Functional and Sequence Characterization of Coagulation Factor IX/Factor X-Binding Protein from the Venom of *Echis carinatus leucogaster*†

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ABSTRACT: A new coagulation factor IX/factor X-binding protein (IX/X-bp) from *Echis carinatus leucogaster* venom has been purified and designated ECLV IX/X-bp. ECLV IX/X-bp binds factor IX and X in a Ca^{2+}-dependent manner and is devoid of thrombin-inhibitory and platelet-aggreagating activities. The apparent dissociation constants (K_d) for binding of ECLV IX/X-bp to factor IX and factor X are 6.6 and 125 nM, respectively. Upon the addition of Mg^{2+}, the required Ca^{2+} concentration for optimal binding of ECLV IX/X-bp to factor IX and factor X was prominently reduced. Mg^{2+} also increases the affinity of factor X for the venom protein. Direct binding of IX/X-bp to factor IX and X could also be detected by far-Western blotting, and results of the experiment ruled out the lectin-like mechanism of ECLV IX/X-bp. The complete amino acid sequence and the disulfide pattern of ECLV IX/X-bp was deduced by enzymatic hydrolysis and automated sequencing of the S-pyridylethylated protein. The venom protein is a heterodimer with one subunit of 131 amino acid residues and another of 125 residues. Both subunits are homologous to each other and to other snake venom proteins of the C-type lectin superfamily.

Snake venoms contain a variety of proteins which affect vertebrate blood coagulation and platelet function (Kornalik, 1991; Teng & Huang, 1991) such as fibrinogenase (Willis & Tu, 1988), platelet aggregation inducers (Teng *et al*., 1993; Huang *et al*., 1995), and inhibitors (Chen *et al*., 1995). An anticoagulant has been isolated from the venom of the habu snake *Trimeresurus flavoviridis* and designated as habu IX/X-bp[1] (Atoda & Morita, 1989) since this protein forms a 1:1 complex with factor IX or X in the presence of Ca^{2+} ions and prolongs the clotting time (Atoda & Morita, 1989). Similar molecules have also been purified from the venom of *Deinagkistrodon acutus* (i.e. acutus IX/X-bp; Cox, 1993; Morita & Atoda, 1994) and *Bothrops jararaca* (i.e. jararaca IX/X-bp; Sekiya *et al*., 1993). Recent study indicated that habu IX/X-bp binds to the Gla domain of factor IX and X in the presence of calcium ions (Atoda *et al*., 1994). These IX/X-bps from crotalid venoms are all heterodimer proteins covalently linked by a disulfide bridge. Moreover, the amino acid sequence (Atoda *et al*., 1991) and disulfide pattern (Atoda & Morita, 1993) of habu IX/X-bp have been determined.

The venom of *B. jararaca* contains not only IX/X-bp but also other structurally similar but functionally distinct proteins: two-chain botrocetin (Fujimura *et al*., 1991), bothrojaracin (Zingali *et al*., 1993), and jararaca GPIb-BP (Fujimura *et al*., 1995). Botrocetin induces vWF binding to the glycoprotein Ib (GPIb) of platelet (Fujimura *et al*., 1991). Bothrojaracin is a thrombin inhibitor binding the exosite of α-thrombin (Zingali *et al*., 1993). Jararaca GPIb-BP is an antagonist specific to platelet GPIb (Fujimura *et al*., 1995). Amino acid sequences of all four venom proteins bear similarity to the animal C-type lectins (Drickamer, 1988).

Echicetin, a heterodimeric GPIb binding protein, was previously isolated from the venom of the saw-scaled or carpet viper, *Echis carinatus*. It inhibited platelet agglutination induced by GPIb agonists (Peng *et al*., 1993). The amino acid sequence has been determined for its β subunit which presumably contains the binding site of echicetin (Peng *et al*., 1994). Since several members of the C-type lectin family are present in the venom of some Crotalidae studied, we would like to know whether any C-type lectin-like anticoagulants other than echicetin might also exist in the venom of *Echis carinatus leucogaster*, an African subspecies of this genus of Viperidae or true viper. In the present study, a IX/X-bp named ECLV IX/X-bp was purified for the first time from a Viperid venom. The complete amino acid sequences of both subunits of ECLV IX/X-bp were determined and compared with those of other homologous venom proteins.

EXPERIMENTAL PROCEDURES

Materials. E. carinatus leucogaster venom was purchased from Latoxan (Rosans, France). Sephadex G-75 was from Pharmacia LKB (Sweden). Trifluoroacetic acid (TFA),

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[1] Abbreviations: ECLV IX/X-bp, factor IX/factor X-binding protein isolated from the venom of *Echis carinatus leucogaster*; habu IX/X-bp, IX/X-bp isolated from the venom of *Trimeresurus flavoviridis*; vWF, von Willebrand factor; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; RP-HPLC, reversed-phase high-performance liquid chromatography; TBS, 20 mM Tris-HCl/140 mM NaCl, pH 7.5; TBS-Ca, TBS containing 5 mM $CaCl_2$; TBST, TBS containing 0.05% of Tween 20; TBST-Ca, TBST containing 5 mM $CaCl_2$; BSA, bovine serum albumin; Gla, γ-carboxyglutamic acid; GPIb, glycoprotein Ib; Pe, S-pyridylethylated; PVDF, poly(vinylidene difluoride); RVVXL1, factor X-activating enzyme light chain 1 from Russell's viper venom; RSL, rattlesnake lectin from *Crotalus atrox* venom; TFA, trifluoroacetic acid; BVPRMCA, *tert*-butyloxycarbonyl-L-Val-L-Pro-L-Arg-4-methylcoumaryl-7-amine; SBHP, streptavidin−biotinylated horseradish peroxidase complex; BAC-sulfo NHS, biotinamidocaproate *N*-hydroxysulfosuccinimide ester.

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4-vinylpyridine, and Fractogel TSK CM-650 (S) were purchased from Merck (Germany). Streptavidin−biotinylated horseradish peroxidase complex (SBHP) was obtained from Amershan (England). *N*-Glycosidase F, neuroaminidase, and *O*-glycosidase were from Boehringer Mannheim (Germany). *tert*-Butyloxycarbonyl-L-Val-L-Pro-L-Arg-4-methylcoumaryl-7-amine (BVPRMCA) was from Peptide Institute (Japan). Anti-human factor IX antiserum, an immunoprobe biotinylation kit, human α-thrombin, hirudin, *p*-nitrophenyl phosphate, *o*-phenylenediamine dihydrochloride, and other reagents were obtained from Sigma Chemical Co. (St. Louis, MO).

Proteins. Bovine coagulation factor IX, factor X, prothrombin, and protein S were purified from bovine plasma as previously described (Hashimoto *et al*., 1985). Bovine vWF was purified according to the method of Mascelli *et al*. (1986).

Factor IX Binding Assay. The binding of IX/X-bp to bovine factor IX was determined by enzyme-linked immunosorbent assay. The microtiter plates (Greiner, Germany) were coated with 50 μL of samples (10 μg/mL) purified from the venom at 4 °C overnight. After the wells were blocked with 100 μL of 1% BSA in TBST (Tris saline buffer containing 0.05% Tween 20) at room temperature for 1 h, to the wells was added 50 μL of bovine factor IX in TBST (5 μg/mL) in the presence or absence of 5 mM $CaCl_2$, incubated for 1 h at room temperature. The wells were washed eight times with TBST-Ca, and to each well was then added 50 μL of anti-human factor IX antiserum ($^1/_{2000}$ diluted with TBST-Ca) and the mixture incubated for 1 h. The wells were washed, and to them was added 50 μL of alkaline phosphatase-conjugated antibodies raised in goat against rabbit IgG (diluted $^1/_{1000}$ with TBST-Ca) and the mixture further incubated 1 h. After eight washes with TBST-Ca, 100 μL of a solution of substrate [1 mg/mL *p*-nitrophenyl phosphate in 0.2 M Tris buffer (pH 8.0)] was added. After 30 min, the reaction was stopped by the addition of 50 μL of 3 M NaOH and the absorbance at 405 nm was measured with a micro-plate reader (Model 450, Bio-Rad, U.S.A.).

Platelet Agglutination Assay. Formaldehyde-fixed human platelets were prepared by the published method (Brinkhous & Read, 1989). The inhibitory effect of purified venom protein on the agglutination of fixed platelets induced by bovine vWF was measured using an aggregometer (JASCO CAF-100 Ca^{2+} analyzer) as previously described (Peng *et al*., 1993).

α-*Thrombin Inhibition Assay*. The inhibition of thrombin amidolytic activity by hirudin was measured with 10 μM BVPRMCA in TBS as previously described (Morita *et al*., 1977). α-Thrombin (0.5 nM) was preincubated with the venom proteins (about 0.2 μM) for 2 min, and then hirudin (20 nM) was added 1 min before BVPRMCA was added. Hydrolysis of BVPRMCA was followed fluorometrically at 25 °C in 700 μL of TBS with $\lambda_{excit} = 280$ nm and $\lambda_{emis} = 360$ nm.

Purification of ECLV IX/X-bp. About 250 mg of the curde venom of *E. carinatus leucogaster* was dissolved in 1.5 mL of ammonium acetate (pH 6.8) and loaded onto a Sephadex G-75 column (1.6 × 100 cm). The column was eluted at a flow rate of 0.25 mL/min, and fractions of 2.5 mL were collected. The fractions containing proteins of 20−40 kDa were collected, then loaded onto a TSK CM-650 (S) column (3 × 10 cm) preequilibrated with 10 mM phosphate buffer (pH 6.8), and eluted with a linear gradient of NaCl (0 to 0.4 M) in the buffer, and each fraction was assayed for factor IX-binding activity, the ability to agglutinate fixed platelet, and the effect on α-thrombin inhibition by hirudin. Active fractions were pooled, dialyzed against distilled water, then lyophilized, and stored at −20 °C.

SDS−PAGE was carried out according to Laemmli (1970). The midrange molecular mass markers (Promega Co., U.S.A.) used were phosphorylase B (97 400), bovine serum albumin (66 200), glutamate dehydrogenase (55 000), ovalbumin (42 700), aldolase (40 000), carbonic anhydrase (31 000), soybean trypsin inhibitor (21 500), and lysozyme (14 400). Proteins were stained with Coomassie Brilliant Blue.

Biotinylation of ECLV IX/X-bp. An immunoprobe biotinylation kit was used; 0.5 mg of ECLV IX/X-bp from a TSK CM650 column in 0.5 mL of 0.1 N phosphate buffer (pH 7.2) was mixed with 5 μL of BAC-sulfo NHS solution (10 mg/mL in 0.1 M phosphate buffer) and incubated with gentle stirring for 30 min at room temperature. The biotinylated protein was purified or desalted by a Sephadex G-25 column (1 × 10 cm) preequilibrium with PBS and stored at −20 °C until used.

Binding of IX/X-bp to Immobilized Coagulation Factors. Ninety-six-well plates were coated separately with 50 or 100 nM purified bovine coagulation factor IX, X, prothrombin, and protein S in TBS at 4 °C overnight. After the wells were blocked with 1% BSA in TBST for 1 h at room temperature, various concentrations (0.2−100 nM) of biotinylated ECLV IX/X-bp were added to the wells before 1 h incubation at room temperature. For competition experiments, each well was incubated with 10 nM biotinylated IX/X-bp plus samples in 50 μL of TBS containing 5 mM $CaCl_2$ for 1 h at room temperature. The plate was washed with TBST-Ca, followed by addition of 10^3-diluted streptavidin−biotinylated horseradish peroxidase complex (SBHP) in TBST-Ca, and incubated for 1 h. After the wells were washed with TBST-Ca, to them were added *o*-phenylenediamine dihydrochloride (0.4 mg/mL) and H_2O_2 (0.012%) in TBS. The reaction was stopped with 3 N H_2SO_4 after 30 min. Absorbance at 490 nm was measured with a microplate reader (Model 450, Bio-Rad). The apparent affinity constant (K_d) for ECLV IX/X-bp binding to coagulation factor was calculated by the published method (Suzuki & Nishioka, 1988).

Far-Western Blotting. Bovine coagulation factors were purified and subjected to 10% SDS-PAGE (Laemmli, 1970). Proteins in the gel were transferred to a PVDF membrane (MSI, U.S.A.) by a semidry transblotter (Hoefer, U.S.A.), followed by incubation for 1 h in the blocking solution (TBST containing 1% BSA). Subsequently, the membrane was incubated in TBST-Ca with 0.5 μg/mL biotinylated ECLV IX/X-bp for 1 h at room temperature. After three 5 min washes with TBST-Ca, bound biotinylated ECLV IX/X-bp was visualized by addition of SBHP (diluted $^1/_{2000}$ in TBST-Ca) for 1 h and the color developed with a solution containing 3,3′-diaminobenzidine (0.6 mg/mL), 0.03% $NiCl_2$, and H_2O_2 (0.03%) in TBS.

Protein and Amino Acid Analysis. The protein concentration was measured spectrophotometrically at 205 nm and calculated by the following equation: concentration (milligrams per milliliter) = absorbance at 205 nm/31 (Scopes,

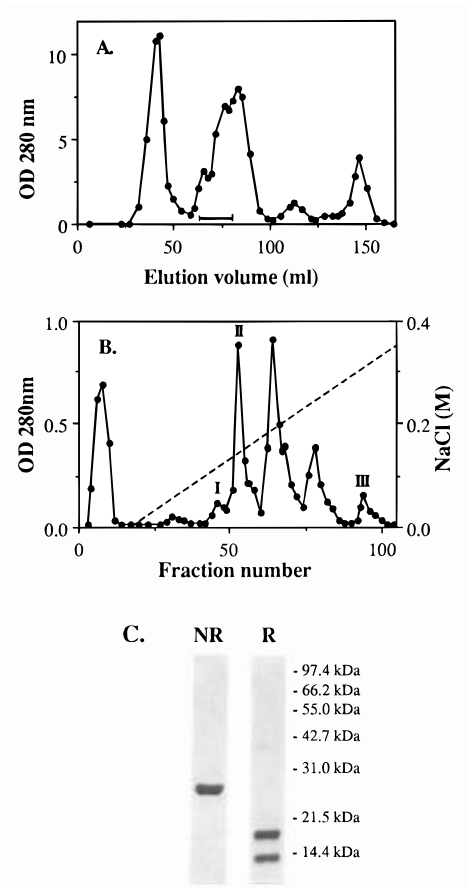

FIGURE 1: Purification of ECLV IX/X-bp. (A) Gel filtration by a Sephadex G-75 column. (B) Cation exchange chromatography on a TSK CM-650 column. Proteins were monitored by absorbance at 280 nm (●). The dashed line indicates the salt gradient. Peaks I and II, and III contained α-thrombin-binding activity, activity to inhibit platelet agglutination by bovine vWF, and factor IX-binding activity, respectively. (C) SDS−PAGE analysis of the purified ECLV IX/X-bp on gel under nonreducing (NR) or reducing (R) conditions (with 0.1 M DTT). Molecular masses of the markers are shown on the right.

1974). Amino acid composition was analyzed by vapor-phase hydrolysis at 158 °C for 30 min using 7 M HCl/10% TFA/0.1% phenol (Chang & Liu, 1988), and the hydrolysate was derivatized to dimethylaminoazobenzenesulfonyl amino acids before separation by reverse-phase HPLC (Kencht & Chang, 1986). The hydrolysate of egg white lysozyme was used as a reference.

N- and O-Deglycosylation of Factor IX. Bovine coagulation factor IX (20 μg) was incubated with 0.5 unit of neuraminidase, 0.5 unit of *N*-glycosidase, or 0.5 unit of *O*-glycosidase in 20 μL of 0.02 M sodium phosphate buffer for 18 h at 37 °C. Reaction was stopped by addition of 2 μL of 10% SDS containing 1 M dithiothreitol to the sample and boiling for 3 min. The products were subjected to far-Western blotting analysis.

RESULTS

Purification of ECLV IX/X-bp. ECLV IX/X-bp was purified to homogeneity from the venom of *E. carinatus leucogaster* in two steps: first by gel filtration chromatography and then by cation exchanger chromatography (panels A and B of Figure 1). The gel filtration of the crude venom by Sephadex G-75 resulted in the separation of six protein fractions (Figure 1A). The fractions corresponding to molecular mass of 20−40 kDa were collected (indicated by bar) and further purified by TSK CM-650 ion exchange chromatography. During purification of these anticoagulants, we found three different activities associated with separated peaks. The content of peak I reduced the α-thrombin-inhibitory effect of hirudin. The content of peak II inhibited the platelet agglutination induced by bovine vWF, while factor IX-binding activity was eluted later in peak III. The isolated factor IX-binding protein also bound to factor X and was thus designated ECLV IX/X-bp. SDS−PAGE analysis of the purified ECLV IX/X-bp showed a single band with an apparent molecular mass of 27 kDa under nonreducing conditions (Figure 1C, lane NR) and showed a doublet of 17 and 14 kDa under reducing conditions (Figure 1C, lane NR) and showed a doublet of 17 and 14 kDa under reducing conditions (lane R). Its yield was about 0.5% (w/w) of the crude venom. Purity of the ECLV IX/X-bp was also checked by RP-HPLC on a Cosmosil C_8 column, and a single peak was eluted at 37% acetonitrile. Both peak I and II from the cation exchanger contain protein subunits of 14 ± 2 kDa as shown by the result of reducing SDS−PAGE.

Binding Specificity and Affinity of ECLV IX/X-bp. In order to investigate the dose dependency of the binding of ECLV IX/X-bp to several Gla-containing plasma proteins, we biotinylated the venom protein without loss of its factor IX-binding activity. Factor IX, factor X, protein S, and prothrombin were fixed on the wells of the microtiter plate separately, and the amount of biotinylated IX/X-bp that bound to the wells in the presence of 5 mM $CaCl_2$ was measured using the SBHP system. The apparent dissociation constant (K_d) of ECLV IX/X-bp to the solid-phase coagulation factor was calculated by a double-reciprocal plot analysis (Suzuki & Nishioka, 1988). The biotinylated IX/X-bp bound to immobilized factor IX and X in a concentration-dependent manner, but the extent of its binding to prothrombin and protein S was very low by similar treatment (Figure 2A). The apparent dissociation constants (K_d) for binding of ECLV IX/X-bp to factor IX and factor X were calculated to be 6.6 and 125 nM, respectively. The binding to factor IX was effectively competed off by a high concentration of unlabeled IX/X-bp (Figure 2B). These results indicated that the binding is specific and saturable. We further examined the binding ability of separated subunits of IX/X-bp. The reduced and S-pyridylethylated subunits (Figure S2 in the supporting information) no longer inhibited the binding of the biotinylated IX/X-bp to factor IX, apparently (Figure 2B).

Effect of Metal Ions. The binding of biotinylated ECLV IX/X-bp to solid-phase factor IX (Figure 3A) and factor X (Figure 3B) requires the presence of Ca^{2+} ion; half-maximal binding to both factors occurred at 0.4 and 1.1 mM Ca^{2+}, respectively. The affinity of binding between the IX/X-bp and factor IX was lowered when Sr^{2+} instead of Ca^{2+} was added. However, Sr^{2+} was not able to substitute Ca^{2+} in the binding to factor X. Other metal ions tested (Mg^{2+}, Ba^{2+}, Zn^{2+}, Cu^{2+}, and Mn^{2+}) could not substitute Ca^{2+} for inducing factor IX or factor X to bind the venom protein.

Mg^{2+} ions were found to affect the Ca^{2+} dependency of binding of the venom protein with factor IX or factor X. For example, the Ca^{2+} concentrations required for half-maximal binding of ECLV IX/X-bp to factor IX were 400, 130, 45, and 11 μM in the presence of 0, 0.125, 0.5, and 2 mM Mg^{2+}, respectively (Figure 4A). In the case of factor X binding, the Ca^{2+} concentrations required for half-

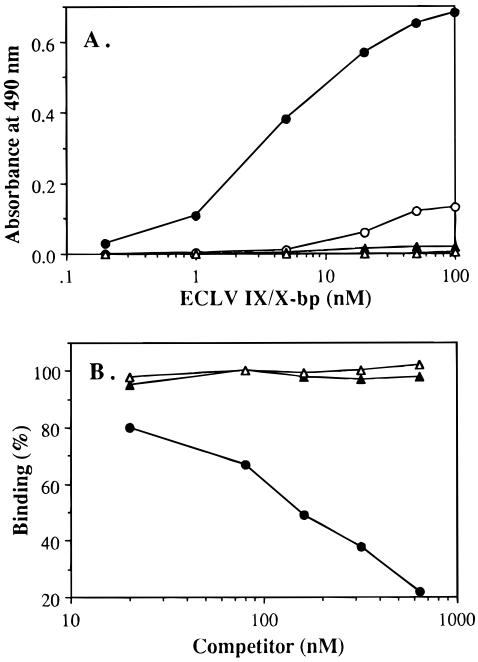

FIGURE 2: (A) binding of ECLV IX/X-bp to bovine plasma coagulation factors. Microwells were coated with 50 nM factor IX (●), 100 nM factor X (○), prothrombin (▲), or protein S (△) before addition of various concentrations of biotinylated IX/X-bp in TBS containing 5 mM $CaCl_2$ and incubation at room temperature for 1 h. Bound IX/X-bp was determined with the SBHP system as described in Experimental Procedures. (B) Competitive effect of unlabeled ECLV IX/X-bp and its subunits on binding of ECLV IX/X-bp. Microwells were coated with factor IX (50 nM) and incubated with biotinylated IX/X-bp (10 nM) plus various concentrations of the unlabeled IX/X-bp (●), alkylated α subunit (▲), or alkylated β subunit (△). Bound IX/X-bp was measured with the SBHP system. Each point represents the average of triplicate determinations.

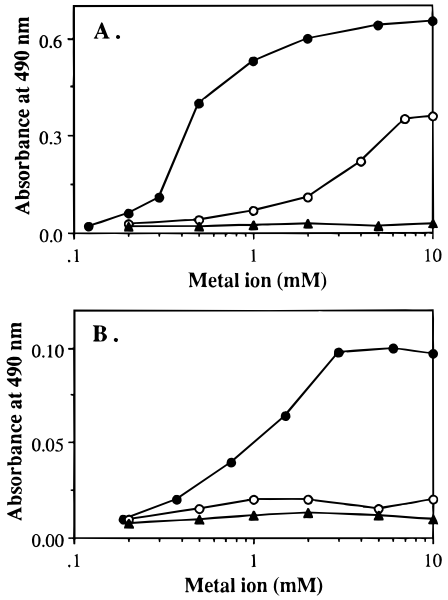

FIGURE 3: Effects of divalent metal ions on the binding of biotinylated ECLV IX/X-bp to factor IX (A) and factor X (B). Microwells were coated with factor IX (50 nM) or factor X (100 nM) before incubation with biotinylated IX/X-bp in the presence of different metal ions: (●) Ca^{2+}, (○) Sr^{2+}, and (△) Mg^{2+}. Bound IX/X-bp was measured with the SBHP system.

maximal binding of the IX/X-bp were 1.1, 0.25, and 0.19 mM in the presence of 0, 0.5, and 2 mM Mg^{2+}, respectively (Figure 4B). At 2 mM Mg^{2+}, the maximal binding of

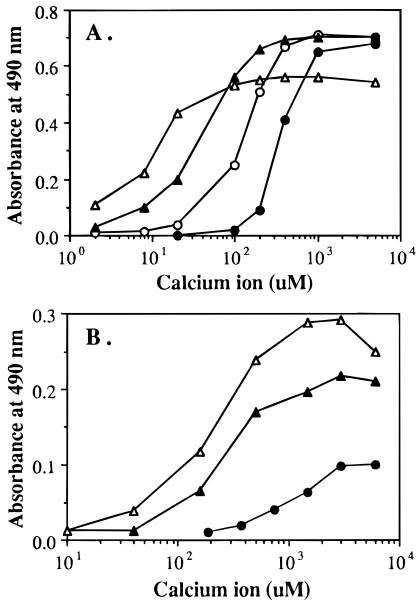

FIGURE 4: Effects of Mg^{2+} on the Ca^{2+} dependency of the binding of the venom protein to factor IX (A) and factor X (B). Microwells were coated with factor IX (50 nM) or factor X (100 nM) and incubated with biotinylated IX/X-bp in the presence of various Ca^{2+} concentrations as well as 0.125 mM (○), 0.5 mM (▲), 2 mM (△), or 0 mM (●) Mg^{2+}. Bound IX/X-bp was measured with the SBHP system. Each point represents the mean value from duplicate determinations.

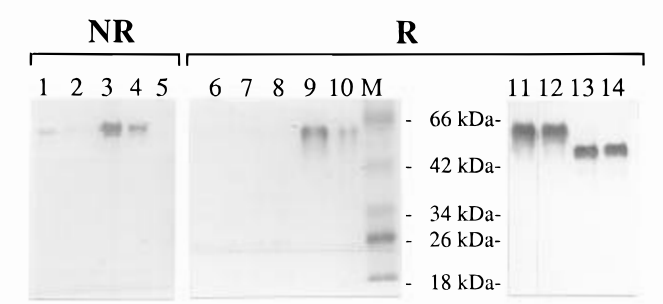

FIGURE 5: Far-Western blotting analysis of binding of the venom protein to plasma coagulation factors and deglycosylated factor IX. Purified bovine coagulation factors were separated by 10% SDS−PAGE under reducing conditions (R) or nonreducing conditions (NR). After electrophoretic transfer of protein to a PVDF membrane, the plasma proteins were probed with 0.5 μg/mL biotinylated ECLV IX/X-bp and stained with the SBHP system: lanes 1 and 6, 5 μg of factor X; lanes 2 and 7, 5 μg of prothrombin; lanes 3, 9, and 11, 2 μg of factor IX; lanes 4 and 10, 0.2 μg of factor IX; lanes 5 and 8, 5 μg of protein S; lanes 12−14, 2 μg of O-deglycosylated, N-deglycosylated, and desialylated plus N-deglycosylated, respectively; and lane M, prestained molecular mass markers.

biotinylated ECLV IX/X-bp to factor IX is slightly reduced, but its binding to factor X was greatly potentiated by Mg^{2+} (see Figure 4B, i.e. increase of maximal binding corresponding to an optical density increase from 0.1 to 0.29 at 490 nm).

Far-Western Blotting To Detect Binding of Biotinylated ECLV IX/X-bp. Purified bovine plasma proteins containing γ-carboxyglutamic acid were subjected to SDS−PAGE on a 10% gel and then electroblotted onto a PVDF membrane. The blot was incubated with biotinylated ECLV IX/X-bp, and the binding of the biotinylated IX/X-bp was detected with the SBHP system (Figure 5). Under nonreducing conditions, its binding to factor IX and factor X could be easily detected (lanes 1 and 3) but not its binding to the other

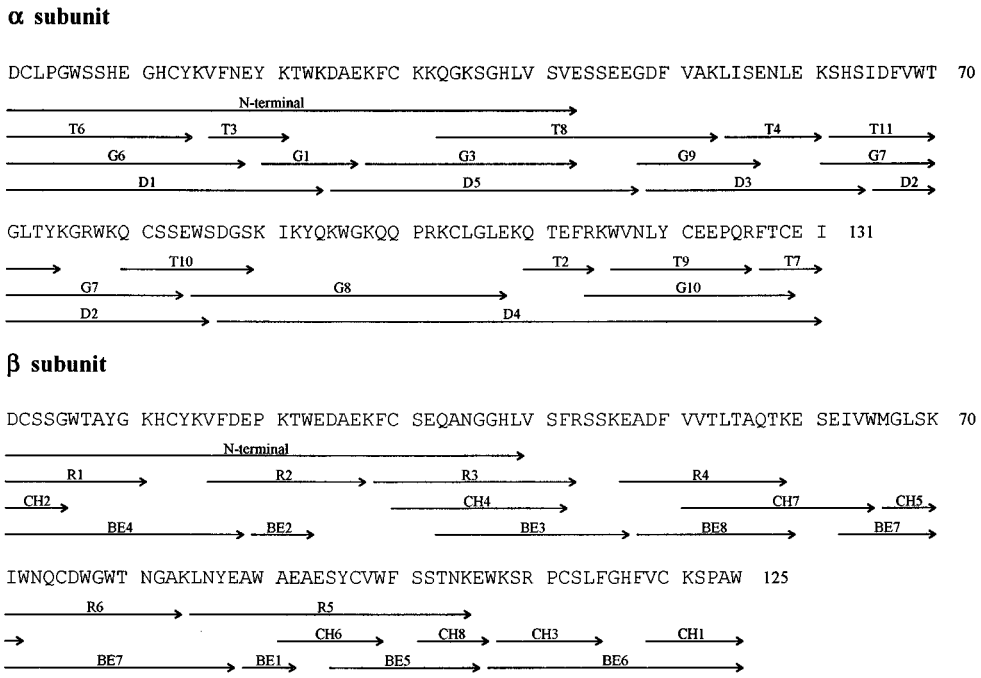

FIGURE 6: Complete amino acid sequence of ECLV IX/X-bp. Amino acid residues are given in single-letter code. Residues determined by Edman degradation are indicated by arrows. Peptide designations are according to the digestion enzyme used: T, trypsin; G or BE, Glu-C endoproteinase; D, Asp-N endoproteinase; CH, α-chymotrypsin; and R, Arg-C endoproteinase.

coagulation factors (lanes 2 and 5), nor when 5 mM EDTA was included in the incubation solution (data not shown). Under reducing conditions, reduced factor IX bound ECLV IX/X-bp (lanes 9 and 10), but both the reduced heavy chain and light chain of factor X could not bind to the bitoinylated IX/X-bp (lane 6).

To address the question of whether the binding of ECLV IX/X-bp is mediated by its lectin-like activity, factor IX was deglycosylated by *O*-glycosidase, *N*-glycosidase F, or neuraminidase plus *N*-glycosidase F and then subjected to far-Western blotting using the biotinylated IX/X-bp and SBHP. Results in Figure 5 show that the N-deglycosylation resulted in a shift of M_r of factor IX to 42.5 kDa, and that is consistent with a previous report on the complete removal of N-linked sugars of factor IX (Mizuochi *et al*., 1983). When a gel containing deglycosylated factor IX was electroblotted, those bands corresponding to 57 kDa after O-deglycosylation, 42.5 kDa after N-deglycosylation, and 42.5 kDa after desialylation and N-deglycosylation were all stained, like the native factor IX (Figure 5, lanes 12−14). Various sugars (including galatose, mannose, *N*-acetylglucosamine, *N*-acetylgalactosamine, etc.) up to 100 mM did not affect the binding of ECLV IX/X-bp (3.5 nM) to factor IX and X (data not shown). These results suggest that the binding to factor IX was not affected by deglycosylation and ruled out a lectin-like mechanism for IX/X-bp.

Amino Acid Sequences. The subunits of ECLV IX/X-bp were isolated by RP-HPLC (Figure S2 in the supporting information) and subjected to protein sequencing. The alkylated α subunit was hydrolyzed with trypsin (T), Glu-C endoproteinase (G), and Asp-N endoproteinase (D). The alkylated β subunit was hydrolyzed with Arg-C endoproteinase (R), α-chymotrypsin (CH), and Glu-C endoproteinase (BE), respectively. The fragments were separated by RP-HPLC (Figures S3−S8 in the supporting information). The complete amino acid sequences of both subunits were elucidated by sequencing of each overlapping peptide (Figure 6). The sequence data are consistent with the results of amino acid analysis (Table S1 in the supporting information). The molecular masses of the α and β subunits calculated from their sequences are 15 438 and 14 371 Da, respectively, and their p*I* values are estimated to be 8.9 and 5.5, respectively.

Disulfide Bridge of ECLV IX/X-bp. Proteolytic digestion of ECLV IX/X-bp with trypsin and Glu-C endoproteinase separately, followed by RP-HPLC separation (Figures S9−S10 in the supporing information) and amino acid analyses gave total of six cystine-containing fragments. One of the Glu-C endoproteinase fragments containing two disulfide bridges was further digested with α-chymotrypsin (Figure S11 in the supporting information). Results from the sequence analysis of all seven peptides indicated the presence of $Cys^{2}-Cys^{13}$, $Cys^{30}-Cys^{129}$, and $Cys^{104}-Cys^{121}$ in the α subunit and $Cys^{2}-Cys^{13}$, $Cys^{30}-Cys^{120}$, and $Cys^{97}-Cys^{112}$ in the β subunit. One interchain disulfide bond was identified between Cys^{81} of the α subunit and Cys^{75} of the β subunit. The disulfide pattern is similar to that of habu IX/X-bp (Atoda & Morita, 1993).

DISCUSSION

A new coagulation factor IX and factor X-binding protein, ECLV IX/X-bp, was purified from the venom of *E. carinatus leucogaster* by a combination of gel filtration and ion exchange chromatographic steps. This IX/X-bp is free of platelet-aggregating activity and apparently differs from echicetin, a platelet glycoprotein Ib antagonist isolated from the *E. carinatus* venom (Peng *et al*., 1993). The coexistence of dimeric C-type lectin-like proteins with diversified biological activities was previously found in several Crotalid venoms. For example, the venom of South American *B. jararaca* contains at least five of these: jararaca IX/X-bp (Sekiya *et al*., 1993), two-chain botrocetin (Fujimura *et al*., 1991), bothrojaracin (Zingali *et al*., 1993), homodimeric lectin (Ozeki *et al*., 1994), and jararaca GPIb-binding protein

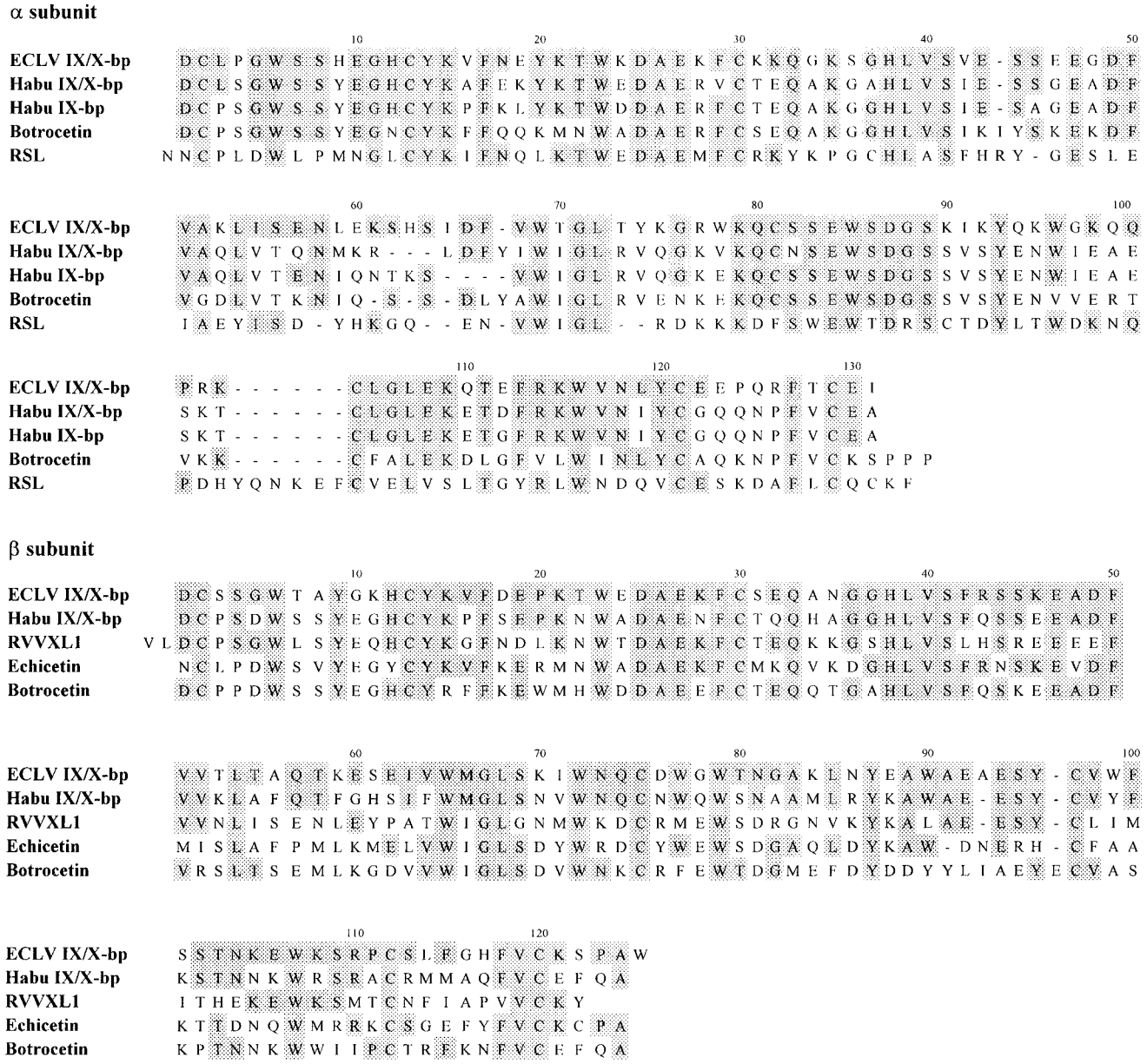

FIGURE 7: Sequence comparison of ECLV IX/X-bp with related proteins. References are as follows: habu IX/X-bp from *T. flavoviridis* venom (Atoda *et al*., 1991), botrocetin from *B. jararaca* venom (Usami *et al*., 1993), the homodimeric rattlesnake lectin (RSL) from *C. atrox* venom (Hirabayashi *et al*., 1991), echicetin from *E. carinatus* venom (Peng *et al*., 1994), and light chain 1 of the factor X-activating enzyme (RVVXL1) from Russell's viper venom (Takeya *et al*., 1992). The amino acid numbering used is that of ECLV IX/X-bp. Residues identical to those of ECLV IX/X-bp are shaded. Gaps (−) are introduced to improve alignment.

(Fujimura *et al*., 1995); the Asian *D. acutus* contains at least three venom proteins of this superfamily: acutus IX/X-bp (Cox, 1993; Morita & Atoda, 1994), agkicetin, and α-thrombin binding protein (Chen & Tsai, 1995). We found that *E. carinatus leucogaster* venom contains a IX/X-bp, a GPIb-bp, and a thrombin inhibitor (Figure 1B). On the basis of preliminary results of their SDS−PAGE and N-terminal sequencing (data not shown), it appears that at least three functional categories of the venom proteins of the C-type lectin family evolve independently, not only in crotalid venoms but also in the venom of true viper.

The binding of factor IX and X with ECLV IX/X-bp could be demonstrated by far-Western blotting experiments (Figure 5). The reduced factor IX (but not X) retained the binding activity for the venom protein. On the other hand, ECLV IX/X-bp after the SDS-PAGE and electroblotting lost its binding activity regardless of whether it was reduced, suggesting that an intact conformation of the venom protein was crucial for its function.

The amino acid sequences of both subunits of ECLV IX/X-bp are compared with those of other homologous venom proteins of the C-type lectin superfamily (Figure 7). Both sequences are 43−59% identical to the other sequences listed. The α subunit of ECLV IX/X-bp had the highest structural similarity to those of habu IX-bp and habu IX/X-bp, with identities of 58 and 57%, respectively. The β subunit of ECLV IX/X-bp also bears respectively 59 and 45% identity to the habu IX/X-bp β subunit and the light chain of factor X activator from Russell's viper venom. We also notice that residues 111−116 of the α subunit and residues 91−96 of the β subunit of these venom IX/X-bp are highly conserved in contrast to their low similarity to the corresponding regions in botrocetin, echicetin, or rattlesnake lectin. The disulfide bond patterns of ECLV IX/X-bp and three other C-type

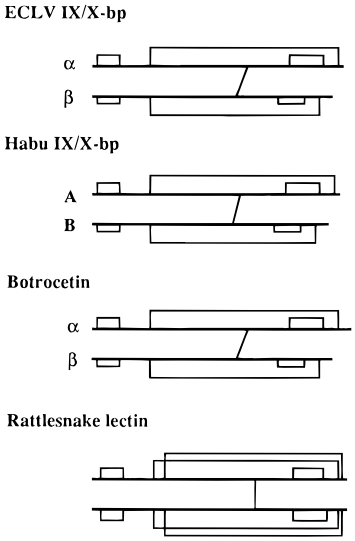

FIGURE 8: Location of intrachain and interchain disulfide bonds in ECLV IX/X-bp and other C-type lectin-like proteins. Bold lines indicate peptide chains, and fine lines indicate disulfide bridges.

lectin-like venom proteins are depicted in Figure 8. The locations of the disulfide bonds are all similar in these proteins except that the rattlesnake lectin has two more intrachain disulfide bridges.

We have studied the effects of Ca^{2+} and Mg^{2+} on the conformation of ECLV IX/X-bp by circular dichroism (data not shown) and intrinsic tryptophan fluorescence spectroscopies. The intrinsic tryptophan fluorescence (at λ_{emis} = 338 nm) of the venom protein was significantly increased by Ca^{2+} but not by Mg^{2+} (data not shown). The half-maximal enhancement of emission fluorescence was observed at approximately 310 μM Ca^{2+}, and this is close to the concentration required for half-maximal binding of IX/X-bp to factor IX. Mg^{2+} also did not affect the Ca^{2+} titration curve of the intrinsic fluorescence of IX/X-bp. Thus, the venom protein binds Ca^{2+} but probably not Mg^{2+}. Each subunit of the habu IX/X-bp was found to bind a Ca^{2+} ion (Atoda *et al*., 1995). It was reported recently (Sekiya *et al*., 1995) that Mg^{2+} has a special binding site on factor IX, and the Ca^{2+} concentration required for the binding of habu IX/X-bp to factor IX was affected by Mg^{2+} but not in the case of factor X. In the present study, however, the required Ca^{2+} concentration for the binding of ECLV IX/X-bp to both factor IX and factor X was prominently reduced by a millimolar concentration of Mg^{2+} (Figure 4), implying an enhancement of the Ca^{2+} affinity by Mg^{2+}. This is in agreement with the previous report that factor IX and factor X both have Mg^{2+} binding sites within their Gla domain (Astermark *et al*., 1991; Persson *et al*., 1991). The binding of Mg^{2+} to factor X possibly induced a further conformational change of the Ca^{2+} − loaded factor X. This change seems to facilitate the interaction of factor X with ECLV IX/X-bp but not that with habu IX/X-bp (Sekiya *et al*., 1995). The difference in the effect of Mg^{2+} on factor X binding by the two venom IX/X-bp may be due to some structural differences between the two venom proteins.

Sunnerhagen *et al*. (1995) compared the structures of the Ca^{2+}-free and the Ca^{2+}-loaded forms of a Gla domain from factor X by two-dimensional NMR and found that the buried hydrophobic residues of the Gla region (Phe[4], Leu[5], and Val[8]) became exposed and engaged in membrane binding upon the addition of Ca^{2+}. It was also demonstrated that acutus IX/X-bp in the presence of Ca^{2+} prevented bovine factor Xa from binding to phospholipid surfaces (Cox, 1993). While binding possibly to the Gla domain of both factor IX and X, ECLV IX/X-bp is more specific for factor IX (Figure 2A). The ratio of K_d values for the binding of factor IX to the binding of factor X is 1:19 for ECLV IX/X bp, 1:2 for jararaca IX/X-bp (Sekiya *et al*., 1993), and 1:3 for habu IX/X-bp (Atoda *et al*., 1994). Why the ECLV protein binds the Gla domain of factor IX much stronger than that of factor X remains to be studied in detail. Recently, a factor IX-bp from *T. flavoviridis* venom was found to have a β subunit identical to that of the IX/X-bp isolated from the same venom, and the major differences in structure of their α subunits are at region 57−68; this region probably is crucial for the specificity toward factor X (Atoda *et al*., 1995). The amino acid sequence at region 57−68 in ECLV IX/X-bp appears to be a combination of those in the IX-bp and IX/X-bp from the habu venom (Figure 7). On the other hand, The N-terminal Gla domains in most of the Gla-containing plasma proteins have rather conserved sequence, except that a reverse sequence of the hydrophobic residues is present in the Gla domain of factor IX (Katayama *et al*., 1979); it is likely that ECLV IX/X-bp and habu IX-bp are specific toward this reversed hydrophobic sequence.

In conclusion, we have first isolated and completed the primary structure of a coagulation factor IX/X-bp from a Viperid venom. It prolonged the coagulation time *in vivo* by inhibiting prothrombin activation reversibly and without toxic effects (Ouyang & Teng, 1973, 1978). It might be a useful anticoagulant agent for pharmaceutical use. Further studies on the binding site and three-dimensional structures of the venom anticoagulant proteins are necessary for a better understanding of their structure−function relationship.

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SUPPORTING INFORMATION AVAILABLE

Eleven figures showing the RP-HPLC patterns of subunit isolation and peptide mapping of ECLV IX/X-bp and one table showing the amino acid composition of ECLV IX/X-bp (16 pages). Ordering information is given on any current masthead page.

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