The Factor VIIIa C2 Domain (Residues 2228–2240) Interacts with the Factor IXa Gla Domain in the Factor Xase Complex*

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Factor VIIIa functions as a cofactor for factor IXa in the phospholipid surface-dependent activation of factor X. Both the C2 domain of factor VIIIa and the Gla domain of factor IXa are involved in phospholipid binding and are required for the activation of factor X. In this study, we have examined the close relationship between these domains in the factor Xase complex. Enzyme-linked immunosorbent assay-based and surface plasmon resonance-based assays in the absence of phospholipid showed that Glu-Gly-Arg active site-modified factor IXa bound to immobilized recombinant C2 domain (rC2) dose-dependently ($K_d = 108 \text{ nM}$). This binding ability was optimal under physiological conditions. A monoclonal antibody against the Gla domain of factor IXa inhibited binding by ~95%, and Gla domainless factor IXa failed to bind to rC2. The addition of monoclonal antibody or rC2 with factor VIIIa inhibited factor IXa-catalyzed factor X activation in the absence of phospholipid. Inhibition was not evident, however, in similar experiments in the absence of factor VIIIa, indicating that the C2 domain interacted with the Gla domain of factor IXa. A fragment designated C2-(2182–2259), derived from V8 proteasome-cleaved rC2, bound to Glu-Gly-Arg active site-modified factor IXa. Competitive assays, using overlapping synthetic peptides encompassing residues 2182–2259, demonstrated that peptide 2228–2240 significantly inhibited both this binding and factor Xa generation, independently of phospholipid. Our results indicated that residues 2228–2240 in the factor VIIIa C2 domain constitutes an interactive site for the Gla domain of factor IXa. The findings provide the first evidence for an essential role for this interaction in factor Xase assembly.

Factor VIII, a plasma protein that participates in the blood coagulation cascade, is deficient or defective in individuals with hemophilia A. Factor VIII circulates in plasma as a noncovalent complex with VWF, which stabilizes the synthesis and activity of the cofactor. Mature factor VIII is synthesized as a single chain polypeptide of ~300 kDa consisting of 2,332 amino acid residues (1, 2). Based on internal homologies of the amino acid sequence, factor VIII has three types of domains arranged in the order of A1-A2-B-A3-C1-C2 (3). Factor VIII circulates in the plasma as a heterodimer of a heavy chain, consisting of the A1, A2, and heterogeneous fragments of partially proteolyzed B domains, together with a light chain consisting of the A3, C1, and C2 domains (1, 3).

The carboxyl-terminal 159 amino acids of factor VIII comprise the C2 domain, which is involved in binding to both VWF (4–6) and phospholipid membrane surfaces (6, 7). Binding in this domain appears to be competitive and mutually exclusive (4, 5, 8, 9). The C2 domain has also been shown to participate in binding to factor Xa and thrombin (10, 11). Additionally, a major epitope for allo- and autoantibodies and for monoclonal antibodies has been located within the C2 domain (4, 6, 12), indicating that this region could be an antigenic “hot spot.” Consequently, important aspects of the expression and regulation of factor VIII appear to be governed by the structure and function of the C2 domain.

Factor VIIIa functions as a cofactor for factor IXa in the anionic, phospholipid surface-dependent conversion of factor X to Xa. In intrinsic factor Xase, factor VIIIa binds to factor IXa and increases the $K_{\text{cat}}$ for factor Xa formation by several orders of magnitude compared with factor IXa alone (13). The A2 domain of factor VIIIa interacts with the catalytic domain of factor IXa, and the A3 domain interacts with the first epidermal growth factor domain (14). Although the affinity of isolated A2 for factor IXa is low ($K_d \sim 300 \text{ nM}$), it amplifies the enzyme activity of factor IXa by modulating an active site in the catalytic domain, and this interaction defines the cofactor activity of factor VIIIa (15). Factor IXa-interactive sites in the A2 domain are located in at least three regions, within residues 484–509 (16), 558–565 (17), and 708–717 (18), respectively.

In contrast, the high affinity ($K_d \sim 15 \text{ nM}$) of the isolated factor VIIIa light chain for factor IXa provides the majority of the binding energy for this interaction. To date, one region within the A3 domain, residues 1804–1818, has been identified.
as a factor IXa-interactive site (19, 20). Recently, Blostein et al. (21) demonstrated that the light chain of factor VIIIa interacts with the Gla domain of factor IXa, which contains 12 post-translationally modified glutamic acid residues (γ-carboxyglutamic acid) and functions in calcium-dependent phospholipid binding (22). However, the site in the light chain of factor VIIIa responsible for interaction with the Gla domain of factor IXa remains to be identified. The C2 domain of factor VIIIa and the Gla domain of factor IXa are involved in phospholipid binding, and both bound sequences could be aligned structurally close. We speculated, therefore, that the C2 domain of factor VIIIa might interact with the Gla domain of factor IXa in the factor Xase complex.

In the present study, we have examined the interaction between the C2 domain of factor VIIIa and the Gla domain of factor IXa in the factor Xase complex, using a combination of functional and binding assays employing recombinant C2 domain, V8 protease-digested C2 fragments, synthetic peptides, and monoclonal antibodies. Our results indicated that residues 2228–2240 in the C2 domain contain an interactive site for the Gla domain of factor IXa. The findings provide the first evidence for an essential role of this interaction in factor Xase assembly.

**MATERIALS AND METHODS**

**Reagents**—Purified recombinant factor VIII was a generous gift from Bayer Corp. (Osaka, Japan). Two monoclonal antibodies, mAb IXa-GD, against the Gla domain of factor IXa and specific for calcium-dependent conformation, and mAb 3A6 against the heavy chain of factor IXa, were prepared (23, 24). A monoclonal antibody, ESH8, against the C2 domain of factor VIII and recognizing residues 2248–2285 (4, 5) was purchased from American Diagnostica Inc. (Stamford, CT). A horseradish peroxidase (HRP)-labeled monoclonal antibody was prepared using Peroxidase Labeling Kit-NH2 (Dojindo Molecular Technologies, Osaka, Japan) in 100 mM Tris-Tricine, pH 8.4, 150 mM NaCl, and 0.01% Tween 20 (HBS buffer). Unbound EGR-ck was removed by extensive dialysis at 4 °C in the same buffer. Chromogenic assays demonstrated less than 0.2% residual activity of factor IXa or GDless factor IXa, respectively.

**Preparation of EGR-GDless Factor IXa**—Factor IXa (10 μM) or GDless factor IXa (2.3 μM) was inactivated overnight at 4 °C by the addition of a 20-fold molar excess of EGR-ck in 20 mM HEPES, pH 7.2, 150 mM NaCl, and 0.01% Tween 20 (HBS buffer). Unbound EGR-ck was removed by extensive dialysis at 4 °C in the same buffer. Chromogenic assays demonstrated less than 0.2% residual activity of factor IXa or GDless factor IXa, respectively.

**Preparation of rC2 Proteolytic Fragments**—The rC2 (16.7 μM) was digested for 96 h at 37 °C with Staphylococcus aureus V8 protease (5.4 μM; Wako Pure Chemical Industries Ltd., Osaka, Japan) in 100 mM Tris-Tricine, pH 8.4, 150 mM NaCl, and 0.1% SDS. The digest was treated with the SDS-Out™ precipitation kit (Pierce) to remove the SDS and was fractionated by reverse phase HPLC using TSKgel ODS-100Z (5 μM; Tosoh Corp.). The reaction mixture was loaded onto a column equilibrated with 90% distilled H₂O, 10% acetonitrile in 0.1% trifluoroacetic acid and eluted with a linear gradient of 10–50% acetonitrile over 60 min. Fragments were detected at 216 nm and automatically collected in 500-μl aliquots and lyophilized. The fragments exhibited excellent solubility following their resuspension in HBS buffer. Protein concentrations were determined by the method of Bradford (28). Electrophoresis of the purified fragments followed by staining with GelCode Blue Stain Reagent (Pierce) showed >95% purity.

**ELISA-based Binding Assay**—Microtiter wells were coated with 200 nM rC2 (100 μl) in 100 mM sodium bicarbonate, pH 9.6, overnight at 4 °C. The wells were washed with HBS buffer and were blocked with the same buffer containing 5% BSA for 2 h at 37 °C. EGR-factor IXa or EGR-GDless factor IXa was then added and incubated in HBS buffer containing 1 mM CaCl₂ and 5% BSA for 2 h at 37 °C. Bound EGR-factor IXa was quantified by the addition of HRP-labeled anti-factor IXa mAb 3A6 and α-phenylenediamine dihydrochloride substrate. Reactions were quenched by the addition of 2 mM H₂SO₄, and absorbances were measured at 492 nm using a Labsystems Multiskan Multiscan microplate reader (Labsystems, Helsinki, Finland). Control experiments demonstrated that mAb 3A6 did not affect the reaction between factor VIII and factor IXa (data not shown).

The amount of nonspecific binding of HRP-labeled IgG in the absence of factor VIII and factor IXa (data not shown). The percentage of inhibition was calculated using the equation, (bound absorbance – non-
specific absorbance)/(maximum − nonspecific) × 100 (%). Absorbance in the absence of competitive protein or immobilized rC2 was regarded as maximum or nonspecific, respectively.

**SPR-based Binding Assay**—The kinetics of rC2 and EGR-factor IXa interaction were determined in SPR-based assays using a Biacore X instrument (BIAcore AB, Uppsala, Sweden). EGR-factor IXa was covalently coupled to the surface of a CM5 chip at a coupling density of 5 ng/mm². Binding (association) of the ligand was monitored in 10 mM HEPES, pH 7.4, 150 mM NaCl, 1 mM CaCl₂, and 0.005% surfactant P20, at a flow rate of 20 μl/min for 2 min at 37 °C. The dissociation of bound ligand was monitored for a 2-min period by replacing the ligand-containing buffer with buffer alone. The level of nonspecific binding corresponding to ligand binding to the uncoated chip was subtracted from the signal. The rate constants for association (kₐ) and dissociation (kₐ) were determined by nonlinear regression analysis (29, 30) using the evaluation software provided by Biacore AB. Equilibrium dissociation constants (K_d) were calculated as kₐ/kₐ.

**Factor IXa Generation Assays**—The rate of conversion of factor X to factor IXa was monitored in a purified system (18, 31). Factor Xa was generated at 22 °C in HBS buffer containing 1 mM CaCl₂ and 0.1% BSA. For assays in the absence of phospholipid, 200 nM factor VIII was activated by 10 nM thrombin. Thrombin activity was inhibited after 1 min by the addition of 2.5 units/ml hirudin, and factor Xa generation was initiated by the addition of 5 nM factor IXa and 1 μM factor X. Experiments in the absence of factor VIIIa were performed under the same conditions except for 20 nM factor IXa. For assays in the presence of phospholipid, 30 nM factor VIII was activated by 10 nM thrombin in the presence of 20 μM phospholipid. Thrombin activity was inhibited after 1 min by hirudin, and factor Xa generation was initiated by the addition of 0.5 μM factor IXa and the indicated amounts of factor X. Aliquots were removed at appropriate times to assess initial rates of product formation and added to tubes containing EDTA (100 mM final concentration) to stop the reaction. Rates of factor Xa generation were determined at 405 nm using a microtiter plate reader after the addition of chromogenic substrate, S-2222 (0.46 mM final concentration). Factor Xa generation was quantified by extrapolation from a standard curve prepared using known amounts of factor Xa.

**ELISA for Factor VIII or EGR-factor IXa Binding to Phosphatidylserine**—ELISA were performed using a minor modification of a previously reported method (7). Briefly, 50 μM 1,2-dioleoyl-sn-glycero-3-phospho-L-serine (Sigma) in methanol was immobilized onto each well of a microtiter plate and allowed to dry at 4 °C overnight. After washing with HBS buffer, the wells were blocked for 2 h at 37 °C with HBS buffer containing 5% BSA. Factor VIII or EGR-factor IXa was added to each well in HBS buffer containing 1 mM CaCl₂, and 5% BSA and incubated for 2 h at 37 °C. Bound factor VIII was detected using anti-factor VIII mAb ESH8, followed by HRP-labeled antimouse second antibody. Bound EGR-factor IXa was detected using HRP-labeled mAb 3A6.

**NH₂-terminal Sequence Analysis**—The C2 fragments were blotted onto polyvinylidene difluoride membranes, stained with Gelcode Blue, and excised. NH₂-terminal sequence analyses of the purified fragments were performed using an Applied Biosystems model 491 sequencer (Foster City, CA). Samples were subjected to 5 or 7 cycles of automated sequencing.

**Solvent-accessible Surface Area Analysis**—The solvent-accessibilities at the interface for the residues 2182–2259 of the C2 domain were calculated from the atomic coordinates using Marc Gerstein’s calc-surface program (32) available from the Helix Systems Web site. The atomic coordinates of human factor VIII and C2 domain were retrieved from the Protein Data Bank (code 2R7E and 1D7P, respectively). Values that are more positive represent a greater probability of surface exposure.

**RESULTS**

**Binding of EGR-factor IXa to the C2 Domain**—Blostein et al. (21) have recently reported that the Gla domain of factor IXa interacts with the light chain of factor VIIIa. The C2 domain of factor VIIIa and the Gla domain of factor IXa are involved in phospholipid-binding, and we surmised, therefore, that they could be juxtaposed in the factor Xase complex and that the C2 domain might associate directly with the Gla domain of factor IXa. To investigate this hypothesis, we initially examined the direct binding of factor IXa to immobilized rC2 using microtiter-based, solid phase binding assays. An active site-modified EGR-factor IXa preparation was used in these experiments to eliminate difficulties of interpretation in the presence of enzymatically active factor IXa. Various concentrations of EGR-factor IXa were incubated with immobilized rC2 (200 nM). Bound EGR-factor IXa was detected using anti-factor IXa mAb 3A6, recognizing the heavy chain of its protease. EGR-factor IXa bound to immobilized rC2 in a dose-dependent manner (Fig. 1A). Control experiments using an anti-C2 mAb demonstrated that immobilized rC2 was not affected by the ionic strength of the wash buffer or the duration of the wash and incubation steps subsequent to C2 binding (data not shown). To confirm the specificity of this binding, various concentrations of factor VIII or rC2 were preincubated with EGR-factor IXa (100 nM) in
the fluid phase prior to addition to the immobilized rC2. Factor VIII and rC2 inhibited EGR-factor IXa binding to immobilized rC2 by ~90 and ~60%, respectively (data not shown), confirming specificity of the assay.

We further evaluated interactions by an alternative approach using real time SPR-based assays. This technique provides information on kinetic and equilibrium binding constants (29, 30). A range of concentrations of rC2 were added to EGR-factor IXa immobilized onto a sensor chip. Fig. 1B shows representative curves corresponding to the association/dissociation of rC2. The data could be comparatively well fitted by nonlinear regression using a 1:1 binding model with drifting baseline line. Kinetic constants showed that rC2 bound to EGR-factor IXa with mild affinity ($K_d = 108 \pm 27 \text{ nM}$, $k_d/k_a = 2.45 \times 10^{-2}$ s$^{-1}$/2.36 $\times$ 10$^{5}$ M$^{-1}$ s$^{-1}$). In ELISA-based assays, the apparent $K_d$ value appeared to be higher (~400 nM), compared with that obtained by SPR-based assays. Since ELISA is not an equilibrium binding assay, the multiple steps of incubation and wash may affect the detection for lower concentrations of EGR-FIXa. The results indicated that the C2 domain of factor VIII interacted directly with factor IXa.

Characterization of the Interaction between the C2 Domain and EGR-factor IXa—The light chain of factor VIIa interacts with factor IXa in electrostatic and calcium-dependent mechanisms (19). To further characterize this interaction, factor IXa (100 nM) was mixed with various amounts of NaCl and incubated with immobilized rC2. Control experiments showed that the amount of immobilized rC2 or the reactivity of antibody was not affected even at a higher concentration of NaCl (data not shown). Binding of EGR-factor IXa to rC2 was maximal at physiological concentrations of NaCl (~150 mM; Fig. 2A). Higher concentrations of NaCl incrementally weakened this interaction, however, and consequently binding was significantly decreased by ~95% at elevated ionic strengths, supporting the salt sensitivity of this interaction. The Na$^+$-bound factor IXa drastically enhances catalytic activity toward factor X and increases the affinity for factor VIIa (33, 34). However, other monovalent cations, K$^+$ and Li$^+$, also inhibited this binding similarly, suggesting that this effect was not due to a specific interaction of Na$^+$-factor IXa.

Ca$^{2+}$ is known to be required for the structural and functional integrity of factor IXa, and hence the effect of Ca$^{2+}$ on factor IXa-rC2 interaction was also examined in the current experiments. Binding of factor IXa to immobilized rC2 was investigated in buffer containing various amounts of CaCl$_2$. EDTA (10 mM) was added to the reaction mixtures to assess binding in the absence of Ca$^{2+}$. The presence of Ca$^{2+}$ up to ~1.0 mM markedly increased factor IXa binding by ~6-fold compared with that in the absence of Ca$^{2+}$ (Fig. 2B). Optimal binding was observed at approximately physiological concentrations of free Ca$^{2+}$ (~1.3 mM). Binding was significantly inhibited by increments of Ca$^{2+}$ >1 mM. The data were consistent, therefore, with a role for Ca$^{2+}$ in C2-factor IXa interaction, although it was not possible to distinguish between a direct or indirect role for Ca$^{2+}$ in mediating this effect.

Binding of the Gla Domain of Factor IXa to the C2 Domain—To investigate whether the Gla domain of factor IXa participates in direct interactions with the C2 domain of factor VIII, EGR-GDless factor IXa was prepared by chymotrypsin digestion and EGR-ck labeling, as described under “Materials and Methods.” Control experiments demonstrated that EGR-GDless factor IXa and EGR-factor IXa were similarly reactive with anti-factor IXa mAb 3A6 in the ELISA (data not shown). The binding of EGR-GDless factor IXa to immobilized rC2 was markedly lower than that of EGR-factor IXa even at the maximum concentration employed (500 nM; Fig. 3A). SPR-based assays also showed that rC2 failed to react with EGR-GDless factor IXa (data not shown). In addition, competitive experiments using an anti-factor IXa mAb, mAb IXa-GD, recognizing the Gla domain of factor IXa and dependent on the presence of Ca$^{2+}$, demonstrated that the monoclonal antibody blocked binding of EGR-factor IXa to rC2 (up to ~95%) in a dose-dependent manner ($IC_{50} = 758 \pm 93$ nM) (Fig. 3B). These findings were in keeping with a significant role for the Gla domain of factor IXa in direct binding to the C2 domain of factor VIII.

To assess the functional role of the interaction between the C2 domain and Gla domain of factor IXa in the factor Xase complex, we examined the effect of rC2 or mAb IXa-GD on factor VIIa/factor IXa-mediated activation of factor X in an
amidolytic assay. For this assay, 200 nM factor VIII was activated by thrombin and incubated with mixtures of 5 nM factor IXa and various concentrations of rC2 or mAb IXa-GD. Factor Xa generation was initiated by the addition of 1 μM factor X. The rC2 competes with factor VIII for binding to phospholipid membranes, and for this reason, the assays were performed in the absence of phospholipid. The addition of rC2 and mAb IXa-GD markedly decreased the rates of factor Xa generation (by >90%) in a dose-dependent manner, with the IC_{50} values of 10.9 ± 3.8 μM and 43.2 ± 15.3 nM, respectively (Fig. 4, A and B).

To exclude the possibility that rC2 and mAb IXa-GD directly affected factor IXa-catalyzed activation of factor X, factor Xa generation was further examined in the absence of factor VIIIa. As expected, there was little inhibition of factor Xa generation in the presence of rC2 or mAb IXa-GD (Fig. 4, inset), confirming that the reactions were governed by factor VIIIa. The results indicated that association between the C2 domain of factor VIII and the Gla domain of factor IXa played a significant role in the assembly of the factor Xase complex and hence factor IXa-catalyzed activation of factor X in the presence of factor VIIIa.

**Purification and Characterization of rC2-digested Fragments—**To locate factor IXa-interactive regions within the C2 domain, limited *Staphylococcus aureus* V8 protease digests of rC2 were prepared. SDS-PAGE analysis demonstrated the presence of two large fragments of apparent mass 7.5- and 6.2-kDa that were significantly smaller than the initial rC2 (~16 kDa) (Fig. 5B). The digestion of rC2 by V8 protease required denaturing conditions with 0.1% SDS, and we confirmed that the binding of SDS-treated, uncleaved rC2 to EGR-factor IXa was similar to that of the non-SDS-treated product in the ELISA-based assay (data not shown). EGR-factor IXa bound to immobilized, unfraccionated V8 protease-cleaved rC2 in a dose-dependent manner (data not shown), prompting us to further isolate and purify the cleaved fragments. The two fragments were not able to be separated by ion exchange HPLC using CM, Mono-Q, and Mono-S under any salt and pH conditions but were resolved by reverse phase HPLC (Fig. 5). SDS-PAGE confirmed that these fractions, designated as peak 1 and peak 2, represented the 7.5- and 6.2-kDa fragments, respectively (Fig. 5, inset). Some smaller peaks were observed, but the bands were poorly detectable in SDS-PAGE and appeared to represent further minor degradation of rC2. The peak 1 and peak 2 fractions were further characterized by size exclusion chromatography, and each was shown to elute as a single peak, indicating that the fragments were monomeric in solution (data not shown).

Automated NH2-terminal sequence analysis identified that the sites of cleavage responsible for the generation of the two C2 fragments were located at residues Glu2181-Ser2182 and Glu2259-Phe2260 for the ~7.5- and ~6.2-kDa fragments, respectively (Table 1). In addition, LC/MS analysis indicated that the molecular mass of fragment 1 (7.5 kDa) and fragment 2 (6.2 kDa) was 8827.25 ± 5.70 and 7355.63 ± 0.68 Da, respectively. On the basis of the previous molecular masses determined by LC/MS, NH2-terminal sequence analysis, and specificity of V8 protease cleavage site (Glu-X and/or -Asp-X), the deduced protein sequences of the two C2 fragments matched Sei2182...Glu2259 (expected mass, 8823.08 Da) and Phe2260-Glu2322 (expected mass, 7354.39 Da). Hence, the ~7.5- and ~6.2-kDa C2 fragments were designated as C2-(2182–2259) and C2-(2260–2322), respectively.

**Binding of the Isolated C2 Fragments to EGR-factor IXa and the Effects on Factor Xa Generation—**To determine whether the C2-(2182–2259) and/or C2-(2260–2322) were able to bind to factor IXa, we investigated direct binding in ELISA-based assays, as described above. EGR-factor IXa bound directly to immobilized C2-(2182–2259) (600 nM) in a dose-dependent manner, although in this instance, the binding efficiency was weaker than that of the uncleaved rC2 (Fig. 6A). In contrast, very limited binding of EGR-factor IXa to immobilized...
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TABLE 1

Amino-terminal sequence analysis of the C2 fragments

| Cycle number | Residues 2182–2188 | C2 fragment 1 | Residues 2260–2264 | C2 fragment 2 |
|--------------|-------------------|--------------|-------------------|--------------|
| 1            | Ser Lys Ala Ile Ser Asp Ala | 10.4 17.7 14.4 14.3 8.4 6.6 7.9 | Phe Leu Ile Ser Ser | 10.7 10.3 11.0 5.5 5.4 |
| 2            |                   |              |                   |              |
| 3            |                   |              |                   |              |
| 4            |                   |              |                   |              |
| 5            |                   |              |                   |              |
| 6            |                   |              |                   |              |
| 7            |                   |              |                   |              |

FIGURE 6. Binding of EGR-factor IXa to C2 fragments and the inhibitory effects on factor Xa generation. A, binding of EGR-factor IXa. Various concentrations of EGR-factor IXa were incubated with immobilized C2-(2182–2259) (600 nM; open circles), C2-(2260–2232) (600 nM; closed circles), and intact rC2 (100 nM; open squares) for 2 h at 37 °C in an ELISA-based assay. Absorbance values were plotted as a function of the concentration of EGR-factor IXa. B, inhibition of factor Xa generation in the absence of phospholipid. Various amounts of C2-(2182–2259) (open circles) or C2-(2260–2232) (closed circles) were preincubated with 5 nM factor Xa for 2 h at 37 °C, and factor Xa generation was initiated with the addition of thrombin-activated factor VIIIa (200 nM) and 1 μM factor X. The initial rate of factor Xa generated in the absence of competitor (100% level) was 1.46 ± 0.19 pmol/min. Initial rates of factor Xa generation were plotted as a function of C2 fragment concentration. Experiments were performed at least three separate times, and average ± S.D. values are shown.

C2-(2260–2232) was observed. In control experiments, EGR-GDless factor IXa did not bind to either immobilized C2 fragment (data not shown). To assess the functional capacity of the two C2 fragments in factor Xase assembly, amidolytic assays were again repeated in the absence of phospholipid. Factor VIII (200 nM) was activated by thrombin and incubated with factor IXa (5 nM)/C2 fragment mixtures and factor X (1 μM). The C2-(2182–2259) competitively inhibited factor Xa generation by ~80% at the maximum concentration employed (IC50 = 1.2 μM; Fig. 6B). The effect of C2-(2260–2232) was significantly lower than that of C2-(2182–2259), however, and inhibited factor Xa generation by ~30%. Collectively, these data suggest that an interactive site(s) for the Gla domain of factor IXa was likely to be located within residues 2182–2259 of the C2 domain.

Effects of Synthetic C2 Peptides on rC2 and EGR-factor IXa Interaction and on Factor Xa Generation in the Absence of Phospholipid—On the basis of the competitive binding assays and ELISA, we focused on the 2182–2259 region in the C2 domain to further identify the potential factor IXa-interactive site. The C2-factor IXa interaction is electrostatically dependent (see Fig. 2A), suggesting that both interactive sites are surface-exposed. The analysis of solvent-accessible surface area was utilized, therefore, to examine regions within residues 2182–2259 exhibiting a high probability of being surface-exposed. The solvent accessibilities at the interface were calculated from atomic coordinates in the structures of factor VIII and C2 (Protein Data Bank code 2R7E and 1D7P, respectively), and they were similar. Using this approach, overlapping synthetic peptides encompassing the 2182–2249 region were prepared (Fig. 7). Since the C2-factor IXa interaction was not affected by anti-C2 mAb ESH8 with epitopes 2248–2285 (data not shown), the 2248–2259 region was excluded. Effects of peptides to block C2-factor IXa interaction and to inhibit factor Xa generation were examined.

The synthetic peptide corresponding to residues 2228–2240 (designated peptide 2228–2240) inhibited binding of EGR-factor IXa to rC2 by ~75% at the maximum concentrations employed (at 1 mM) (Fig. 8A). The IC50 value was ~400 μM. The other six peptides, corresponding to residues 2182–2195, 2192–2204, 2201–2213, 2210–2222, 2219–2231, and 2237–2249, demonstrated no inhibitory effects. Moreover, a control peptide (VKMTKQFDVQLWE), comprising the 2228–2240 residues in a random sequence, completely lost the ability to inhibit this interaction (data not shown). The inhibitory effects of these peptides were further studied in the factor Xa generation assay. The peptide 2228–2240, which blocked C2-factor IXa interaction, depressed factor Xa generation by ~75% at the maximum concentration employed (IC50 ~ 25 μM) (Fig. 8B). The other C2 peptides and the scrambled peptide had little effect. The ability of peptide 2228–2240 to inhibit factor Xa generation appeared to be more significant than that in the binding assay. It seemed likely, therefore, that peptide 2228–2240 not only affected interactions between the Gla domain of factor IXa and the C2 domain but also allosterically modulated other reactions. Nevertheless, the findings suggest that interactive site for the Gla domain of factor IXa was located within residues 2228–2240 of the C2 domain.

Effects of peptide 2228–2240 on Factor Xa Generation in the Presence of Phospholipid—Intact C2 and isolated C2 fragments contain phospholipid-binding regions, and to preclude interference by these reactions, our current factor Xa generation...
assays were performed in the absence of phospholipid. Crystal structure analysis has demonstrated that binding of the C2 domain to phospholipid membranes involves three hydrophobic “feet” containing residues Met2199/Phe2200, Val2223, and Leu2251/Leu2252 and four basic residues, Arg2215, Arg2220, Lys2227, and Lys2249 (35, 36). The factor IXa-interacting site that we have identified within residues 2228–2240 appears, therefore, to be in close proximity to, but not likely to be overlapping, the phospholipid-binding region. In support of this contention, we found that binding of factor VIII and factor IXa to phosphatidylerine was not significantly inhibited by peptide 2228–2240 (data not shown). Nevertheless, to further examine the physiological role of peptide 2228–2240 binding to the Gla domain of factor IXa, factor Xa generation was measured in the absence of factor VIIIa and phospholipid. Factor VIII (30 nM) was activated by thrombin and incubated with factor IXa (0.5 nM) in a phospholipid micelle mixture with various concentrations of factor X. Since rC2-factor IXa interaction was optimal at relatively low concentrations of Ca2+ (~1 mM), under these circumstances, the Vmax was 20-fold lower than that previously reported (31). Nevertheless, in the presence of peptide 2228–2240, the Km value remained unchanged, whereas the Vmax was decreased, dependent on the concentration of the peptide (Fig. 9). Factor Xa generation was completely inhibited (>95%) in the presence of 15 µM peptide. These results suggested that peptide 2228–2240 inhibited factor Xa generation on phospholipid micelles by noncompetitive inhibitory mechanisms. Furthermore, peptide 2228–2240 did not affect factor Xa generation using Gdless factor IXa in place of factor IXa in these assays (data not shown), again indicating that peptide 2228–2240 specifically bound to the Gla domain of factor IXa but did not moderate interactions between factor VIIIa and factor X.

**DISCUSSION**

The enzyme factor IXa and its cofactor factor VIIIa are assembled on phospholipid membranes for the activation of factor X. In previous reports, factor IXa recognition sites were identified within the A2 and A3 domains of factor VIIIa. In the A2 domain, the extended surface, centered on residues 484–509 (16), 558–565 (17), and 708–717 (18), appeared to interact with the factor IXa with weak affinity (~300 nM) (15). In contrast, in the A3 domain, the light chain, including residues 1804–1818 (20), interacted with the protease with high affinity (~15 nM) (19). The structural model of factor VIIIa-factor IXa on phospholipid membranes reported by Blostein et al. (21) proposed that the C2 domain of factor VIIIa and the Gla domain of factor IXa bound to phospholipid would be in close proximity, suggesting that both domains might bind to each other. In the present study, we show for the first time that the residues 2228–2240 in the C2 domain and the Gla domain of factor IXa bind to each other.

This conclusion is based on several novel findings using the established models. (i) Direct binding studies demonstrated that active site-modified EGR-factor IXa bound to the C2 domain with mild affinity (~100 nM), whereas Gdless EGR-factor IXa failed to bind. In addition, mAb IXα-GD, recognizing the Gla domain of factor IXa, blocked C2-factor IXa interaction. (ii) A factor Xa generation assay without phospholipid showed that rC2 and mAb IXα-GD inhibited factor IXα-medi-
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The factor X activation in the presence of factor VIIIa. (iii) A C2-(2182–2259) fragment, derived from V8 protease-cleaved rC2, directly bound to EGR-factor IXa and inhibited factor Xa generation, whereas the C2-(2260–2322) did not bind. (iv) Competitive assays, using overlapping synthetic peptides encompassing residues 2182–2259, showed that peptide 2228–2240 significantly inhibited both factor IXa binding and factor Xa generation, independently of phospholipid. These data identified amino acid residues 2228–2240 within the C2 domain as essential for factor IXa docking.

In the present study, we utilized EGR-factor IXa, a catalytically inactive derivative of factor IXa, in direct binding experiments. Modified factor IXa prepared with EGR-crk is well known to minimize enzyme-catalyzed degradation, but conformational changes and/or steric hindrance due to incorporation of EGR-crk into the active site of factor IXa may cause difficulties. Nevertheless, Lenting et al. (19) reported that thrombin-cleaved factor VIII light chain bound to modified factor IXa with high affinity (~15 nM), and we also analyzed direct binding of the C2 domain using untreated factor IXa and EGR-factor IXa. Binding patterns were similar using active factor IXa and EGR-factor IXa (data not shown), suggesting that any potential effects of conformational changes and/or steric hindrance induced by EGR-crk were minimal. The results also indicated that the C2 domain does not participate in docking to the active site pocket of factor IXa.

We obtained direct evidence for a restricted factor IXa-interactive site in the C2 domain using solvent-accessible surface area analysis with overlapping peptides encompassing residues 2182–2259. The sequence 2228–2240 appeared to be specific for this interaction, and a scrambled peptide confirmed this specificity. The peptide 2228–2240 did not affect factor VIII binding to phospholipid, however, in keeping with an earlier study using similar C2 peptides (7). Our observations add significantly to understanding the nature of the factor Xase complex involving factor IXa and the C2 domain of factor VIII. Our suggestion that this region is not related to phospholipid binding by experiments using peptide can be supported by the following reasons.

First, based on the ability of synthetic peptides encompassing residues 2303–2332 in C2 to inhibit factor VIII-phospholipid binding, a major site was previously located within this region (7). In addition, earlier elegant examination of the 1.5 Å x-ray structure of the C2 domain revealed the presence of three hydrophobic “feet” (Met2199/Phe2200, Val2223, and Leu2251/Leu2252) that penetrate the membrane and four basic residues (Arg2215, Arg2220, Lys2227, and Lys2249) that lie underneath the “feet” and stabilize binding by electrostatic interaction with phospholipid (35, 36). These findings show that the 2228–2240 region in C2 is in close proximity to, but is not likely to overlap, the phospholipid-binding region. Second, The interaction between factor IXa and thrombin-cleaved factor VIII light chain, lacking the acidic region of the A3 domain involved in high affinity VWF binding, was not affected by the presence of VWF (19). Our present study also showed that the C2-factor IXa interaction was not affected by VWF (data not shown). Since the C2 domain is involved in VWF binding at a site that overlaps the phospholipid-binding site (8, 9), the 2228–2240 region does not overlap this site. Last, factor VIIIa contacts with residue Phe25 and/or Val46 of the Gla domain of factor IXa but not with the membrane-binding ω loop (residues 1–11) (21). Furthermore, a naturally occurring mutation (G12R) within the Gla domain is associated with reduced activity of the factor Xase complex but does not affect phospholipid binding (37). These results are consistent with the view that interaction between the Gla domain and the C2 domain is not dependent on phospholipid binding. Taken together, our findings imply that interactions between both domains facilitate a tight ternary complex with phospholipid.

Binding of C2 to the Gla domain of factor IXa was governed by electrostatic and/or calcium-dependent interactions. This mechanism was similar to that observed between the light chain of factor VIII and factor IXa (19). Furthermore, peptide 2228–2240 significantly inhibited (>95%) factor Xa generation in the presence of phospholipid through noncompetitive inhibitory mechanisms, similar to those observed using peptide 1804–1818, previously reported as a factor IXa-binding site in A3. These data strongly indicated that the properties of both interactions were common and that both peptides inhibited the enzyme activity of factor IXa by binding at a site distinct from the substrate binding pocket. Of interest, the binding affinity of C2 for factor IXa (~100 nM) was ~7-fold lower than that of the light chain (~15 nM). In the absence of phospholipid, the inhibitory effect of peptide 2228–2240 on C2-factor IXa interaction was not significantly different from that of peptide 1804–1818 on light chain-factor IXa interaction. In the presence of phospholipid, however, peptide 2228–2240 appeared to inhibit factor Xa generation more strongly than peptide 1804–1818 (IC50 ~ 5 and ~600 μM, respectively). The binding affinity of the A3 domain for factor IXa is not known; nevertheless, the high affinity of the light chain appears to make an essential contribution to reactions involving not only the A3 domain but also the C2 domain. Furthermore, the data indicate that peptide 2228–2240 predominantly participates in factor IXa docking for catalyzing the activity of the factor Xase enzyme.

Recently, two groups have reported the intermediate resolution x-ray crystallographic structure of B-domainless factor VIII (38, 39). Factor IXa-interactive sites within factor VIII based on crystal structure reveal that residues 558–565 and 708–717 in A2 and 1804–1818 in A3 are located on one face of factor VIII, whereas residues 484–509 in A2 and our identified 2228–2240 in C2 are located on another face. Ngo et al. (39) have constructed a model of the factor VIIIa-factor IXa complex with x-ray crystal structure of human factor VIII and porcine factor IXa backbone with the following constraints. Residues 558–565, 708–717, and 1804–1818 of factor VIIIa interact with the residues 330–339, residues 301–303, and the putative binding region, including EGF domains (Tyr69 and Asn92) and Gla domain (Phe25) of factor IXa, respectively. Although this differed from our data in the binding site of C2 for the Gla domain, a factor IXa-interactive site comprising residues 2228–2240 in C2 is unlikely to contact the Gla domain simultaneously according to this model. This discrepancy may be due to conformational change of the C2 domain. Conformational changes in C2 of factor VIIIa upon removal of the NH2 terminus of the light chain (residues 1649–1689) (40) probably
leads to enhancement of the factor VIIIa affinity for phospholipid membrane (41). This may affect the Gla domain binding. In addition, the C2 domain is relatively loosely docked to the remainder of factor VIII molecules (38, 39); consequently, the position of this domain within active form factor VIIIa on the phospholipid surface may change easily. These findings can be supported by the case of residues 484–509 in A2. The model proposed by Ngo showed that this region did not interact with factor IXa despite the factor IXa-interactive model proposed by Ngo showed that this region did not interact with factor IXa. Furthermore, Stolova-McPhie et al. (43) found that it was unable to modify the factor VIII-factor IXa binding model, including the 484–509 region. The following possibilities are raised for this reason: the conformational change in A2 upon binding of the catalytic domain of factor IXa and different A2 arrangement between inactive form factor VIII and active form factor VIIIa. Therefore, it is not so surprising that the 2228–2240 region in factor VIIIa interacts with factor IXa Gla domain.

An earlier report by Nogami et al. (11) demonstrated that residues 2253–2270 within the C2 domain of factor VIII contribute to a unique factor Xa-interactive site within the light chain that promotes factor Xa docking during cofactor activation and cleavage of the light chain at Arg1689. Binding of factor Xa to the C2 domain was independent of binding to phospholipid or VWF, indicative of a distinct factor Xa-binding site in the C2 domain. This binding was remarkably similar to that of the C2-factor IXa interaction observed in this study. In addition, interaction between the light chain of factor VIIIa and factor IXa was not inhibited by active site-modified factor Xa (19). However, the C2-factor IXa interaction was not inhibited by anti-C2 mAb ESH8 (data not shown), which recognizes residues 2248–2285 and inhibits the factor VIII-factor Xa interaction (11). These findings suggest that the factor IXa-interactive site in the C2 domain does not overlap the factor VIII-Xa interactive site.

Comparisons of amino acid sequences among human, porcine, murine, and canine factor VIII molecules indicate that residues 2228–2240 within the C2 domain are well conserved, in keeping with the suggestion that this region could be fundamental for interaction with the Gla domain of factor IXa (44–46). This region appears to be unique, and the specific sequence of residues is distinct from those of the factor IXa-interactive sites within the A2 and A3 domains of factor VIIIa (16–18, 20). Naturally occurring mutations of residues 2228–2240 (W2229C, W2229S, Q2231H, V2232A/E, and M2238V) have been reported in the hemophilia A data base (HAMSteRS), and are seen in mild/moderate hemophilia A. It is tempting to speculate that the pathogenetic mechanism for these point mutations might be associated with dysfunctional blood coagulation by moderating interactions between the C2 domain of factor VIIIa and the Gla domain of factor IXa. Furthermore, substitutions at Trp2229 to Cys and Val2232 to Ala are related to the development of inhibitors (47, 48), consistent with our suggestion that the 2228–2240 region in C2 is surface-exposed and influences antigenicity.

In conclusion, we provide the first evidence for an essential role of the association between the 2228–2240 region of the C2 domain and the Gla domain of factor IXa in the factor Xase complex. Further studies using site-directed mutagenesis are warranted to further clarify the functional role of residues 2228–2240 in the C2 domain.

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