Structural Requirements for Ca\textsuperscript{2+} Binding to the \(\gamma\)-Carboxyglutamic Acid and Epidermal Growth Factor-like Regions of Factor IX

STUDIES USING INTACT DOMAINS ISOLATED FROM CONTROLLED PROTEOLYTIC DIGESTS OF BOVINE FACTOR IX*

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Blood coagulation factor IX is composed of discrete domains with an NH\textsubscript{2}-terminal vitamin K-dependent \(\gamma\)-carboxyglutamic acid (Gla)-containing region, followed by two domains that are homologous with the epidermal growth factor (EGF) precursor and a COOH-terminal serine protease part. Calcium ions bind to the Gla-containing region and to the NH\textsubscript{2}-terminal EGF-like domain. To be able to determine the structure and function of the Gla- and EGF-like domains, we have devised a method for cleaving factor IX under controlled conditions and isolating the intact domains in high yield, either separately or linked together. The Ca\textsuperscript{2+} and Mg\textsuperscript{2+} binding properties of these fragments were examined by monitoring the metal ion-induced changes in intrinsic protein fluorescence. A fragment, consisting of the Gla region linked to the two EGF-like domains, bound Ca\textsuperscript{2+} in a manner that was indistinguishable from that of the intact molecule, indicating a native conformation. The Ca\textsuperscript{2+} affinity of the isolated Gla region was lower, suggesting that the EGF-like domains function as a scaffold for the folding of the Gla region. The Gla-independent high affinity metal ion binding site in the NH\textsubscript{2}-terminal EGF-like domain was shown to bind Ca\textsuperscript{2+} but not Mg\textsuperscript{2+}. A comparison with similar studies of factor X (Persson, E., Björk, I., and Stenflo, J. (1991) J. Biol. Chem. 266, 2444–2452) suggests that the Ca\textsuperscript{2+}-induced fluorescence quenching is due to an altered environment primarily around the tryptophan residue in position 42.

Factor IX is a vitamin K-dependent plasma zymogen that upon activation by factor VIIa-tissue factor or factor XIa is converted to a serine protease active in blood coagulation (1–4). Together with the closely related factors VII, X, and protein C, factor IX forms a subgroup among the vitamin K-dependent clotting factors that is characterized by identical domain structure, a pronounced sequence homology, and identifiable positioning of introns separating the exons that code for the various domains (2, 5–9). The mature factor IX molecule consists of an NH\textsubscript{2}-terminal \(\gamma\)-carboxyglutamylacidic region, followed by two domains that are homologous to the epidermal growth factor (EGF) precursor and a COOH-terminal serine protease part. In factor IX, as well as in prothrombin and factor X, the Gla region has an ordered structure in the presence of Ca\textsuperscript{2+} (10–12). It can easily be removed by limited proteolytic cleavage in factor IX and related proteins, suggesting that it is quite mobile relative to the EGF-like domains, at least in the absence of Ca\textsuperscript{2+} (13–18). The EGF-like domains are independently folded structural units, a conclusion that derives from the observations that synthetic EGF-like domains fold spontaneously into their native conformation and that isolated EGF-like domains of factors IX and X and protein C retain a Ca\textsuperscript{2+} binding site (19–24). The NH\textsubscript{2}-terminal EGF-like domain of bovine factor IX (BfIX) has one \(\beta\)-hydroxyaspartic acid (Hya) residue, whereas the corresponding residue of human factor IX is only partially hydroxylated (25, 26). In addition, this domain has a unique O-linked carbohydrate side chain in both bovine and human factor IX (27–29).

The Gla region of factor IX binds Ca\textsuperscript{2+} and phospholipid and is also involved in binding of factor IX to a putative endothelial cell receptor (30–33). The NH\textsubscript{2}-terminal EGF-like domain binds one Ca\textsuperscript{2+} and also seems to interact with the same endothelial cell receptor (20, 21, 33). The serine protease part of activated factor IX has been implicated in the interaction with the cofactor, factor VIIa (34), although indirect evidence suggests that there is also a weak interaction between the COOH-terminal EGF-like domain and factor VIIa (35).

To elucidate the function of the EGF-like domains of factor IX, proteolytic fragments that consist of one or two EGF-like domains linked to the Gla region, to ensure phospholipid affinity, would be most useful. We have therefore exploited the putative interdomain mobility, the compact structure of the EGF-like domain, and the fact that the highly anionic Gla-containing region is quite resistant to degradation by proteolytic enzymes and developed a procedure to cleave factor IX under controlled conditions and to isolate the various domains. In contrast to methods that depend on the expression of recombinant proteins in pro- or eukaryotic
systems or the chemical synthesis of the domains, the current approach has the advantage that the disulfide bond pairing and the postribosomal modifications do not pose a problem. Furthermore, considerable amounts of the highly purified intact domains can be obtained if bovine proteins are used as starting material.

In this communication, we describe the isolation and characterization of a fragment, containing the two EGF-like domains, from a controlled proteolytic digest of bovine factor IX, as well as a fragment containing these two domains linked to the Gla region. We also demonstrate that the Gla region, linked to the EGF-like domains, has a native conformation as judged by its normal Ca\textsuperscript{2+} binding properties. Moreover, we report that the Gla-independent high affinity Ca\textsuperscript{2+} binding site does not bind Mg\textsuperscript{2+}.

**EXPERIMENTAL PROCEDURES\textsuperscript{2}**

**RESULTS**

Isolation and Characterization of Factor IX Domains—The strategy for the isolation of fragments of bovine factor IX (BfIX) is shown in Fig. 1. Cleavage of BfIX with chymotrypsin was monitored by SDS-PAGE, which revealed the formation of several discrete fragments (Fig. 2). One of these fragments, with an apparent molecular weight of 26,000, gave two sequenced in equimolar amounts: Tyr-Asn-Ser-Gly-Lys-Leu, corresponding to residues 1–6; and Val-Thr-Pro-Ile-Cys-Ile corresponding to residues 286–291 of intact BfIX (45). This fragment was isolated from a digest of BfIX (50–90 mg) by chromatography on a column of Q-Sepharose Fast Flow followed by gel filtration on a Sephadex G-75 column (Figs. 3 and 4). Sequence analysis indicated that the isolated fragment (BfIX-GlaEGFNc) was at least 95% homogeneous and gave no evidence for internal peptide bond cleavage (less than 5%). The recovery was between 25 and 35% in three experiments. The isolated BfIX-GlaEGFNc was reduced and alkylated and the two peptides were separated by HPLC. The long peptide, derived from the NH\textsubscript{2}-terminal part of the molecule, contained 11.8 mol of Gla and 1.1 mol of Hya per mol of protein and had an amino acid composition corresponding to residues 1–144 of intact BfIX (Table I). The small peptide, derived from the serine protease part, had a composition in agreement with residues 286–296. The absorption coefficient (A\textsubscript{280} nm) of the intact fragment was determined to be 10.5, based on a molecular weight of 18,170 for the apoprotein (calculated from the amino acid composition).

Cleavage of BfIX-GlaEGFNc (4–8 mg) with lysyl endopeptidase in an EDTA-containing buffer gave a fragment with an apparent molecular weight of 20,000 (Fig. 11B). This fragment, which was isolated as described above (Fig. 5), gave two sequences in equimolar amounts: Gin-Tyr-Val-Asp-Gly-Asp, corresponding to residues 44–49 of intact BfIX, and one sequence corresponding to residues 286–291 (see above) (45). The fragment was at least 95% homogeneous. Approximately 10% of the material was cleaved in the COOH-terminal EGF-like domain, at Lys-96. The fragment (BfIX-EGF(c)) contained no detectable Gla (<0.02 mol/mol of protein), but 1.2 mol of Hya per mol of protein, and had an amino acid composition corresponding to residues 286–296 of BfIX (Table I). The recovery relative to BfIX-GlaEGFNc was 30–40%. The absorption coefficient (A\textsubscript{280} nm at 280 nm) of the intact fragment was determined to be 9.5 based on a calculated molecular weight of 12,390.

The Gla peptide (BfIX-Gla), which was eluted together with BfIX-GlaEGFNc from the ion exchange column (Fig. 5), was isolated by gel filtration on a Sephadex G-75 column (not shown). Sequence analysis of the isolated BfIX-Gla corresponded to residues 1–6 of intact BfIX (see above) and indicated that the digestion was performed at 37 °C with 0.5% (w/w) chymotrypsin in 50 mM Tris-HCl, pH 7.5, containing 0.1 M NaCl and 5 mM EDTA. Aliquots of 50 μg were withdrawn from the incubation mixture and subjected to SDS-PAGE on a 10–15% gradient gel. Twenty μg of intact BfIX was applied in lane 0. Below each lane the digestion time in minutes is given, and on both sides of the gel molecular weight markers are shown. The samples were not reduced.


**Ca^{2+} Binding to Factor IX Domains**

**TABLE I**

| Amino acid | Intact | Light chain (1-144) | Heavy chain (286-296) | GlaEGF NC (44-144; 286-296) | Gla (1-43) |
|------------|--------|---------------------|-----------------------|-----------------------------|------------|
| Asp        | 19.2 (19) | 17.2 (17)          | 2.0 (2)               | 15.7 (16)                   | 3.0 (3)    |
| Thr        | 8.1 (8)   | 7.0 (7)             | 1.0 (1)               | 5.2 (5)                     | 2.4 (3)    |
| Ser        | 9.0 (10)  | 9.3 (10)            | 0 (0)                 | 7.7 (8)                     | 2.3 (2)    |
| Glu        | 22.9 (23) | 23.3 (23)           | 0 (0)                 | 11.2 (11)                   | 11.9 (12)  |
| Pro        | ND (5)    | ND (4)              | ND (1)                | ND (5)                      | ND (0)     |
| Gly        | 10.7 (10) | 10.3 (10)           | 0 (0)                 | 2.2 (2)                     | 8.7 (8)    |
| Ala        | 6.2 (6)   | 5.3 (5)             | 1.0 (1)               | ND (14)                     | 1.1 (1)    |
| Cys        | ND (16)   | 13.2 (15)           | 0.5 (1)               | ND (14)                     | ND (2)     |
| Val        | 8.9 (9)   | 8.1 (8)             | 1.0 (1)               | 6.7 (7)                     | 2.0 (2)    |
| Met        | 0.9 (1)   | 0.8 (1)             | 0 (0)                 | 1.0 (1)                     | 0 (0)      |
| Ile        | 5.2 (5)   | 3.3 (3)             | 1.8 (2)               | 5.0 (5)                     | 0 (0)      |
| Leu        | 6.1 (6)   | 5.6 (6)             | 0 (0)                 | 3.5 (4)                     | 0 (0)      |
| Tyr        | 4.8 (5)   | 3.8 (4)             | 1.0 (1)               | 3.7 (4)                     | 0 (0)      |
| Phe        | 7.3 (7)   | 6.6 (7)             | 0 (0)                 | 3.5 (3)                     | 3.6 (4)    |
| His        | 1.3 (1)   | 1.1 (1)             | 0 (0)                 | 1.2 (1)                     | 0 (0)      |
| Lys        | 11.9 (13) | 12.3 (13)           | 0 (0)                 | 7.7 (8)                     | 5.3 (5)    |
| Arg        | 7.1 (8)   | 7.0 (7)             | 1.0 (1)               | 4.7 (5)                     | 3.0 (3)    |
| Trp        | ND (2)    | ND (2)              | ND (0)                | ND (2)                      | ND (1)     |
| Hya        | 1.1 (1)   | 1.1 (1)             | 0 (0)                 | 1.2 (1)                     | 0 (0)      |
| Gla        | 12.6 (12) | 11.8 (12)           | 0 (0)                 | 0 (0)                       | 12.0 (12)  |

- Values obtained by extrapolation to zero hydrolysis time.
- ND, not determined.
- Determined as carboxymethylcysteine.
- WD, not determined.
- Determined as carboxymethylcysteine.
- 72-h hydrolysis.

Fig. 6. **Fragments isolated from bovine factor IX.** Intact BfIX (lanes A and F), BfIXα' (lanes B and G), Gla-domainless BfIXα" (lanes C and H), BfIX-GlaEGFNc (lanes D and I), and BfIX-GlaEGFNc (lanes E and J) were subjected to SDS-PAGE on a 10-15% gradient gel. The protein band in lane B with an apparent molecular weight of 40,000 corresponds to BfIXα", lacking the COOH-terminal region (residues 139-235 of the heavy chain) due to cleavage at Arg-138. The samples in lanes F-J were reduced and alkylated prior to electrophoresis. The light chain of BfIXα" has an apparent molecular weight of 30,000 (lanes G and J), whereas the Gla-domainless counterpart has a molecular weight of approximately 25,000 (lanes H and J). Molecular weight markers are shown on both sides of the gel. BfIX-Gla is very poorly stained by Coomassie Brilliant Blue and is not visible on the gel.

Cated at least 95% homogeneity. The fragment contained 12.0 mol of Gla per mol of protein, but no detectable Hya (<0.02 mol/mol of protein), and had an amino acid composition in agreement with residues 1-43 of the zymogen (Table I). The absorption coefficient (A<sub>ε</sub> at 280 nm) was calculated to be 10.0 (M<sub>r</sub> = 5,779). Fig. 6 shows the SDS-PAGE of the fragments used in this investigation.

**Measurements of Intrinsic Protein Fluorescence**—Two classes of binding sites, with differing affinities for Ca<sup>2+</sup>, have previously been identified in BfIX and BfIXα" by Morita *et al.* (13, 46). Two of the sites are Gla-independent with a K<sub>d</sub> of approximately 60 μM, whereas the sites with lower affinity, presumably Gla-dependent, have an average K<sub>d</sub> of approximately 0.8 mM. The isolated BfIX fragments allowed us to characterize these Ca<sup>2+</sup> binding sites and to investigate whether the Ca<sup>2+</sup> binding properties of the free Gla region are identical with those of the Gla region bound to the EGF-like domains. To this end, we measured the effects of Ca<sup>2+</sup> and Mg<sup>2+</sup> on the intrinsic protein fluorescence of BfIX-GlaEGFNc, BfIX-EGFNc, and BfIX-Gla and compared the observed changes with those induced by these ions in BfIXα" and Gla-domainless BfIXα".

Fluorescence emission spectra showed a significant quenching of tryptophan fluorescence in all proteins, except BfIX-EGFNc, on addition of saturating concentrations of Ca<sup>2+</sup>, whereas only a small blue shift was observed (Fig. 7). In addition, an additional increase of the fluorescence of BfIX-GlaEGFNc, and BfIX-Gla-domainless BfIXα" was observed with either fragment (Fig. 7A). At higher Ca<sup>2+</sup> concentrations, an additional fluorescence quenching, to approximately 15%, was observed in BfIXα" (Fig. 7A), due to binding of Ca<sup>2+</sup> to low affinity sites (half-maximum at approximately 1 mM). This fluorescence was not seen in Gla-domainless BfIXα" (not shown). On addition of Mg<sup>2+</sup>, no initial fluorescence quenching could be observed with either fragment (Fig. 7B). At higher Mg<sup>2+</sup> concentrations, there was a 15% fluorescence quenching in BfIXα" due to binding of Mg<sup>2+</sup> to sites that were half-saturated at 3-5 mM Mg<sup>2+</sup>, whereas only a small (2-3%) quenching was observed in Gla-domainless BfIXα" (Fig. 7B).
Ca$^{2+}$ Binding to Factor IX Domains

FIG. 7. Fluorescence emission spectra of BfIXa$^{\beta'}$ and BfIX fragments in the presence and absence of Ca$^{2+}$. Normalized emission spectra of BfIXa$^{\beta'}$ (A), Gla-domainless BfIXa$^{\beta'}$ (B), BfIX-GlaEGFNc (C), BfIX-EGFNc (D), and BfIX-Gla (E) in the presence (----) and absence (—) of Ca$^{2+}$. The protein concentrations were 2 μM (A–D) and 5 μM (E), and the spectra shown correspond to the Ca$^{2+}$ concentrations at which a final plateau was reached on titration of each fragment, i.e., at 4 mM (A and C), 0.2 mM (B), 1 mM (D), and 20 mM (E) Ca$^{2+}$. In A and C, the fluorescence spectra at 0.2 mM Ca$^{2+}$ (-----), at which an intermediate plateau was observed in the titrations, are also shown (see Fig. 8A).

10% increase of the intrinsic fluorescence due to binding of Ca$^{2+}$ to sites that were half-saturated at approximately 40 μM Ca$^{2+}$ (Fig. 8B), i.e., with an affinity similar to that of BfIXa$^{\beta'}$ and Gla-domainless BfIXa$^{\beta'}$. At higher Ca$^{2+}$ concentrations, there was a 45–50% quenching that was half-saturated at about 0.8 mM Ca$^{2+}$ (Fig. 8A), to be compared with the half-saturated quenching at approximately 1 mM Ca$^{2+}$ for BfIXa$^{\beta'}$. A similar pattern was observed on addition of Mg$^{2+}$, although the binding affinity was lower (Fig. 9). The initial increase of the fluorescence was approximately 17%, with half-saturation at approximately 150 μM Mg$^{2+}$. At higher Mg$^{2+}$ concentrations, a 45% quenching, half-saturated at approximately 2.5 mM Mg$^{2+}$, was observed.

To further characterize the ion binding properties of the factor IX molecule, the Gla residues in BfIX-GlaEGFNc were decarboxylated. After 16 h of heating, the fragment was intact as judged by sequence analysis, but retained only 13% of the Gla residues. On titration with Ca$^{2+}$, there was a 6% initial increase in tryptophan fluorescence, due to binding of Ca$^{2+}$ to sites that were half-saturated at approximately 60 μM (Fig. 10), with no additional fluorescence change being observed at higher Ca$^{2+}$ concentrations (up to 2.5 mM). In contrast, Mg$^{2+}$ did not induce any significant change in the fluorescence emission of the fragment (Fig. 10).

On titration of BfIX-Gla with Ca$^{2+}$, an initial plateau was observed, followed by a decrease of the intrinsic fluorescence to about 40% at >20 mM Ca$^{2+}$ (Fig. 8A). Half-saturation occurred at approximately 4 mM Ca$^{2+}$, i.e., at a concentration 4 times higher than that observed for BfIXa$^{\beta'}$ and BfIX-
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[Graph showing Ca\textsuperscript{2+} Binding]

**Fig. 10.** Effect of metal ions on intrinsic tryptophan fluorescence of decarboxylated BfIX-GlaEGF\textsubscript{NC}. BfIX-GlaEGF\textsubscript{NC} was decarboxylated and titrated with Ca\textsuperscript{2+} (■) and Mg\textsuperscript{2+} (□). The protein concentration was 2 µM, and the data were plotted as described in the legend to Fig. 8.

**Fig. 11.** Effect of Ca\textsuperscript{2+} on the proteolytic cleavage carboxy-terminal of Lys-43. Digestion of BfIX\textalpha' (A) and BfIX-GlaEGF\textsubscript{NC} (B) was performed at 37 °C with 0.2% (w/w) lysyl endopeptidase in 50 mM Tris-HCl, pH 7.5, containing 0.1 M NaCl and either 5 mM Ca\textsuperscript{2+} or 5 mM EDTA. Aliquots (30 µg) were withdrawn from the incubation mixtures and subjected to SDS-PAGE on a 10-15% gradient gel. Twenty µg of intact BfIX\textalpha' and BfIX-GlaEGF\textsubscript{NC} were applied in the lanes marked 0. Below each lane the digestion time in minutes is given, and on the left side of each gel, molecular weight markers are shown. The protein band in A with an apparent molecular weight of 40,000 corresponds to BfIX\textalpha' lacking the COOH-terminal region (residues 139-255 of the heavy chain) due to cleavage at Arg-138. The samples were not reduced.

GlaEGF\textsubscript{NC}. All fluorescence changes were reversible on addition of EDTA.

**Metal Ion Dependence of Cleavage at Lys-43**—During development of the procedure for isolation of BfIX-GlaEGF\textsubscript{NC}, it was noticed that the rate of cleavage, as well as the cleavage pattern, depended on the presence of Ca\textsuperscript{2+} in the buffer. Fig. 11A demonstrates that lysyl endopeptidase (0.2%, w/w) cleaves BfIX\textalpha' completely within 2 min in the presence of EDTA, whereas no cleavage at all can be seen after 20 min in a Ca\textsuperscript{2+}-containing buffer. The same protection against cleavage was achieved with a buffer containing Mg\textsuperscript{2+} (not shown). This Ca\textsuperscript{2+} and Mg\textsuperscript{2+} dependence was also observed when the enzyme cleaved BfIX-GlaEGF\textsubscript{NC} to produce BfIX-Gla and BfIX-EGF\textsubscript{NC}, indicating a similar metal-ion-induced conformational transition in the fragment and in intact BfIX\textalpha' (Fig. 11B).

**DISCUSSION**

Binding of Ca\textsuperscript{2+} to BfIX-GlaEGF\textsubscript{NC}, which has 2 Trp residues, results in changes of intrinsic protein fluorescence indicative of at least two binding processes, one with high affinity (half saturation at ~40 µM Ca\textsuperscript{2+}) and one with low affinity (half-saturation at ~0.8 mM Ca\textsuperscript{2+}). The high affinity Ca\textsuperscript{2+} binding is observed as an increase in fluorescence emission, whereas Ca\textsuperscript{2+} binding to the low affinity sites results in a fluorescence quenching. A similar initial increase in fluorescence also occurs upon binding of Ca\textsuperscript{2+} to the high affinity sites in the GlaEGF fragments from protein C and factor X (47, 48). As judged from the increase in the fluorescence emission, a high affinity Ca\textsuperscript{2+} binding site is also present in the decarboxylated form of BfIX-GlaEGF\textsubscript{NC}, and thus this site seems to be Gla-independent. On the other hand, no change in intrinsic tryptophan fluorescence was observed in decarboxylated BfIX-GlaEGF\textsubscript{NC} on addition of Mg\textsuperscript{2+}, indicating that the Gla-independent site does not bind Mg\textsuperscript{2+}. The Ca\textsuperscript{2+}-induced alteration of fluorescence emission of the GlaEGF fragments from factors IX and X and protein C must be caused primarily by changes in the environment of Trp-42, Trp-84, and Trp-113. These observations suggest that changes in the environment of Trp-72 in factor IX and Trp-42, correspondingly, is the only conserved Trp residue in all three fragments. Moreover, in factor X it is the only Trp residue in the light chain. The initial increase in tryptophan fluorescence, that takes place on titration of intact BfIX-GlaEGF\textsubscript{NC} with Ca\textsuperscript{2+}, probably does not solely depend on binding of Ca\textsuperscript{2+} to the Gla-independent site, since a similar increase was observed on titration with Mg\textsuperscript{2+}, which does not bind to this site. Hence, it is likely that part of the observed increase in tryptophan fluorescence of intact BfIX-GlaEGF\textsubscript{NC} on addition of Ca\textsuperscript{2+} is due to binding of Ca\textsuperscript{2+} to the Gla-dependent binding sites and part to the Gla-independent site. The Gla dependence of the low affinity Ca\textsuperscript{2+} binding sites was clearly demonstrated by the absence of fluorescence changes in the decarboxylated fragment. In this context, it is noteworthy that factor IX has two classes of Mg\textsuperscript{2+} binding sites (Fig. 9). However, the affinity for binding of Ca\textsuperscript{2+} is higher and, most importantly, there appears to be no Gla-independent binding of Mg\textsuperscript{2+}.

Binding of Ca\textsuperscript{2+} to Gla-domainless BfIX\textalpha', which lacks Trp-42, is accompanied by a small quenching of intrinsic protein fluorescence, also described by Morita et al. (13, 46). A similar quenching is observed on Ca\textsuperscript{2+} binding to Gla-domainless bovine factor C (51). In contrast, Ca\textsuperscript{2+} binding to the Gla-domainless bovine factor X, which has no Trp residue in the light chain, results in a very small fluorescence quenching (49, 52, 53). These observations suggest that changes in the environment of Trp-72 in factor IX and Trp-84 in protein C are responsible for the fluorescence quenching that occurs in the Gla-domainless proteins, in spite of the fact that no fluorescence quenching can be seen on Ca\textsuperscript{2+} binding to BfIX-EGF\textsubscript{NC} and the corresponding fragment from bovine protein C (47). The latter finding would imply that quenching of the fluorescence from Trp-72 in factor IX and Trp-84 in protein C only occurs when these domains have an adjacent serine protease region. However, it cannot be excluded that the fluorescence quenching that accompanies Ca\textsuperscript{2+} binding to Gla-domainless BfIX\textalpha' and protein C is caused by Trp residues in the heavy chain of the molecule. It should be emphasized that both the increase and decrease of intrinsic protein fluorescence occur at the same Ca\textsuperscript{2+} concentration in BfIX-GlaEGF\textsubscript{NC} and intact BfIX\textalpha', indicating that the fragment has a native conformation. In contrast, much higher Ca\textsuperscript{2+} concentrations are needed to induce a fluorescence quenching in BfIX-Gla, which suggests that this...
region alone cannot attain a native conformation. It appears, therefore, that the EGF-like domains in factor IX provide a scaffold for the normal folding of the Gla region. Furthermore, a comparison with the factor X fragments suggests that only the NH$_2$-terminal EGF-like domain is required for this purpose (48).

The large quenching of fluorescence on addition of high concentrations of Ca$^{2+}$ to BfIX-GlaEGF$_{NC}$ indicates a marked change in the environment of Trp-42. This is reflected in the different accessibility of Lys-43 to cleavage by lysyl endopeptidase in metal ion- and EDTA-containing buffers (Fig. 11). This peptide bond is cleaved very rapidly in both BfIX-GlaEGF$_{NC}$ and BfIXa$^{ag}$ in the absence of Ca$^{2+}$ and Mg$^{2+}$, but is resistant in the presence of these ions. These ions thus induce a large conformational change not only in the Gla region but also in the connecting peptide. The latter peptide has a cluster of aromatic amino acids and is coded on a small exon in factors VII, IX, X, protein C, and prothrombin (Fig. 12). It is noteworthy, that the crystal structure of prothrombin fragment 1 has revealed that in prothrombin the corresponding region forms an α-helix (11).

Certain patients with hemophilia B and normal antigen levels have circulating factor IX molecules with very low biological activity due to point mutations in the NH$_2$-terminal EGF-like domain (3, 54, 55). Some of these mutated amino acids have been implicated in the Gla-independent Ca$^{2+}$ binding site of factor IX and protein C, i.e. Asp-47 and -49 and Hya-64 in factor IX and Hya-71 in protein C (56, 57). Recently, it was also demonstrated that a Gla-independent Ca$^{2+}$ binding site in factors IX and X is located in the NH$_2$-terminal EGF-like domain (20–22). These findings, which suggest an important role of the NH$_2$-terminal EGF-like domain in the clotting activity of factor IX, have to be reconciled with the observations made by Lin et al. (35), who have exchanged the EGF-like domains between recombinant factors IX and X, expressed factor IX in mammalian tissue culture, and studied its activity in clotting assays. The evidence obtained so far suggests that the NH$_2$-terminal EGF-like domain can be exchanged between the two clotting factors with only little loss of biological activity in factor IX. On the other hand, when the COOH-terminal EGF-like domain was exchanged, the activity was dramatically reduced (residual activity <5%). It is conceivable that the NH$_2$-terminal EGF-like domain of factor X can function as a scaffold not only for the folding of the factor X Gla region, but also for the folding of the factor IX Gla region in the hybrid protein. Moreover, Ca$^{2+}$ binding to these domains seems to confer a native conformation to the serine protease part and full biological activity. Our results are entirely consistent with the notion that the Gla region in factor IX is folded in a similar manner as the Gla region in prothrombin fragment 1 as recently determined by x-ray diffraction methods by Soriano-Garcia et al. (11).

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SUPPLEMENTAL MATERIAL TO:

STRUCTURAL REQUIREMENTS FOR Ca2+ BINDING TO THE γ-CARBOXY-GLUTAMIC ACID AND EGF-LIKE REGIONS OF FACTOR IX.

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EXPERIMENTAL PROCEDURES

Materials. Russell’s viper venom, α-chymotrypsin, aprotinin, thrombin (DTT) and phenylmethylsulfonyl fluoride (PMSF) were from Sigma. Lysozye phosphatase was purchased from Wako Chemical Co. Disopropylfluorophosphate (DFP) and threo-β-hydroxyasparaginase were from Dako, *γ*-carboxyglutamic acid (Gla) from Bachem Finechemie, whereas exoa-γ-carboxyglutamic acid (γC) and δ-carboxyglutamic acid were synthesized as previously described (38,39). Acrylamide, bisacrylamide and sodium dodecyl sulfate (SDS) were obtained from British Drug House andindocteinol, bovine acedia. Coomassie Brilliant Blue R 250 and acrylamide from Merck AG. O-Sepharose Fast Flow, Sephadex G-75 Superfine. Sepharose 4B-200 HR and molecular weight markers for electrophoresis were from Pharmacia LKB Biotecology. Chelatex™ 180 was obtained from Bio-Rad Laboratories and guanidine·Cl from Bethesda Research Laboratories. Chelatex used for amino acid analysis was obtained from Beckman and chemicals for protein sequencing analysis were from Applied Biosystems.

Proteins. Bovine factor IX was purified as described by Fujikawa et al. (28). The factor K-activating coagulant protein from Russell’s viper venom (KRV-CCP) was isolated as described by Kasai et al. (39). BSA was prepared according to Morris et al. (40). Glutathione (BSA) was made by cleavage of BSA with lysyl endopeptidase (10 mg/mg BSA) for 10 min in 20 mM Tris-HCl (pH 7.5), containing 5 mM EDTA, at 37°C for 10 min followed by chromatography on a Sepharose Fast Flow column as described in the legend to Fig. 5. This fragment consists of a light chain (B1, terminal residue: Glu 44) linked to the same sequence part (Glu·2·3·40) terminal residue: Val 180) by a doublet bond: BSA·KRV·BSA was deacetylated as described by Bajaj et al. (40). For the determination of protein concentrations the following absorptions coefficients (A280 nm) and molecular weights were used: BSA, 1.49 and 66,400 (38,41). BSA: 14.1 and 47,700 (13). Glutamatese BSA, 1.4 and 41,300 (13).
Gels electrophoresis. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was run according to判决 the Dodebrah (46), but with the buffer system of Maue (44). Samples were reduced and alkylated with DTT and iodoacetamide. The gels were stained with Coomassie Brilliant Blue R-250.

Amino acid analysis and sequence determinations. Amino acid analysis was performed as previously described (24). The different B11x variants were first localized by SDS-PAGE, followed by electrophoretic onto polyvinylidene fluoride (PVDF) membranes as described by Mattson et al. (44), and direct sequence determination on an Applied Biosystems 477 A Pulsed Liquid Phase Sequencer according to the manufacturer's instructions. The spotted gels were sequenced with previous electrophilic.

Isolation of B11x-GlaEGFNC. Purified B11x (5 mg/ml) was dialyzed against 50 mM Tris-HCl, pH 7.5, containing 0.1 M NaCl, and then dispersed with c-chemotactic (0.5 % w/v) in the presence of 5 mM EDTA at 37°C. Aliquots were removed from the incubation mixture at different times and subjected to SDS-PAGE (Fig. 2). For preparative purposes, 50-500 mg B11x was digested for 30 minutes under the same conditions. After termination of the reaction by addition of DTT and PMSE to a final concentration of 2 mM each, the samples was chromatographed on a column of Q-Sepharose Fast Flow (1.6x10 cm), equilibrated with 50 mM Tris-HCl, pH 7.5, containing 0.1 M NaCl (Fig. 3). The flow rate was 35 ml-h and 4-mi fractions were collected. Elution was accomplished with a linear gradient of NaCl from 0 to 0.8 M (250 ml) in the same buffer. The fractions containing B11x-GlaEGFNC (as judged by SDS-PAGE and HPLC analyses) were pooled and concentrated using a filtration module (cut-off 5 kDa) and a Cartridge (10 kDa). Two contaminating fragments were then removed by chromatography on a Sephadex G-75 column (1 x 600 cm) equilibrated with 0.1 M NaHCO3 (Fig. 4). The flow rate was 5 ml/h and 1.3-ml fractions were collected. The fractions containing B11x-GlaEGFNC were identified by SDS-PAGE and characterized with respect to NH2-terminal amino acid sequence and amino acid composition.

Isolation of B11x-GlaEGFNC and B11x-Gla. B11x-GlaEGFNC was dialyzed against 50 mM Tris-HCl, pH 7.5, containing 0.1 M NaCl and then digested with trypsin endopeptidase (0.2 % w/v) at 37°C, in the presence of 5 mM EDTA. The rate of digestion was monitored by SDS-PAGE (Fig. 1B). For preparative purposes, 4-8 mg B11x-GlaEGFNC was digested for 2 minutes. The reaction was terminated by addition of DTT and PMSE to a final concentration of 2 mM each, and the samples was chromatographed on a column of Q-Sepharose Fast Flow (1.6x10 cm) equilibrated with 50 mM Tris-HCl, pH 7.5 (Fig. 5). The flow rate was 25 ml/h and 2.1-ml fractions were collected. The column was eluted with a linear NaCl gradient from 0 to 0.8 M (2 x 100 ml) in the same buffer. The fractions containing B11x-GlaEGFNC were pooled separately and chromatographed using a filtration module (cut-off 4 kDa and 1 kDa, respectively) and chromatographed on a column of Sephadex G-75 superfine (1 x 600 cm) equilibrated with 0.1 NH4HCO3 (not shown). The flow rate was 5 ml/h and 1.3-ml fractions were collected.

Separation of the two chains of B11x-GlaEGFNC. The isolated B11x-GlaEGFNC (0.1 M NaHCO3, about 50 mg) was evaporated to dryness and dissolved in 50 µl of 0.5 M Tris-HCl, pH 8.0, containing 6 M guanidine-HCl, 5 mM EDTA and 25 mM DTT. After incubation at 37°C for 2 hours, 50 µl of 2.5 M Tris-HCl, pH 8.5, containing 6 M guanidine-HCl, 5 mM EDTA and 50 mM iodoacetamide, was added, and the sample incubated in the dark for 4 hour at room temperature. Separation was obtained on a C4 column (2.1 x 20 mm Aquaprep, Brownlee Labs, Santa Clara, CA) equilibrated with 50 mM phosphate buffer, pH 6.8, and kept at 4°C. An anion-exchange gradient, 0.15 % (v/v) in 4 minutes, followed by 15-40 % (v/v) in 45 min in the same buffer, was used for elution at a flow rate of 0.1 ml/min. The absorbance of the column effluent was monitored at 215 nm. The HPLC system consisted of a Beckman System Gold PSM 126 connected to a Waters 590 Photodiode Array Detector. The effluent was collected manually and analyzed of metal sequenced and subjected to amino acid analysis.

Fluorescence Measurements. Trypsinogen fluorescence was measured in an SLM 4800S spectrophotofluorimeter (SLM-Aminco Instruments, Urbana, IL) at 25°C. The protein concentration were 2 µM, except for B11x-Gla which was analyzed at a concentration of 5 µM. To remove any excess carboxylic buffer solutions were passed through a Chelex 100 column (Bio-Rad) and the protein samples dialyzed against 50 mM Tris-HCl, pH 7.5, containing 0.1 M NaCl and 0.1 M NaCl, 100%. Fluorescence emission spectra were recorded between 300 and 400 nm in the absence and presence of Ca2+. The excitation wavelength was 280 nm and the emission and emission bandwidths were 2 and 8 nm, respectively. Aliquots were normalized to a value for a calcium in the presence of Ca2+ of 1.0 at the wavelength of emission maximum.

Titration of Ca2+ and Mg2+-induced changes in fluorescence intensity were monitored at an excitation wavelength of 280 nm and an emission wavelength corresponding to the maximum fluorescence intensity shown by the emission spectra in the absence of Ca2+. i.e., 324 nm for B11x, 332 nm for B11x-GlaEGFNC, 350 nm for B11x-GlaEGFNC, B11x-GlaEGFNC, B11x-Gla and B11x-Gla. The excitation and emission bandwidths were 2 and 16 nm, respectively. CaCl2 was added from 3 different stock solutions (1 M, 30.0 mM and 9.0 mM, as determined by atomic absorption spectrophotometry) and MgCl2H2O 1.0 mM stock solution, in portions ranging from 0.5 to 8.0 µM. The emission intensity was measured 2 minutes after each addition by averaging 30 signal readings of 0.2% second each. The samples were illuminated to exciting light for a minimum of time (~30 for a each addition of titrant) to avoid possible photodegradation. At the end of the titrations, the excess of EDTA was added and the emission intensity measured to make sure that the changes were reversible. After correction for dilution, the data were plotted as F/F0, where F0 is the fluorescence intensity of the sample and F the emission intensity in the absence of metal ions.