Localization of the specific binding site for magnesium(II) ions in factor IX

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Abstract **We demonstrated recently that coagulation factor IX has a specific binding site(s) for Mg^{2+} ions, independent of the Ca^{2+}-binding sites, and that binding of Mg^{2+} ions is very important for expression of the functional conformation of this protein. We report here the localization of this Mg^{2+}-specific binding site. We prepared three Gla-containing fragments of bovine factor IX, namely $GlaEGF_{NC}$ (residues 1–144+286–296), $GlaEGF_N$ (1–83) and the Gla domain peptide (1–46). Fragments $GlaEGF_{NC}$ and $GlaEGF_N$ retained the ability to undergo a conformational change upon binding of Mg^{2+} ions in the presence of excess Ca^{2+} ions. This change could be detected by a conformation-specific antibody. Furthermore, the Gla domain peptide was capable of binding Mg^{2+} ions, as determined by the metal ion-induced quenching of the intrinsic fluorescence. It appears that the Mg^{2+}-specific binding site of factor IX is located in the N-terminal Gla domain.**

Key words: Factor IX; Mg^{2+} ion; Ca^{2+} ion; Gla domain

1. Introduction

Coagulation factor IX is a serine protease zymogen that is indispensable for normal hemostasis [1]. Factor IX, resembling other vitamin K-dependent coagulation proteins, requires Ca^{2+} ions at physiological concentrations (in the mM range) for maintenance of its native functional conformation [2,3]. We demonstrated recently that Mg^{2+} ions, present normally in blood plasma, also play an important role in the stabilization of the native conformation of factor IX and thus in the expression of its biological activities [4]. Various Gla domain-directed probes (several preparations of conformation-specific antibodies and IX/X-bp, a snake venom anticoagulant protein [5,6]) specifically recognize the Ca^{2+}-bound conformation of factor IX. We showed that the binding of such probes was much enhanced by Mg^{2+} ions at levels in plasma in the presence of Ca^{2+} ions, even though Mg^{2+} ions alone did not promote the binding of each probe. It is known that Ca^{2+}-binding sites of factor IX can interact with other polyvalent cations, but our results clearly indicated that this protein has a specific binding site(s) for Mg^{2+} ions, independent of Ca^{2+}-binding sites, and that binding of Mg^{2+} ions induces an additional conformational change even when all the Ca^{2+}-binding sites are already occupied. The biological activity of factor IX was potentiated by the cation, concomitant with its effect on the conformation. We revealed that Ca^{2+}-dependent conversion by factor XIa of factor IX to factor IXa was accelerated by the addition of Mg^{2+} ions [4]. More recently, we also showed that all the factor IX/IXa-dependent reaction steps in coagulation are greatly augmented by Mg^{2+} ions and definitively proved that the cation is a crucial constituent of the blood coagulation cascade [7].

Binding sites for Ca^{2+} ions in vitamin K-dependent coagulation factors, including factor IX, have been extensively studied. The N-terminal Gla domains are the common Ca^{2+}-binding sites in all cases [2,3]. Seven Ca^{2+} ions and the amino acid residues that act as Ca^{2+}-binding ligands have been identified by X-ray crystallography of prothrombin fragment 1 [8]. In addition to the Gla domain, factor IX (as well as factors VII, X and protein C) has two other Ca^{2+}-binding sites. The primary structures and the domain organizations of these proteins resemble one another; each of them has, adjacent to a Gla domain, two tandem repeats of an EGF domain, an activation peptide and a serine protease catalytic domain. The first EGF domain [9] and the catalytic domain [10] are capable of binding Ca^{2+} ions. Although various polyvalent metal ions can also interact with some of these Ca^{2+}-binding sites, specific binding sites for metal ions other than Ca^{2+} ions, such as Mg^{2+} ions, have not yet been identified. In the present study, we tried to locate the Mg^{2+}-specific binding site in factor IX in order to establish a biochemical basis for the interaction between factor IX and Mg^{2+} ions.

2. Experimental

2.1. Preparation of factor IX fragments

Bovine factor IX was prepared by the published method [11]. The fragments designated $GlaEGF_{NC}$ and $GlaEGF_N$ were prepared by the method of Valcarce et al. [12]. In the original report [12], it was stated that $GlaEGF_N$ underwent stoichiometric internal cleavage at Glu^{70}/Cys^{71}. We failed to detect such a cleavage upon sequence analysis; little if any such intramolecular cleavage occurred in our preparation.

The Gla domain (1–46) peptide was prepared from $GlaEGF_{NC}$ as follows. One mg of $GlaEGF_{NC}$ was dissolved in 1 ml of 20 mM Tris·HCl, pH 7.5, and digested with endoproteinase Asp-N (E/S = 1 : 500, w/w; Boehringer Mannheim) at 37°C for 12 h. The digest was loaded onto Mono-Q 5/5 (Pharmacia) preequilibrated with the same buffer and the column was developed with a gradient of NaCl (0–0.8 M) over 40 min at a flow rate of 1 ml/min. The Gla domain (1–46) fragment was eluted in 0.4 M NaCl, as the last protein peak. The yield was 0.2 mg.

2.2. ELISA

The ELISA was performed as reported previously [4]. We used a monoclonal antibody (4A4; subclass IgG1, κ), that recognizes Ca^{2+}-bound conformation of the Gla domain of factor IX. The presence of the epitope of this antibody in the Gla domain was confirmed by immunoblotting analysis with the isolated Gla domain peptide (H. Atoda and T. Morita, unpublished observation). The antibody was selected from the panel of monoclonal antibodies, which had been prepared by immunization with the human protein [4], but later shown also to be reactive to the bovine protein.

2.3. Fluorescence quenching

A 1000-μl aliquot of the Gla domain (1–46) peptide (5 μM) in 20 mM Tris·HCl, 140 mM NaCl, pH 7.5, was placed in a micro-quartz cell. Two and one-half microliters of 4 M calcium chloride and/or 2.5

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μl of 0.4 M magnesium chloride solution was added to the sample, and the fluorescence spectrum was recorded after each addition with excitation at 280 nm in a spectrofluorometer (model FP-777; JASCO, Tokyo, Japan).

3. Results

As we showed previously [4], the Gla domain of factor IX (both human and bovine) undergoes further conformational change upon addition of Mg^{2+} ions in the presence of Ca^{2+} ions. We postulated that the Mg^{2+}-specific binding site should be located within or near the Gla domain. To verify this possibility, we prepared several Gla domain-containing proteolytic fragments of bovine factor IX as shown in Fig. 1. The $GlaEGF_{NC}$ and $GlaEGF_N$ fragments were prepared by the published method [12], and the Gla domain (1–46) peptide was prepared by Asp-N digestion of $GlaEGF_{NC}$. The preparation of the Gla domain (residues 1–42) by chymotryptic digestion has been reported [13], but we preferred to use the longer fragment because the aromatic amino acid stack domain (the short linker between the Gla domain and the first EGF domain; residues 41–46) is important for correct folding of the Gla domain [14,15]. The identity of each fragment was confirmed by analysis of the sequence and/or composition of amino acids.

We first examined the binding of the conformation-specific, Gla domain-directed monoclonal antibody, 4A4, the binding of which is absolutely dependent on Ca^{2+} ions. As is shown in Fig. 2, the extent of binding of the probe to $GlaEGF_{NC}$ and $GlaEGF_N$ was augmented by the addition of Mg^{2+} ions as observed in the case of the intact molecule. Magnesium ions were effective even in the presence of excess Ca^{2+} ions but were ineffective in the absence of Ca^{2+} ions. Thus, the Mg^{2+}-specific binding site was located within $GlaEGF_N$. By contrast, we failed detect any binding of the 4A4 antibody to the Gla domain peptide at any tested concentrations of Ca^{2+} ions or of the antibody (data not shown). This failure was probably attributable both to the low efficacy of immobilization of the small peptide on the assay plate and to the decrease in binding affinity of the antibody upon fragmentation of the antigen. Nevertheless, it appeared that the middle and the C-terminal portions of factor IX, i.e., the second EGF domain (EGF_C), the activation peptide and the catalytic domain, were unnecessary for the recognition of Mg^{2+} ions, while the N-terminal portion, the Gla domain or the first EGF domain (EGF_N), included the specific binding site for Mg^{2+} ions.

To narrow down still further the location of the binding site, we employed a physicochemical approach. The Gla domain of factor IX contains a single tryptophan residue at the 42nd position and, upon binding of metal ions, fluorescence derived from this residue is quenched concomitant with the conformational rearrangement of this domain [15]. Changes in the fluorescence spectrum of the Gla domain peptide in an isotonic, neutral buffer were recorded after addition of Ca^{2+} and/or Mg^{2+} ions with excitation at 280 nm (Fig. 3). A decrease in intrinsic fluorescence was observed upon addition of Ca^{2+} ions. Together with the decrease in fluorescence intensity, a blue-shift was seen, indicating that the observed quenching was not due to mere aggregation of the peptide but rather represented a change in the conformation. The quenching reached a plateau when the concentration of Ca^{2+} ions was sufficiently high (>1 mM), in agreement with the reported result with a synthetic Gla domain peptide of human factor IX [15]. Further addition of Ca^{2+} ions did not alter the fluorescence, an indication that all the Ca^{2+}-binding sites had been occupied and the conformational change had been completed. However, when we added a relatively low concentration of Mg^{2+} ions (1 mM) in the presence of a supermaximal concentration of Ca^{2+} ions (10 mM), a significant decrease in fluorescence was observed (Fig. 3). This result indicated that the Mg^{2+}-specific binding site was located in the N-terminal Gla domain (between residues 1 and 46) and that occupation of this site by the cation induced a conformational change in this domain, which could be detected as a change in the micro-environment around Trp^{42}.

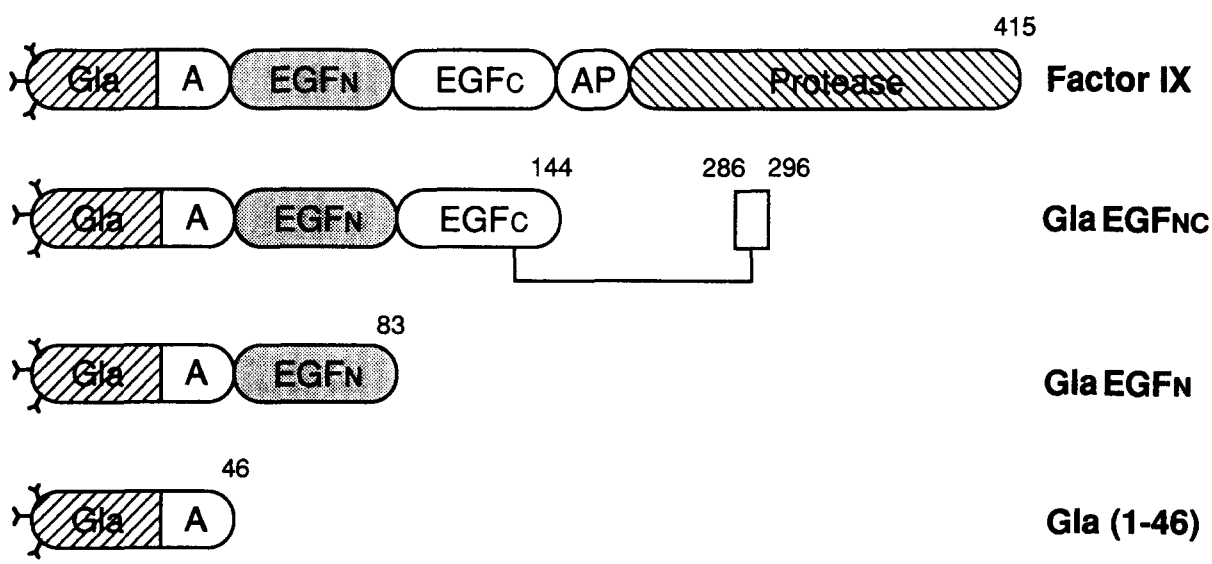

Fig. 1. Structures of factor IX fragments. The fragments used in this study are shown schematically. Gla, the Gla domain; A, the aromatic amino acid stack domain; AP, the activation peptide. The symbol Y denotes Gla residue(s). The domains that contain Ca^{2+}-binding site(s) are shaded.

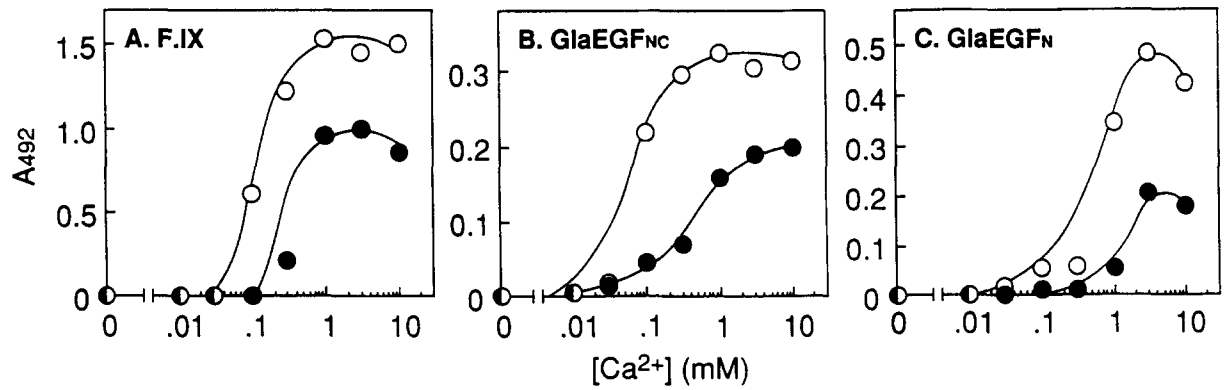

Fig. 2. Enhancement of binding of the conformation-specific antibody by Mg^{2+} ions. The binding of the monoclonal antibody 4A4 that recognized the Ca^{2+}-bound conformation of factor IX to the intact molecule (A), $GlaEGF_{NC}$ (B) and $GlaEGF_N$ (C) was investigated by ELISA in the presence of indicated concentrations of Ca^{2+} ions. ●, Ca^{2+} alone; ○, Ca^{2+} plus 3 mM Mg^{2+}.

4. Discussion

We showed previously that factor IX has a specific binding site(s) for Mg^{2+} ions, which does not interact with Ca^{2+} ions [4]. We showed clearly here that this Mg^{2+}-specific site is located between residues 1 and 46, namely in the Gla domain of factor IX. The fragment $GlaEGF_N$ underwent a conformational change that was detectable as an increase in binding of a conformation-specific probe upon the addition of Mg^{2+} ions even in the presence of excess Ca^{2+} ions, and the binding profile was very similar to that of the intact molecule. Furthermore, the isolated Gla domain peptide still retained the ability to respond to Mg^{2+} ions, as was apparent from the observed changes in intrinsic fluorescence. We were unable to narrow down the site any further because current methodologies cannot be applied to smaller fragments. Deletion of a few C-terminal residues of the Gla domain peptide had a considerable effect on the folding of this module; a synthetic peptide corresponding to residues 1–42 of the human protein no longer responds to metal ions in the fluorescence quenching assay [15]. For unequivocal identification of the ligands of the Mg^{2+} ion, further investigations that can provide more detailed structural information (e.g., X-ray crystallography and NMR spectrometry) are necessary.

The effects of Mg^{2+} ions on factor IX were seen with both the human and the bovine protein. However, we failed to detect any such effect with other vitamin K-dependent coagulation proteins, i.e., prothrombin, factors VII, X and protein C [4]. This result is noteworthy, since the primary structures of these proteins, in particular those of Gla domains, are very similar. Furthermore, in view of their common function, namely the Ca^{2+}-dependent binding to anionic phospholipids, we would expect their tertiary structures also to be similar. With these considerations in mind, we compared the primary structure of the Gla domain of factor IX with those of the other proteins (Fig. 4). Although a very high degree of similarity was found when we compared the entire sequences of each protein, significant differences unique to factor IX were found mainly in the N-terminal portion. (1) The first residue of factor IX is tyrosine, while the others all have alanine at this position. (2) Insertion of glycine as the fourth residue occurs in factor IX. (3) A cluster of three hydrophobic residues followed by a basic residue (in human factor X, FLγγVK, residues 4–9), which constitute the putative binding site for phospholipids [16], is well conserved in this family but, in factor IX, it is changed to KLγγFV in the human and bovine proteins. It is thus possible that these residues unique to factor IX act as ligands for a Mg^{2+} ion, though we cannot exclude possibility of the participation of the C-terminal portion, where some difference is also found. Changes in amino acid residues should induce changes in tertiary structure. It is possible that the size of metal-binding pocket formed by these residues is reduced such that a Ca^{2+} ion can no longer gain

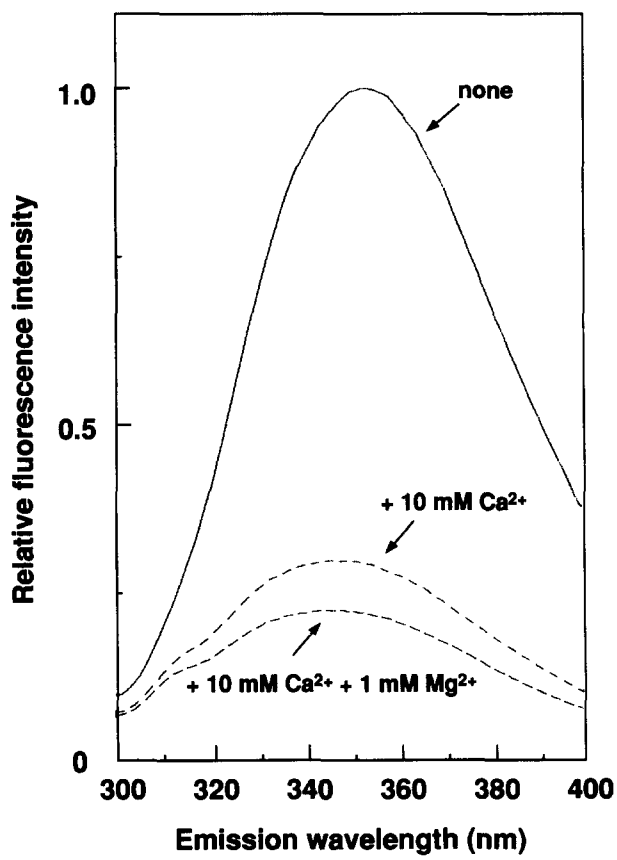

Fig. 3. Metal ion-induced quenching of the intrinsic fluorescence of the Gla domain (1–46) peptide. Fluorescence spectra of the peptide (5 μM) were recorded after excitation at 280 nm with no additions (top trace), with 10 mM Ca^{2+} (middle) and with Ca^{2+} plus 1 mM Mg^{2+} (bottom). The decrease in fluorescence due to changes in volume was negligible.

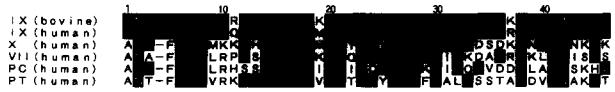

Fig. 4. Primary structures of Gla domains of various vitamin K-dependent coagulation factors. N-Terminal sequences of bovine [20] and human [21] factor IX, human factor X [22], human factor VII [23], human protein C (PC) [24] and human prothrombin (PT) [25] are aligned. Residues identical to those in both human and bovine factor IX are shaded. γ, Gla residue.

access but a Mg^{2+} ion, which has a smaller ionic radius (0.86 Å) than a Ca^{2+} ion (1.14 Å), can bind within the pocket.

Upon binding of Mg^{2+} ions, the biological activities of factor IX are considerably enhanced [4,7]. The rate of activation of factor IX by factor XIa is increased with a striking reduction in the apparent K_m [4]. Although the activation by factor XIa requires Ca^{2+} ions at around 1 mM, the concentration at which the conformational change of the Gla domain occurs, the Gla domain does not participate directly in the interaction of the enzyme and the substrate [15]. Therefore, it appears that the conformational rearrangement of the Gla domain upon binding of Ca^{2+} ions induces a secondary conformational rearrangement of the whole molecule such that the cleavage sites (Arg^{145}/Ala^{146} and Arg^{180}/Val^{181}) in factor IX become more accessible to the activator. Our finding that the Mg^{2+}-specific binding site is located in the Gla domain indicates, therefore, that the Mg^{2+}-induced change in the conformation of the Gla domain also modulates the tertiary structure of the entire molecule. The structure of factor IX that is stabilized by both Ca^{2+} and Mg^{2+} ions should represent the actual conformation in blood plasma. Recently, the solution structures of a synthetic peptide that corresponded to the Gla domain of human factor IX (residues 1–47) both in Ca^{2+}-free and Ca^{2+}-bound forms were resolved by NMR spectrometry [17,18]. Furthermore, a X-ray crystallographic structure of porcine factor IXaβ was also reported (in this case, the N-terminal portion was invisible as a consequence of the absence of metal ligands that stabilize the conformation of this region) [19]. Our investigations indicate, however, that these structural studies must be re-examined in view of the demonstrated effect of Mg^{2+} ions (i.e. in the presence of both Ca^{2+} and Mg^{2+} ions) for the clarification of the exact 'native' conformation of this important protein.

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