenching; whereas Factor IX(1-47) undergoes calcium-induced fluorescence quenching under conditions identical to intact Factor IX. Moreover, protein C(1-48) shows a phospholipid binding affinity that is only 3-fold greater than protein C(1-38) (31). In contrast, Factor IX(1-47) binds to phospholipids with high affinity, but Factor IX(1-42) shows poor binding to phospholipid, and this binding is not reversible upon the addition of EDTA. In sum, these results serve to indicate that the structures of the Gla domains of the vitamin K-dependent proteins share many structural elements but that differences in structure reflect differences in function.

Based upon the characterization of Factor IX(1-47), this peptide but not Factor IX(1-42) will serve as a model for the NMR study of the structure of the Factor IX Gla domain and its interaction with membrane surfaces. Recent results of two-dimensional NMR experiments have led to the complete assignment of protons in Factor IX(1-47) in the absence of metal ions (42). Preliminary structural models of Factor IX(1-47) based upon the NMR constraints indicate the presence of alpha-helical structure and other local structures about the disulfide loop.

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