Factor IX is an indispensable protein required in the blood coagulation cascade. It binds to the surface of phospholipid membrane by means of a γ-carboxyglutamic acid (Gla) domain situated at the N terminus. Recently, we showed that physiological concentrations of Mg\(^{2+}\) ions affect the native conformation of the Gla domain and in doing so augment the biological activity of factor IXa and binding affinity with its binding protein even in the presence of Ca\(^{2+}\) ions. Here we report on the crystal structures of the Mg\(^{2+}\)/Ca\(^{2+}\)-bound and Ca\(^{2+}\)-bound (Mg\(^{2+}\)-free) factor IX Gla domain (IXGD1–46) in complex with its binding protein (IX-bp) at 1.55 and 1.80 Å resolutions, respectively. Three Mg\(^{2+}\) and five Ca\(^{2+}\) ions were bound in the Mg\(^{2+}\)/Ca\(^{2+}\)-bound IXGD1–46, and the Mg\(^{2+}\) ions were replaced by Ca\(^{2+}\) ions in Mg\(^{2+}\)-free IXGD1–46. Comparison of Mg\(^{2+}\)/Ca\(^{2+}\)-bound with Ca\(^{2+}\)-bound structures of the complexes showed that Mg\(^{2+}\) ions, which formed a bridge between IXGD1–46 and IX-bp, forced IXGD1–46 to rotate 4° relative to IX-bp and hence might be the cause of a more tight interaction between the molecules than in the case of the Mg\(^{2+}\)-free structure. The results clearly suggest that Mg\(^{2+}\) ions are required to maintain native conformation and in vivo function of factor IX Gla domain during blood coagulation.

Factor IX is a pivotal participant in the early stages of the blood coagulation cascade. Its deficiency leads to hemophilia B, which is characterized by severe bleeding (1). Factor IX binds to phospholipid membrane by means of a γ-carboxyglutamic acid (Gla) domain located at its N terminus, which is also found in other vitamin K-dependent proteins such as factors VII and X and prothrombin. A Gla domain consists of 10–13 γ-carboxyglutamic acid residues and requires Ca\(^{2+}\) ions for stabilization of the active conformation for membrane binding (2–4). The enzymatically activated factor IX, factor IXa, forms a complex with factor VIIIa, which in turn activates factor X to form factor Xa. These enzymatic reactions eventually lead to fibrin clot formation. In the classical cascade model of coagulation, factors VIII and IX are classified as components of the “intrinsic pathway” and are placed downstream from factor XI (5, 6). Today, however, a sole mechanism, the so-called “extrinsic pathway” is said to govern blood coagulation, which starts with the exposure of tissue factor (TF), an extravascular component, to the bloodstream and the formation of the active factor VIIa/TF complex. It has been shown previously that activation of factor X by the factor IXa/factor VIIa complex is ~50-fold more efficient than activation by the factor VIIa/TF complex (7), even though factor X had been thought to be activated directly mainly by factor VIIa/TF. Komiyama et al. (8) showed that factor IX is preferred over factor X as a substrate of factor VIIa/TF. Further, Mann and co-workers (7, 9) have demonstrated that the factor VIIa/TF-induced generation of thrombin is strongly dependent on factor VIII. These studies have clearly demonstrated that factors VIII and IX are required for the chain reaction to proceed by the extrinsic pathway.

Recently, we showed that the Ca\(^{2+}\)-dependent activation of factor IX by the factor VIIa/TF complex is accelerated by Mg\(^{2+}\) ions (10), as well as by factor XIa (11), and that Mg\(^{2+}\) ions also accelerate the activation of factor X by the factor IXa/factor VIIIa complex (10). Such acceleration by Mg\(^{2+}\) ions has been observed only in the case of the reactions involving factors IX and IXa. In blood plasma, magnesium is present in relatively high concentrations (0.8–1.2 mM; 0.4–0.6 mM as free ions) as is also calcium (2.2–2.6 mM; 1.1–1.3 mM as free ions) (12). Because of its abundance, we hypothesized that Mg\(^{2+}\) ions might play a significant role in blood coagulation, such as in augmenting the activity of factor IXa. Based on known facts, a model illustrating the principal elements of the principal chain reaction has been proposed (10).

In addition to the acceleration in activity by Mg\(^{2+}\) ions, folding of the native tertiary structure of factor IX Gla domain appears to require not only Ca\(^{2+}\) but Mg\(^{2+}\) ions (11, 13). Snake venom obtained from Trimeresurus flavoviridis contains a protein that can bind to either factor IX or factor X and is called a factor IX/factor X-binding protein (IXX-bp) (14, 15). The affinity of IXX-bp for factor IX Gla domain is significantly increased above that of Ca\(^{2+}\) ions by Mg\(^{2+}\) ions (11). Metal-ion-induced intrinsic fluorescence quenching studies also indicate that the factor IX Gla domain has specific Mg\(^{2+}\) ion-binding site(s) and that the binding of Mg\(^{2+}\) ions promotes further conformational changes in the Ca\(^{2+}\)-bound molecule (13) enhancing the ability of the protein to function. At present, a total of seven Ca\(^{2+}\)-bridging sites have been identified within the Ca\(^{2+}\)-prothrombin fragment-1 (16) and in...
the Gla domain of factor VIIa (17). A recent crystallographic study of the complex between factor X Gla domain-containing peptide 1–44 (XGD1–44) and snake venom factor X-binding protein (X-bp) has revealed an eighth Ca$^{2+}$-binding site in the Gla domain of factor X (18). Even though the structures of the Ca$^{2+}$-ion-bound (19) and Mg$^{2+}$-ion-bound (20) factor IX Gla domain peptides (residues 1–47) have been investigated by NMR, the coordination sites of Ca$^{2+}$ and Mg$^{2+}$ ions are not known, especially when both ions are present.

Here, we report on the states of Mg$^{2+}$ ions in the crystal structure of factor IX Gla domain 1–46, complexed with its binding protein in the presence of Mg$^{2+}$/Ca$^{2+}$ and Ca$^{2+}$/Ca$^{2+}$ ions. Interpretation of the data supports a role for Mg$^{2+}$ ions in the blood coagulation scheme presented before, whereby the biological activity of factor IXa is enhanced.

**EXPERIMENTAL PROCEDURES**

**Protein Purification**—Factor IX was purified from frozen bovine plasma following standard protocols (21). Gla domain peptide (residues 1–46, IXGD1–46) was prepared as described (13). IX-bp was purified from the venom of T. flavoviridis as previously reported (22). Purified IXGD1–46 and IX-bp were mixed, and the proteins in the mixture were complexed by the addition of MgCl$_2$ and CaCl$_2$ solutions to a final concentration of 1 mM MgCl$_2$ and 3 mM CaCl$_2$, respectively. The mixture of IXGD1–46 and IX-bp was loaded onto a Pharmacon Superdex 75 column (1.6 × 60 cm), pre-equilibrated with 30 mM Tris-HCl, pH 8.0, containing 1 mM MgCl$_2$ and 3 mM CaCl$_2$, and eluted. The IXGD1–46/IX-bp complex was characterized by a peak that moved faster than the IX-bp peak. To obtain a Mg$^{2+}$-free complex, Mg$^{2+}$ salts were omitted in all procedures. The pooled fractions containing the complex were concentrated to ~5 mg/ml before use.

**Crystallization and Data Collection**—Both the Mg$^{2+}$-containing and Mg$^{2+}$-free complexes were crystallized by the microbatch method from a 2-µl solution consisting of 1 µl of 5 mg/ml protein and 1 µl of precipitant (30 mM Tris-HCl, pH 8.0, 14% PEG 6000, 5 mM CaCl$_2$), in the presence or absence of 2 mM MgCl$_2$ at 20 °C.

Crystals having approximate dimensions of 0.3 × 0.1 × 0.1 mm were harvested into a cryoprotectant that contained 30% glycerol in the precipitant and flash frozen in a stream of N$_2$ gas at 100 K. Diffraction data were collected on a MAR CCD detector using synchrotron radiation (λ = 0.72 Å) at Beamline 41XU of SPring-8. Data reduction was performed using a HKL2000 package.

**Phasing and Refinement**—The structures of the IXGD1–46/IX-bp complex were solved by the molecular replacement method using the XGD1–44/X-bp complex structure (18) with the programs AMoRe (23) and ARP/WARP (24). Manual model fitting was done with QUANTA (Molecular Simulations, Waltham, MA), and the structures were refined using program CNS (25) after removing 10% of the measurements for monitoring of the R$_{free}$ factor. In the early stages of model fitting, Mg$^{2+}$ ions were assigned to all-cation-binding sites. In the Mg$^{2+}$-bound complex, after several cycles of refinement and map inspection, Mg$^{2+}$ ions and Ca$^{2+}$ ions were easily distinguishable because of 1) B-factors that calculated Mg$^{2+}$ ions as Ca$^{2+}$ ions, 2) differences in the shapes and numbers of coordinated oxygen atoms, 3) electron density height and radius on the map, and 4) distances from coordinated oxygen atoms. Considering these different factors, Mg$^{2+}$ ions, instead of Ca$^{2+}$ ions, were assigned to sites Ca-1, Ca-7, and Ca-8. The difference in the distances between Mg$^{2+}$–O and Ca$^{2+}$–O, calculated from average distances, was 0.295 Å. This is in good agreement with the published value of 0.28 Å (26). The results of the refinement are summarized in Table I. All of the figures were produced with MOLSCRIPT (27) and Raster3D (28).

**RESULTS**

**Structure of IXGD1–46**—Fig. 1 shows a stereo view of the overall structure of IXGD1–46 in complex with IX-bp. Fig. 2A gives the amino acid sequence alignment of the Gla domains of bovine and human factors IX and X and shows the location of the residues coordinated to Mg$^{2+}$ and Ca$^{2+}$ ions. Three Mg$^{2+}$ ions, labeled Mg-1, Mg-7, and Mg-8, were located on the surface of IXGD1–46 (Fig. 2B), corresponding to the positions of Ca-1, Ca-7, and Ca-8 in XGD1–44 (Fig. 2C) in the crystal structure of XGD1–44/X-bp complex (18). Each Mg$^{2+}$ ion had a typical bipyramidal coordination geometry with a pair of Gla residues and water molecules. Fig. 3 shows a typical region containing both Mg-1 and Ca-2 for comparison. Mg-1 was also coordinated to Glu$^{38}$ of the IX-bp A chain and contributed to one of the major interactions between the two molecules, as found in the case of Ca-1 in the XGD1–44/X-bp complex. In IXGD1–46, the Mg–O distances on average were 2.12 Å, whereas the Ca–O distances on average were 2.41 Å.

To clarify the coordination of Mg$^{2+}$ ions, attempts were made to solve the structure of the IXGD1–46/IX-bp complex by comparing the structures under Mg$^{2+}$-free and Mg$^{2+}$-bound conditions. Under Mg$^{2+}$-free conditions, the three Mg$^{2+}$ ions, Mg-1, Mg-7, and Mg-8, were replaced by Ca$^{2+}$ ions. At site Mg-1, the distance from the coordinated oxygen atoms to Ca$^{2+}$ ion was elongated in the Mg$^{2+}$-free condition (from 2.11 to 2.34 Å), as was the distance from the other two Mg$^{2+}$-binding sites. Although this difference in distance is small, the difference was enough to cause a rotation of IXGD1–46 when IX-bp was bound (see below).
Structure of Factor IX-binding Protein and Induced Fitting—Comparison of the tertiary structures of IX-bp between the complexed and free forms (29) showed that the general shapes were similar before and after binding with IXGD1–46. When the B chains of the complexed and free forms were superimposed, the A chain was displaced toward the concave surface by a 3° rotation around the axis (Fig. 4A), which exists approximately perpendicular to the pseudo 2-fold axis and which passes through the position of the interchain disulfide bond between Cys79 of the A chain and Cys75 of the B chain. The spatial movement of the A chain of IX-bp following binding with IXGD1–46 reached a maximum distance of 4 Å in the direction of the concave surface. Consequently, the A chain globular body shifted as interaction occurred directly with the Lys5 and Arg11 near hydrophobic residues are in yellow at the putative insertion site (20, 40). Basic residues (Lys5 and Arg11) near hydrophobic residues are shown in purple, C, same view of XGD 1–44 with yellow lines representing membrane surface in both models.

**DISCUSSION**

In the crystal structure of factor IX Gla domain in complex with IX-bp, 1-, 7-, and 8-cation-binding sites were occupied by Mg2+ ions in the physiological range of Mg2+ and Ca2+ ion concentration. To our knowledge, this is the first report identifying the binding sites of Mg2+ ions in the Gla domain. It is known that Mg2+ ions greatly enhance the affinity between Gla domain and IX-bp (11). In this study, crystallographic evidence shows that Mg2+ bridging may play an important role in this association. Mg2+ bridging brings the two molecules closer together by 0.58 Å than does Ca2+ bridging alone. It may be assumed that negatively charged groups of IXGD1–46 insert deeply into the concave surface of IX-bp, thereby causing a tighter interaction. In addition to this association, the induced fitting may involve the interaction of subunits of IX-bp caused by rotation around the axis passing in the proximity of the interchain disulfide bond, resulting in water-mediated interactions, as well as direct interaction between a loop of the A chain and Lys30 of IXGD1–46 as mentioned above. On the other hand, in the Mg2+-free (Ca2+ only) complex, Lys30 of IXGD1–46 is too far removed to form hydrogen bonds with the side chains of IX-bp, and hence the affinity between Gla
domain and IX-bp might be lower in agreement with previous findings (10, 11).

It is also known that Mg\(^{2+}\) ions increase the affinity between factor IXa and antibodies (11, 13) and the biological activity of factor IXa in the presence of factor VIIIa (10). The interaction between factors IXa and VIIIa is well documented by experiments indicating that epidermal growth factor and the serine protease domains of factor IXa were involved in the interaction with factor VIIIa (30–35). We consider that the Gla domain also participates in the interaction with factor VIIIa via Mg\(^{2+}\) binding sites as follows. The data in Fig. 5 show a spatial relationship among the Gla, epidermal growth factor, and serine protease domains, forming an arched structure. The C-terminal helix of the Gla domain (named aromatic residues stack domain) contributes to the formation of the lower part of the concave surface on the right side of the factor IXa molecule, which is known as a candidate site for the binding to factor VIIIa. Because the Mg-8-binding site points toward the concave surface, Mg-8 might play an important role in binding to factor VIIIa. Similarly, Mg-8 is expected to contribute to the binding to factors VIIa and X.

In Fig. 2 (B and C), IXGD1–46 and XGD1–44 are shown oriented relative to a phospholipid membrane surface placed schematically at the bottom of the figure. Binding of the Gla domain of factor IX to membrane could then take place by Mg-1 mediated an interaction between the Gla residues of factor IX and the negative head groups of the phospholipids via Mg\(^{2+}\) bridging. In addition, another Mg\(^{2+}\) or Ca\(^{2+}\) ion (not shown) could participate in forming an ion bridge with Gla\(^{33}\) of factor IX, as in the case of factor X (18). Such a protein-membrane interaction involving Ca\(^{2+}\) ion bridging has been shown in the crystal structure of the complex between annexin V and phospholipid head groups (36, 37). The phospholipids at the membrane surface interact through Ca\(^{2+}\) ions, which are able to gain access only from the opposite side of the protein. As in the case with annexin V, the Ca\(^{2+}\) or Mg\(^{2+}\) ions, which are present on the surface of the factor IX Gla domain, may form bridges with the phospholipid head groups. The hydrophobic residues Phe\(^{4}\), Lue\(^{5}\), and Val\(^{6}\) of factor X Gla domain, on the other hand, are considered to be buried in the membrane surface (18, 38). The residue Phe\(^{4}\) in factor X corresponds to Lys\(^{5}\) in factor IX, which may lie on the membrane surface, forming direct interactions with negative head groups of the phospholipid. This type of interaction probably contributes only slightly toward the association of the Gla domain on the phospholipid membrane, because the hydrophilic side chains of Lys\(^{5}\) and Arg\(^{11}\) (Fig. 2B) exist in the proximity of the residues inserted in the putative membrane. These interactions and Mg\(^{2+}\) bridging (which brings the Gla domain and membrane closer together than Ca\(^{2+}\) bridging) cause a tilt in the long axis of the Gla domain relative to the membrane surface, such that a small clockwise rotation is seen if Fig. 2B is compared with Fig. 2C. Even if the tilt angle is small, the location of the active site of the catalytic domain relative to that of the membrane-binding site is significantly affected because the active site, which corresponds to the position of D-Phe-Pro-Arg-chloromethylketone in Fig. 5 is distant from the membrane. Further studies are required to determine whether such positional differences caused by Mg\(^{2+}\) ions are biologically relevant.

Freedman et al. (20) have reported that synthetic factor IX Gla domain (residues 1–47) failed to bind to phospholipids when only Mg\(^{2+}\) ions were present because of disorder in the structure, especially within residues 1–11. Four Ca\(^{2+}\) ions from...
Ca-2 to Ca-5 are essential to stabilize the ω-loop region for membrane-binding (20, 39) because these Ca2+ ions bind to conserved Gla2 and Gla4 residues in this region (Fig. 2B). The replacement by Mg2+ ions may lead to destabilization of the structure of the Gla domain, particularly in this region because of significant differences that exist with Ca2+ ion in both coordination distance and coordination sphere (Mg2+ ions take generally a typical bipyramidal configuration and six coordination). This may explain why the Ca2+ ions are not replaced by Mg2+ ions in the present structure.

In conclusion, three Mg2+ ion-binding sites have been identified in the crystal structure of the factor IX Gla domain 1–46 in complex with its binding protein. Overall, the present results confirm the importance of Mg2+ ions as augmentors of Ca2+ ions in factor IX activity and lend support for a pathway presented before involving Mg2+ ions in the initial stage of the coagulation cascade. Because the activation of factor X is affected by Mg2+ ions when factor VIIIa is present (10), it will be necessary to repeat the experiment with all components present, including phospholipids, to understand how Mg2+ ions accelerate the coagulation cascade.

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