High-Affinity Calcium-Binding Site in the γ-Carboxyglutamic Acid Domain of Bovine Factor VII†

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ABSTRACT: The calcium-mediated interaction of factor VIIa with tissue factor is considered to be the primary trigger of blood coagulation. To determine the role of calcium ions in the action of factor VII, we prepared monoclonal antibodies whose binding to factor VII was calcium-dependent. A monoclonal antibody designated C6 strongly inhibited factor VII-induced clotting at a molar ratio of factor VII to antibody of 1:1. The half-maximal binding of factor VII to the C6 antibody was observed at a concentration of calcium ions of 80 μM. Proteolytic fragments of factor VII were assayed for their ability to inhibit competitively the binding of ^{125}I-factor VII to immobilized C6 antibody. The binding was inhibited by increasing amounts of factor VII, by a fragment that contained the γ-carboxyglutamic acid (Gla) domain linked to first epidermal growth factor-like domain, and by a Gla domain peptide (residues 1−41), over a range of concentration of 10^{-9} to 10^{-7} M. The antigenic site recognized by the monoclonal antibody C6, which was generated upon the high-affinity binding of calcium ions, was located in the Gla domain. The C6 antibody inhibited the activation of factor X and the amidolytic activity of factor VIIa in the presence of tissue factor. These results demonstrate that a high-affinity calcium-binding site(s) is located in the Gla domain of factor VII, which is concerned with the initiation of tissue factor-mediated blood coagulation by factor VIIa.

Factor VII (FVII)[1] is a vitamin K-dependent plasma zymogen which participates in the initiation of the extrinsic pathway of blood coagulation (Davie et al., 1991). The mature protein is composed of a series of domains, including the γ-carboxyglutamic acid-containing (Gla-containing) domain, two epidermal growth factor-like (EGF-like) domains, and a serine protease domain (Hagen et al., 1986; Takeya et al., 1988). The catalytic and proteolytic activity of serine protease FVIIa is regulated by the interaction of FVIIa with its macromolecular cofactor, tissue factor (TF), which is mediated by calcium ions (Broze, 1982). There is evidence that the first EGF-like domain (EGF1) (Toomey et al., 1991; Clarke et al., 1992) and the serine protease domain (Wildgoose et al., 1990, 1993; Kumar et al., 1991) of factor VII participate in direct binding to TF. A contribution to these interactions is also apparently provided by the Gla domain (Sakai et al., 1990; Ruf et al., 1991) or by the region composed of the Gla domain linked to the first EGF-like domain (Gla-EGF1) (Kazama et al., 1993; Higashi et al., 1994) in the presence of calcium ions.

Factor VII contains several Ca^{2+}-binding sites (Strickland et al., 1980; Schiødt et al., 1992). A high-affinity ($K_d \approx$ 200 μM) Ca^{2+}-binding site in the protease domain of factor VII affects the interaction between tissue factor and factor VII (Sabharwal et al., 1995). The binding of Ca^{2+} ions to the Gla domain of factor VII, which contains several low-affinity Ca^{2+}-binding sites, contributes to the recognition of macromolecular substrates on anionic cell-surface phospholipids (Neuenschwander & Morrissey, 1994). In addition to the Gla domain, factor VII as well as factors IX and X and protein C has two other Ca^{2+}-binding sites (Schiødt et al., 1992). The first EGF domain and the catalytic domain have Ca^{2+}-binding sites. High-affinity binding of Ca^{2+} ions to EGF1 induces a conformational change (Stenflo, 1991), and this is also influenced by the conformation of the neighboring Gla domain in vitamin K-dependent proteins (Valcarce et al., 1993). The aromatic amino acid stack domain, the short linker between the Gla domain and the first EGF domain, is important for correct folding of the Gla domain (Jacobs et al., 1994). On the other hand, mutations in the aromatic amino acid stack domain of protein C failed to exert any notable effects on Gla domain-related Ca^{2+}-dependent properties (Christiansen et al., 1995). Thus, binding of Ca^{2+} to the Gla and neighboring domains has specific effects, but the nature of the interaction between Ca^{2+} ions and these domains remains unclear.

In the present study, we prepared factor VII and various proteolytic fragments, including the Gla domain (residues 1−41), EGF1 (residues 42−85), and Gla domain linked to EGF1 (residues 1−85). Immunolabeling using a monoclonal

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[1] Abbreviations: EGF, epidermal growth factor; FVII and FVIIa, factor VII and factor VIIa; Gla, γ-carboxyglutamic acid; MAb, monoclonal antibody; PC/PS, vesicles composed of 70% phosphatidylcholine and 30% phosphatidylserine; TF, tissue factor.

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antibody (MAb) to FVII revealed the Ca^{2+}-dependency and the inhibitory effect of FVII-induced coagulation in plasma. We have localized the epitope for the antibody and its Ca^{2+}-binding properties on the factor VII-related fragments. These studies demonstrated for the first time that the high-affinity Ca^{2+}-binding site of bovine factor VII lies in the Gla domain (residues 1−41) with no requirement for the presence of the aromatic amino acid stack domain and its possible role in the interaction with TF.

MATERIALS AND METHODS

Materials. Materials were purchased from following companies. α-Chymotrypsin treated with *N*-tosyl-L-lysyl chloromethyl ketone and FVII-deficient plasma were from Sigma (St. Louis, MO). Lysyl endopeptidase from *Achromobacter lyticus* was purchased from Wako Pure Chemical Industries (Osaka, Japan). *H*-D-Ile-Pro-Arg-*p*-nitroanilide (S-2288) was from Kabi Vitrum (Stockholm, Sweden). Formyl-Cellulofine and *tert*-butoxycarbonyl-Leu-Gly-Arg-*p*-nitroanilide (Boc-Leu-Gly-Arg-*p*NA) were from Seikagaku Kogyo (Tokyo, Japan). IODO-BEADS was from Pierce (Rockford, IL). Na[^{125}I] was from DuPont/New England Nuclear (Boston, MA). Bio-Beads SM-2, Chelex 100 resin, and the mouse immunoglobulin typing kit (MOUSE TYPER) were from Bio-Rad (Richmond, CA). Phosphatidylcholine (PC) and phosphatidylserine (PS) were from Avanti Polar Lipids (Pelham, AL). Human placental thromboplastin (Thromborel-S) was from Behring Werke (Marburg, Germany). Peroxidase-conjugated goat antibodies against mouse immunoglobulin were from ICN Biomedicals (Costa Mesa, CA). All other reagents were of the highest quality available commercially.

Proteins. Bovine factor X was purified as described previously (Hashimoto et al., 1985). Bovine factor VII was isolated as described by Saisaka et al. (1992) by use of a column of the monoclonal antibody (clone C6), whose interaction with factor VII was Ca^{2+}-dependent, coupled to formyl-Cellulofine. Factor VIIa was prepared by the activation of factor VII with human β-XIIa as described elsewhere (Inoue & Morita, 1993). Tissue factor apoprotein was isolated from an acetone powder of bovine lung by a modified version of the method of Broze et al. (1985a), and it was solubilized in 50 mM Tris-HCl, pH 7.5, that contained 0.1 M NaCl and 0.1% Triton X-100. The homogeneity of all preparations of proteins was evaluated by SDS−PAGE. Tissue factor apoprotein in detergent was relipidated in vesicles prepared from phosphatidylcholine and phosphatidylserine (70:30, by mass) by a 30-min incubation that was followed by the removal of detergent with Bio-Beads SM-2 (Van den Besselaar et al., 1982). The concentration of TF was determined with the BCA Protein Assay Kit (Pierce) with bovine serum albumin (BSA) as the standard. The concentrations of the other proteins were determined from the following molecular weights and extinction coefficients ($E_{280}^{1\%}$): factor X, 55 000, 12.4 (Jackson & Hanahan, 1968); factors VII and VIIa, 53 000, 12.9 (Bach et al., 1986); β-XIIa, 30 000, 15.2 (Fujikawa & McMullen, 1983); mouse IgG, 150 000, 15.5; Gla-domainless factor VII, 47 500, 14.2; factor VII residues 1−85 (referred to hereinafter as Gla-EGF1), 11 000, 7.2; residues 1−41 (Gla domain), 5 500, 10.7; and residues 42−85 (EGF1), 5 500, 4.4. The latter values were estimated from the published amino acid sequence of bovine factor VII (Takeya et al., 1988).

Preparation of Protease-Digested Fragments of Factor VII. Factor VII (5.5 mg) was dialyzed against 0.1 M Tris-HCl, pH 7.5, that contained 5 mM EDTA, 4 M urea and 0.02% NaN_3, and then it was digested with lysyl endopeptidase (1.1 mg) at 37 °C for 8 h. After termination of the reaction by addition of diisopropyl fluorophosphate to a final concentration of 1 mM, the sample was dialyzed against Tris-buffered saline (TBS; 50 mM Tris-HCl, 0.1 M NaCl, pH 7.5) and chromatographed on a column (1.6 cm i.d. × 17 cm) of formyl-Cellulofine to which the monoclonal antibody (clone C6) had been coupled. The column was equilibrated with TBS that contained 3 mM $CaCl_2$, and, after the column was washed of unbound material with 50 mM Tris-HCl, pH 7.5, that contained 3 mM $CaCl_2$ and 1.0 M NaCl, bound proteins were eluted in the same buffer prepared with 5 mM EDTA instead of $CaCl_2$. The flow rate was 16 mL/h, and 2-mL fractions were collected. The fractions containing Gla-EGF1 were pooled and dialyzed against TBS. Gla-EGF1 (0.93 mg) was dialyzed against 50 mM Tris-HCl, pH 8.0, and then digested with α-chymotrypsin (3.1 μg) at room temperature for 20 min. The sample was chromatographed on a column of DEAE-Sepharose Fast Flow (1.0 cm i.d. × 17 cm) that had been equilibrated with the same buffer, at a flow rate of 24 mL/h. Elution was accomplished with a linear gradient of NaCl from 0 to 0.8 M (2 × 250 mL) in the same buffer. The fractions containing the Gla domain and those containing EGF1 were pooled separately and dialyzed against TBS. The Gla domain, EGF1, and Gla-EGF1 were characterized with regard to their respective NH_2-terminal amino acid sequences and amino acid compositions. The purity of Gla-EGF1 was evaluated by SDS−PAGE, but the material showed scarcely any staining with Coomassie Brilliant Blue, probably because the fragment containing the Gla domain was highly acidic. Immunoblotting with MAb C6 identified a single band at 11 kDa. Gla-domainless factor VII was prepared by limited digestion of FVII with α-chymotrypsin. Purified FVII, 5.2 mg in 50 mM Tris-HCl, pH 8.0, plus 1 mM benzamidine was digested with α-chymotrypsin (17 μg) at room temperature for 20 min. The digest was fractionated on a column (1.6 cm i.d. × 15.5 cm) of DEAE-Sepharose Fast Flow that had been equilibrated with the same buffer at a flow rate of 30 mL/h. The column was eluted with a linear gradient of NaCl (0−0.5 M, 2 × 200 mL) in the same buffer. The fractions containing Gla-domainless FVII were pooled and dialyzed against TBS.

Amino Acid Analysis and Sequence Determination. Samples were hydrolyzed by treatment with 5.7 N HCl, in tubes that has been sealed under a vacuum, at 110 °C for 24 h. After evaporation, the hydrolyzates were analyzed on an amino acid analyzer (model L8500; Hitachi, Tokyo, Japan) by the method of Spackman et al. (1958). Sequences were analyzed on a gas-phase instrument with a protein sequencer (model 473A; Applied Biosystems, Foster City, CA) equipped with a data analysis module (model 610A; Applied Biosystems).

Decarboxylation of Gla. The Gla residues in factor VII were decarboxylated by the method of Bajaj et al. (1982). Factor VII, after lyophilization of a solution in 0.1 M NH_4HCO_3, pH 8.0, was heated at 110 °C for the indicated times. The Gla content of heat-decarboxylated and intact factor VII was determined by the method of Kuwada and Katayama (1983).

Preparation of Monoclonal and Polyclonal Antibodies. BALB/c mice were injected intraperitoneally with 200 μg of factor VII in the presence of 2 mM $CaCl_2$ and Freund's complete adjuvant. The immunization with factor VII was repeated after 2 weeks, 4 weeks (with the antigen emulsified in Freund's incomplete adjuvant), and 6 weeks (with the antigen in 10 mM phosphate buffer, 0.15 M NaCl, pH 7.2). 7 days later, spleen cells were fused with the myeloma cell line P3U1 by the method of Köhler and Milstein (1975) in 40% polyethylene glycol 1450. Screening of the hybridomas for anti-factor VII antibodies was performed by enzyme-linked immunosorbent assay (ELISA) in the presence of 5 mM $CaCl_2$. Cell supernatants were diluted with TBS containing 0.1% Tween 20 and 5 mM $CaCl_2$, and added to the factor VII-adsorbed plates. The bound antibody was determined using goat anti-mouse IgG coupled with horseradish peroxidase and *o*-phenylenediamine as described below. Hybridomas producing anti-factor VII antibodies were subcloned by the limiting dilution method. Preparation of ascites fluid and purification of the IgG fraction were carried out as described previously (Strickland et al., 1988). IgG subtyping was performed by ELISA using MOUSE TYPER in accordance with the manufacturer's instructions.

Polyclonal antibodies were raised in rabbits against bovine factor VII, and purified from rabbit serum by affinity chromatography on protein A-Cellulofine (Seikagaku Kogyo, Tokyo, Japan). Anti-factor VII polyclonal antibodies were coupled with horseradish peroxidase (Ishikawa et al., 1983) and used for sandwich assays for factor VII.

Enzyme-Linked Immunosorbent Assay (ELISA). In this assay, wells of microtiter plates were coated with 50 μL of antigen (FVII or related fragments) in TBS at 4 °C overnight. They were then blocked with 50 μL of a 1% solution of gelatin in TBS that contained 0.1% Tween 20 for 30 min at room temperature. After being washed, monoclonal antibodies of various concentrations in TBS that contained 0.1% Tween 20 and 3 mM $CaCl_2$ were added to the coated wells, and the wells were incubated for 1 h at room temperature. The solutions in wells were then removed by aspiration and washed, and the amount of monoclonal antibody that remained was determined by reaction with peroxidase-conjugated antibodies raised in goat against mouse IgG for 1 h. Reaction with the antigen was visualized by incubation with *o*-phenylenediamine and H_2O_2 in 50 μL of 0.1 M citrate buffer, pH 5.5. The half-maximal binding of monoclonal antibodies to the solid-phase protein was calculated by the method by Suzuki and Nishioka (1988). In a variation of the assay, the dependence on Ca^{2+} ions of the binding of monoclonal antibodies to FVII, the Gla domain and Gla-EGF1 was determined as follows. FVII and the digested fragments were dialyzed against a solution of 50 mM Tris-HCl, pH 8.0, 0.1 M NaCl, 1 g of Chelex 100/L. Microtiter plates were incubated with 50 μL of FVII, the Gla domain, or Gla-EGF1 at a respective concentration of 10 nM, 10, or 1 μg/mL in TBS for 2 days at 4 °C. After being blocked with 1% gelatin, monoclonal antibodies were incubated in the wells with varying concentrations of Ca^{2+} ions in TBS that contained 0.1% Tween 20 for 1 h, and then each well was washed with buffer that contained the same respective concentration of Ca^{2+} ions, and the bound antibody was quantified as described above.

In the sandwich assay, microtiter plates were incubated with 100 nM MAb C6 dissolved in 50 mM $NaHCO_3$ for 2 h at 37 °C followed by blocking with 1% gelatin. The wells were washed three times with TBS containing 0.1% Tween 20. Factor VII (10 nM), dissolved in this buffer containing various concentrations of Ca^{2+} ions, was incubated for 2 h at 37 °C. The wells were washed three times with buffer of the same respective concentrations of Ca^{2+} ions. The amount of bound factor VII was subsequently determined by addition of peroxidase-conjugated anti-factor VII polyclonal antibodies followed by reaction with *o*-phenylenediamine described above.

Competitive Inhibition with ^{125}I-FVII. Factor VII was labeled with Na[^{125}I] using IODO-BEADS in accordance with the manufacturer's instructions. Microtiter plates were coated overnight at 4 °C with MAb C6 (100 μg/mL in TBS, 50 μL per well). The wells were then incubated with a 1% solution of BSA in TBS for 1 h. This incubation was followed by two washes with TBS that contained 3 mM $CaCl_2$. Each well was incubated with 40 000 cpm of ^{125}I-FVII (approximately 0.15 μg/mL) plus the sample to be tested in 50 μL of TBS that contained 3 mM $CaCl_2$ and 1 mg of BSA/mL for 3 h at room temperature. The wells were washed twice with TBS that contained 3 mM $CaCl_2$ and 0.1% Tween 20, and the amount of radiolabeled FVII bound to the immobilized antibody was determined in a γ-counter.

Clotting Assay with FVII. The clotting assay with FVII was performed as described previously (Nemerson & Clyne, 1974) using FVII-deficient plasma as substrate, and human placental thromboplastin (Thromborel-S). In brief, FVII (20 nM) in TBS that contained 3 mM $CaCl_2$, 1 mg of BSA/mL, and FVII-deficient plasma were incubated with antibodies at various concentrations for 30 min at 37 °C. The clotting reaction was initiated by the addition of 30 μL of a solution of Thromborel-S in H_2O to 20 μL of the mixture, and the clotting time was recorded. Activities were determined by extrapolation from a standard curve constructed from the results of assays with pooled normal plasma at various dilutions.

Assay of the Activation of Factor X. The inhibition by antibodies of the generation of factor X was monitored as follows. Solutions with the indicated concentrations of FVIIa, TF, and phospholipid were prepared in 50 mM Tris-HCl, pH 8.0, that contained 0.15 M NaCl, 5 mM $CaCl_2$, and 1 mg of BSA/mL, and reaction mixtures were incubated for 10 min at 37 °C in the presence of various concentrations of each antibody. Prewarmed FX was then added to initiate the reaction. The FXa generated during incubations was assayed as described previously (Inoue & Morita, 1993) with the synthetic substrate, Boc-Leu-Gly-Arg-*p*NA. The concentration of FXa was determined by extrapolation from a standard curve prepared from results of assays with purified FXa.

Assay of the Amidolytic Activity of Factor VIIa. The enzymatic activity of FVIIa and cofactor complexes was measured by a one-step chromogenic assay. Reaction mixtures containing the indicated concentrations of FVIIa and TF were mixed with varying concentrations of antibody in 50 mM Tris-HCl, pH 8.0, that contained 0.15 M NaCl, 5 mM $CaCl_2$, and 1 mg of BSA/mL in a microtiter plate, and the plate was then incubated for 10 min at 37 °C. Hydrolysis of the chromogenic substrate was initiated by the addition of S-2288 (final concentration, 0.1−4 mM) and mixing. Initial rates of hydrolysis of the substrate were determined by monitoring absorbance at 405 nm with a kinetic plate

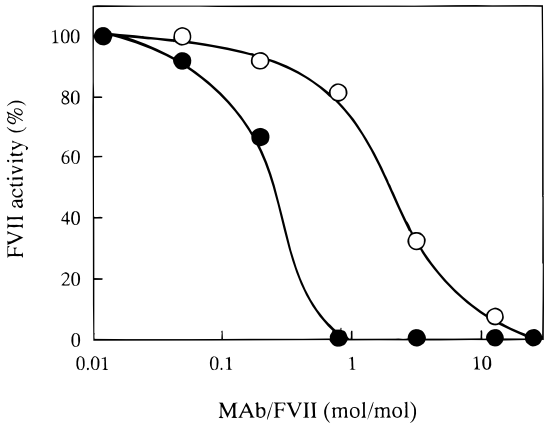

FIGURE 1: Effects of FVII-specific MAbs on factor VII-induced clotting. A clotting system consisting of FVII-deficient plasma, 20 nM FVII, 50 mM Tris-HCl, pH 7.5, that contained 0.1 M NaCl, 3 mM $CaCl_2$, and 1 mg of BSA/mL, and human placental thromboplastin was used to test the effects of FVII-specific MAbs on clotting time. Increasing concentrations of MAb C6 (●) or C7 (○) were added to the reaction mixture, and the prolongation of clotting time was monitored. The results of the control reaction without any antibody were taken to represent 100% activation of FVII, and results for samples that contained antibodies are expressed as percentages of the control value.

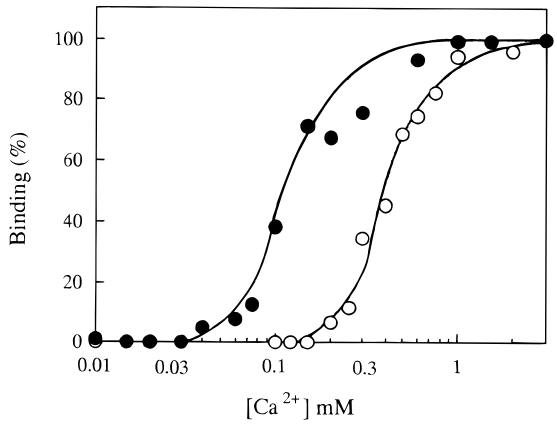

FIGURE 2: Dependence on the concentration of Ca^{2+} ions of the binding of MAbs to FVII. Wells of a microtiter plate were coated with FVII. MAb C6 (●) or C7 (○) was added, followed by solutions prepared with different concentrations of Ca^{2+} ions. The amount of bound antibody was determined by ELISA. The maximum amount of each antibody bound to FVII that had adsorbed to the plate was determined at a saturating level of Ca^{2+} ions. Values for antibody bound at given concentrations of Ca^{2+} ions are expressed as percentages of the maximal binding.

reader (Well Reader; Seikagaku Kogyo). Initial rates were determined essentially as described elsewhere (Krishnaswamy, 1992).

RESULTS

Characterization of Monoclonal Antibodies. Two monoclonal antibodies were selected and cloned from one fusion to examine the role of binding of Ca^{2+} ions in the activity of factor VII. The two antibodies (designated C6 and C7) were of the IgG_1 isotype, and they had K_d values of 2.9×10^{-10} and 7.1×10^{-10} M for factor VII, respectively. These antibodies bound factor VIIa in the same manner as they bound factor VII. The specificity of these antibodies was examined by a solid-phase ELISA, in which the microtiter plate was coated with prothrombin, factor IX, factor X, protein C, or protein Z. None of these proteins bound the C6 antibody, while bovine factor X showed slight cross-reactivity with MAb C7. As shown in Figure 1 each antibody inhibited the clotting activity of factor VII. In particular, C6 completely inhibited this activity at a molar ratio of factor VII to antibody C6 of 1:1.

C6 bound to factor VII in a calcium-dependent manner, with half-maximal binding at 80 μM, below 0.1 mM Ca^{2+} ions (Figure 2). C7 also was found to be specific for a Ca^{2+}-dependent epitope, and exposure of this epitope required more than 0.3 mM Ca^{2+} ions (Figure 2). These results indicate that exposure of the epitopes for C6 and C7 involved a high- and a low-affinity Ca^{2+}-binding site, respectively. In the sandwich assay, factor VII bound to MAb C6 on microtiter plates with a similar K_d value (5.2×10^{-10} M) and in a calcium-dependent manner. The soluble factor VII bound to coated MAb with the same calcium-dependence as was observed when the MAb was offered to coated FVII in the presence of various calcium concentrations. This suggests that native factor VII undergoes a conformational transition in the presence of low concentrations of Ca^{2+} ions and that this altered conformation was detected by MAb C6.

Localization of the Epitopes for Antibodies. We next characterized the C6 antibody in an effort to localize the high-affinity Ca^{2+}-binding site and to examine the effect of binding of Ca^{2+} ions on the function of factor VII. C6 and C7 antibodies recognized factor VII and VIIa with equal affinity and an epitope on the light chain of factor VIIa in Western blotting experiments (data not shown). To localize the epitope of C6 and C7 on factor VII, we isolated proteolytic fragments of factor VII. The proteolytic fragment that contained the epitope for C6 was isolated, by use of a column of C6 coupled to formyl-Cellulofine, from fragments generated from bovine factor VII by digestion with lysyl endopeptidase. Amino acid analysis and sequence analysis indicated that the fragment consisted of residues 1−85, corresponding to the Gla domain linked to first EGF-like domain (Gla-EGF1; Table 1). The fragment was cleaved by α-chymotrypsin and separated by chromatography on DEAE-Sepharose Fast Flow. The resultant fragments were identified as the Gla domain (residues 1−41) and the first EGF-like domain (EGF1; residues 42−85) (Table 1), demonstrating that the parent fragment was comprised of these two domains. These isolated fragments, in addition to Gla-domainless factor VII and factor VII, were then assayed for their ability to inhibit competitively the binding of ^{125}I-labeled factor VII to immobilized C6 antibodies (Figure 3). Gla-EGF1 and native factor VII strongly inhibited the binding of factor VII to C6, with half-maximal inhibition occurring at 10 nM peptide. The Gla domain peptide inhibited the binding to a moderate extent. Other Gla-domainless fragments, such as EGF1 and Gla-domainless factor VII, were not inhibitory. Thus, the epitope for C6 appeared to be located in the Gla domain, but the relative affinity of C6 for the Gla domain was approximately 10-fold lower than its affinity for Gla-EGF1 or native factor VII, suggesting that C6 required the presence of EGF1 together with the Gla domain for maximal binding.

Effect of Ca^{2+} Ions on Binding of Antibodies to FVII and Fragments of FVII. The dependence on Ca^{2+} ions of the binding of antibodies (C6 and C7) to factor VII and fragments of factor VII was examined by a solid-phase

Table 1: Amino Acid Compositions of Gla-EGF1, the First EGF-Like Domain (EGF1), and the Gla Domain Peptide[a]

amino acid	Gla-EGF1 (1−85)		first EGF-like domain (42−85)		Gla domain (1−41)	
Asp	9.4	(10)	8.3	(8)	2.5	(2)
Thr	1.9	(2)	1.2	(1)	1.2	(1)
Ser	6.1	(7)	4.8	(5)	2.5	(2)
Glu	16.4	(18)	6.3	(6)	9.9	(12)
Pro	3.1	(3)	1.7	(2)	1.4	(1)
Gly	7.0	(7)	5.7	(5)	2.7	(2)
Ala	3.0	(3)	1.4	(1)	2.3	(2)
Cys	ND[b]	(8)	ND	(6)	ND	(2)
Val	1.0	(1)	1.1	(1)	0.6	(0)
Met	0	(0)	0	(0)	0	(0)
Ile	1.7	(2)	1.1	(1)	1.0	(1)
Leu	5.8	(6)	1.3	(1)	4.5	(5)
Tyr	1.8	(2)	0.9	(2)	0.1	(0)
Phe	5.6	(6)	2.3	(2)	2.8	(4)
Lys	1.0	(1)	1.0	(1)	0.2	(0)
His	0.9	(1)	0	(0)	1.5	(1)
Trp	ND	(1)	ND	(0)	ND	(1)
Arg	6.6	(7)	1.9	(2)	3.3	(5)

[a] The residue numbers in intact bovine factor VII are given below the designation of each fragment. The composition deduced from the published sequence (Takeya et al., 1988) is shown in parentheses. [b] ND, not determined.

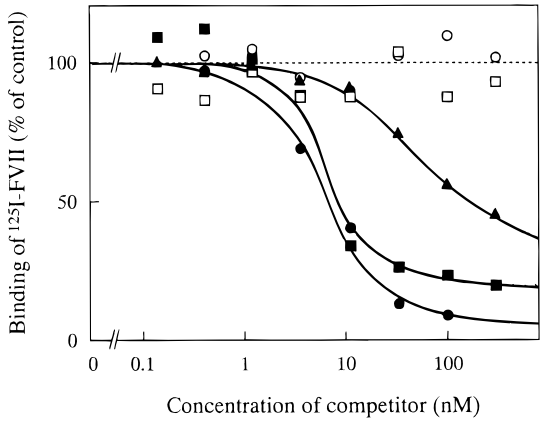

FIGURE 3: Binding of FVII and proteolytic fragments of FVII to MAb C6. The binding of MAb C6 to intact factor VII (●), to Gla-domainless factor VII (○), to Gla-EGF1 (■), to the Gla domain (▲), and to EGF1 (□) was examined in a competitive binding assay with ^{125}I-FVII as tracer. Results are plotted as percentages of radioactivity bound in the absence of competitor.

ELISA. The Gla domain peptide required 145 μM Ca^{2+} for half-maximal binding of C6, resembling in this respect both factor VII and Gla-EGF1 (Table 2). These results indicate that the Gla domain peptide retained the epitope for C6 that included the high-affinity Ca^{2+}-binding site. The generation of the epitope for C7 required a high concentration of Ca^{2+} ions, above 0.3 mM (Table 2). The C7 antibody recognized a Ca^{2+}-induced conformational change in the Gla domain associated with a low-affinity Ca^{2+}-binding site.

For further characterization of the Ca^{2+}-binding properties that exposed the epitopes for C6 and C7 in the Gla domain, the Gla residues in factor VII were decarboxylated. The number of Gla residues remaining in factor VII decreased with time and fell to zero at 5 h (Figure 4). The extent of binding of C6 in the presence of Ca^{2+} ions fell concomitantly with the decarboxylation of Gla residues (Figure 4), suggesting that the expression of the Ca^{2+}-induced epitope specific for C6 involved Gla residues. The epitope for C7

Table 2: Dependence on Ca^{2+} Ions of Binding of MAbs to FVII, to Gla-EGF1, and to the Gla Domain Peptide

peptide	EC_{50} of Ca^{2+} (μM)[a] MAb C6	MAb C7
factor VII	80	325
Gla-EGF1	105	390
Gla domain	145	490

[a] EC_{50} values of Ca^{2+} ions were determined by ELISA.

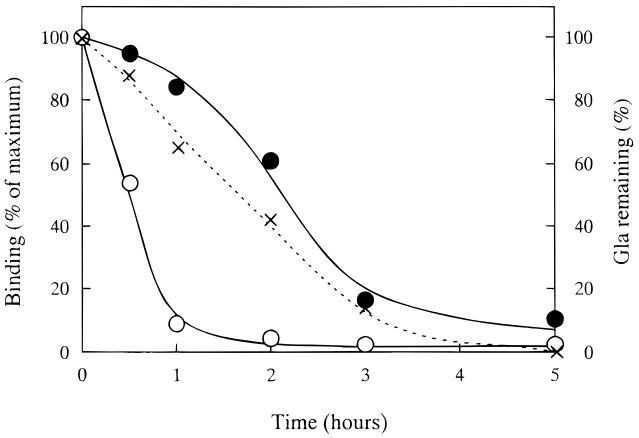

FIGURE 4: Correlation between the Gla residues that were protected during the thermal decarboxylation of FVII and the binding of decarboxylated FVII to MAbs. The percentage of remaining Gla residues (×) is plotted as a function of the duration of heating. The protein was lyophilized in a solution of 0.1 M NH_4CO_3, pH 8.0, and then heated at 110 °C *in vacuo*. The Gla content was determined at the indicated times. The broken line shows the curve for kinetics of thermal decarboxylation. Decarboxylated FVII (15 nM), prepared as described above, was used to coat the wells of microtiter plates. After wells were blocked, MAb C6 (●) or C7 (○) was added to a concentration of 1 nM in the presence of 3 mM $CaCl_2$. The bound antibody was quantified by ELISA, and the results are plotted as percentages of the binding of each antibody to intact factor VII. Solid lines indicate the binding of MAbs to decarboxylated FVII.

was found to be more sensitive to decarboxylation than that for C6. After 2 h, when 40% of Gla residues remained, the extent of binding of the epitope to C6 remained at 60% of the control value, whereas the binding ability of C7 was very considerably diminished. These results, shown in Figure 4, demonstrate that there are two independent epitopes that involve a conformational change in the Gla domain at low and high concentrations of Ca^{2+} ions.

Effects of the FVII-Specific Antibodies on the Proteolytic and the Amidolytic Activity of Factor VIIa. To examine the inhibitory effect of antibodies on the clotting induced by FVII, the effects of antibodies on the rate of activation of factor X by factor VIIa with various cofactors were studied (Table 3). In the presence of relipidated and detergent-solubilized tissue factor, C6 antibodies inhibited the activation of factor X. When phospholipid was added to FVIIa, C6 depressed the generation of factor Xa to a level nearly equivalent to that obtained with factor VIIa alone, and this effect was also observed with the C7 antibody (data not shown).

C6 inhibited the amidolytic activity of factor VIIa in the presence of detergent-solubilized tissue factor. The k_{cat}/K_m value for the amidolytic activity of FVIIa-TF complex was 690 M^{-1} s^{-1} in the presence of MAb C6 at a molar ratio of MAb to FVIIa of 5:1. The value was approximately one-

Table 3: Effects of FVII-Specific MAb C6 on the Kinetic Parameters of the Activation of Factor X by FVIIa in the Presence of Various Cofactors[a]

enzyme	added MAb[b]	k_{cat}/K_m (M^{-1} s^{-1})
FVIIa	−	4
	+	5
FVIIa + PC/PS	−	3 000
	+	7
FVIIa + TF	−	10 500
	+	2 200
FVIIa + TF + PC/PS	−	570 000
	+	150 000

[a] Catalytic efficiencies were determined under the following conditions: 200 nM FVIIa; 200 nM FVIIa + 50 μM PC/PS; 20 nM FVIIa + 20 nM TF in 0.1% Triton X-100; and 2.5 nM FVIIa + 2.5 nM TF + 50 μM PC/PS, all in 50 mM Tris-HCl, 0.15 M NaCl, 5 mM $CaCl_2$, 1 mg of BSA/mL, pH 8.0, at 37 °C, with factor X at concentrations from 0.02 to 50 μM in the presence or absence of the C6 antibody. [b] MAb was added at a molar ratio of MAb to FVIIa of 5:1. In the case of FVIIa + TF + PC/PS, the ratio of MAb to FVIIa was 10:1.

third of that (1910 M^{-1} s^{-1}) in the absence of MAb. The antibody had no effect on the amidolytic activity in the presence of factor VIIa alone or factor VIIa plus phospholipid (data not shown).

DISCUSSION

The Gla domains of vitamin K-dependent coagulation factors contain several low-affinity Ca^{2+}-binding sites which are involved in the interaction of the protease with anionic cell-surface phospholipids (Schwalbe et al., 1989; Pollock et al., 1988). When the Gla domain was removed with a protease, the resultant Gla-domainless proteins each retained at least one high-affinity Ca^{2+}-binding site (Sugo et al., 1984; Morita et al., 1984; Johnson et al., 1983; Dahlbäck et al., 1990), located in the EGF1 and/or the serine protease domain (Handford et al., 1990; Bajaj et al., 1992; Sabharwal et al., 1995). Jacobs et al. (1994) noted that the Gla domain peptide (residues 1−47) of factor IX that contains the "aromatic amino acid stack region" (residues 39−46) undergoes a conformational transition in the presence of a low concentration of Ca^{2+} ions (half-maximal change at 50 μM), while the Gla domain peptide (residues 1−42) that lacks the aromatic amino acid stack region does not. The aromatic amino acid stack region was reported to be correlated with the higher affinity for Ca^{2+} ions of the sites in EGF1 of factor X (Valcarce et al., 1993, 1994) and factor IX (Astermark et al., 1994). The binding of Ca^{2+} ions to factor VII induces conformational changes that can be monitored by changes in fluorescence (Schiødt et al., 1992; Strickland et al., 1980) and the accessibility of previously hidden antigenic determinants (Wildgoose et al., 1992). We examined the Ca^{2+}-binding activity of the Gla domain using a Gla domain-directed monoclonal antibody, C6, indicating the half-maximal binding to factor VII at 80 μM Ca^{2+} ions. The results indicated that the epitope of C6 was located in the Gla domain. The isolated Gla domain peptide (residues 1−41), devoid of the aromatic amino acid stack region, retained an antigenic site recognized at a Ca^{2+} concentration of 145 μM (EC_{50}), close to the value for native factor VII. Our findings provide the first direct evidence for a Ca^{2+}-induced conformational change in the Gla domain of factor VII at a low Ca^{2+} concentration near 100 μM and indicate that the high-affinity Ca^{2+}-binding site is located in the Gla domain of factor VII.

In general, the loss of any one of the various Gla residues causes the loss of the coagulant activity of vitamin K-dependent proteins (Straight et al., 1985; Malhotra et al., 1985) and diminishes the extent of binding of many monoclonal antibodies which recognize the Gla domain (Smith et al., 1987; Broze et al., 1985b). These observations suggest that all of the Gla residues in the Gla domain are essential for maintenance of the conformation of the entire Gla domain. However, we have demonstrated that the epitope recognized by C6 is a novel antigenic site that is dependent on a few Gla residues that remain after decarboxylation. Zapata et al. (1988), studying the effects of chemical modification of Gla residues, observed the binding of three Ca^{2+} ions to high-affinity sites with positive cooperatively that was related to Gla residues 7, 8, and 33 of bovine prothrombin fragment-1. Moreover, the chemically synthesized Gla domain peptide (residues 1−38) of human protein C contains one to two tight binding sites for Ca^{2+} ions (K_d = 0.14−0.37 mM), as determined by Ca^{2+}-selective electrode titrations by Colpitts and Castellino (1994). The epitope of antibody C6 might map to these Gla residues or the first Ca^{2+}-binding site. The half-maximal inhibition by the isolated Gla domain of the binding of factor VII to C6 was observed at a somewhat higher concentration than those of Gla-EGF1 and native factor VII. Thus, the presence of the first EGF-like domain (EGF1) obviously affects the affinity for factor VII of the antigen-binding site of C6. EGF1 stabilizes the structure of Gla-EGF1 (Stenflo, 1991).

MAb C6 completely inhibited the clotting activity of factor VII of a molar ratio of factor VII to antibody of 1:1. It would be that the antigenic site of factor VII for the antibody was functionally important. The Gla domain of factor VII affects the activation of factor X by direct interaction with factor X in its phospholipid-bound conformation (Martin et al., 1993). MAbs C6 and C7, which recognized the Gla domain, were able to inhibit activation of factor X by factor VIIa in the presence of phospholipid but not the activation by factor VIIa alone, suggesting that the Gla domain of FVIIa is of great importance in the activation of factor X on a phospholipid surface. Interestingly, the C6 antibody showed the inhibition of tissue factor-dependent activation of factor X not only in the presence but also in the absence of phospholipid. Furthermore, the increasing amidolytic activity of factor VIIa when tissue factor was present was inhibited by the addition of the C6 antibody. It seemed that the antigenic site recognized by the C6 antibody plays an essential role in promoting the binding of FVIIa to TF via direct protein−protein interaction. The amidolytic activity of the FVIIa-TF complex was also inhibited by the Gla-EGF1 peptide but not by the Gla domain peptide or EGF1 peptide (Kazama et al., 1993; Higashi et al., 1994). The conformational transition of the Gla domain detected by MAb C6 may stabilize the association between the Gla domain and EGF1 for optimum interaction with TF.

In conclusion, the Gla domain of bovine factor VII has a high-affinity Ca^{2+}-binding site(s). A Ca^{2+}-induced conformational change(s) is critical for the initiation of blood coagulation by FVIIa in the presence of TF. Further studies of the interaction between Ca^{2+} ions and the Gla domain of factor VII should provide insight into the structures that bind to phospholipid and the apoprotein of tissue factor.

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