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Binding Site for Blood Coagulation Factor Xa Involving Residues 311–325 in Factor Va*

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Yumi Kojima, Mary J. Heeb, Andrew J. Gale, Tilman M. Hackeng, and John H. Griffin‡

From the Departments of Molecular and Experimental Medicine and of Vascular Biology, The Scripps Research Institute, La Jolla, California 92037

Factor Va inactivation by activated protein C is associated with cleavages at Arg^{306}, Arg^{506}, and Arg^{679} with Arg^{306} cleavage causing the major activity loss. To study functional roles of the Arg^{306} region, overlapping 15-mer peptides representing the sequence of factor Va residues 271–345 were synthesized and screened for anticoagulant activities. The peptide containing residues 311–325 (VP311) noncompetitively inhibited prothrombin activation by factor Xa, but only in the presence of factor Va. Fluorescence studies showed that VP311 bound to fluorescence-labeled 5-dimethylaminonaphthalene-1-sulfonyl-Glu-Gly-Arg factor Xa in solution with a K_d of 70 μM. Diisopropylphosphoryl factor Xa and factor Xa but not factor VII/VIIa or prothrombin bound to immobilized VP311 with relatively high affinity. These results support the hypothesis that residues 311–325, which are positioned between the A1 and A2 domains of factor Va and likely exposed to solvent, contribute to the binding of factor Xa by factor Va. Based on this hypothesis, it is suggested that cleavage by activated protein C at Arg^{306} in factor Va not only severs the covalent connection between the A1 and A2 domains but also disrupts the environment and structure of residues 311–325, thereby down-regulating the binding of factor Xa to factor Va.

Blood coagulation factor Va (FVa)[1] is the essential cofactor for the prothrombinase complex that consists of factor Xa (FXa), phospholipids, calcium ions, and FVa and that is responsible for conversion of prothrombin to thrombin (1–6). FVa generated by limited proteolysis of FV is usually composed of a heavy chain containing the A1-A2 domains in amino acid residues 1–709 and a light chain containing the A3-C1-C2 domains in residues 1546–2196. These two chains are noncovalently associated in the presence of divalent metal ions (3, 7). Protein C is a vitamin K-dependent plasma protein zymogen that is cleaved by thrombin to yield the active serine protease, activated protein C (APC). APC down-regulates blood coagulation by proteolytic inactivation of the cofactors factor Va and factor VIIIa (8, 9). Irreversible proteolytic inactivation of FVa by APC is reported to be associated with three cleavages at Arg^{306}, Arg^{506}, and Arg^{679} in the FVa heavy chain, whereas cleavage at only Arg^{306} in FV causes full loss of its activity (10). The importance of specific cleavages has been studied using purified Gln^{506}-FVa that lacks the Arg^{506} cleavage site (11–14). Inactivation of FVa by APC proceeds via a biphasic reaction that consists of a rapid and a slow phase. The rapid phase is associated with an initial cleavage at Arg^{506} and partial loss of activity (~30%), whereas extensive or complete inactivation of FVa requires cleavage at Arg^{306}. The contribution of cleavage at Arg^{679} to FVa inactivation is presently unclear. All published results suggest that cleavage at Arg^{306} plays the most important role for inactivation of FVa as well as for FV. Inactivation of FVa by APC is associated with loss of the ability of FVa to bind FXa and with dissociation of the A2 domain of FVa from the rest of the cleaved FVa (15, 16). To help clarify why cleavage at Arg^{306} inactivates FV and FVa, overlapping 15-mer peptides representing FVa heavy chain residues 271–345 were synthesized and screened for their ability to inhibit prothrombin activation using purified prothrombinase components. The results presented here suggest that the region between the A1 and A2 domains of FVa involving residues 311–325 of FVa provides a binding site for FXa and implies that APC cleavage at Arg^{306} down-regulates FVa activity, at least in part, by disrupting the immediate environment and/or structure of this FXa-binding site.

EXPERIMENTAL PROCEDURES

Peptide Synthesis—Peptides with amino-terminal α-amino groups and carboxyl-terminal carboxamide moieties were prepared under the supervision of Dr. Richard Houghten of the Torrey Pines Institute for Molecular Studies using the simultaneous multiple synthesis method (17) and were analyzed by reverse-phase high pressure liquid chromatography and mass spectral analysis to verify purity and composition (17, 18). Alternatively, some peptides were synthesized by and purchased from the Peptide Synthesis Group (Beckman Center, Stanford University, Palo Alto CA).

Proteins—Human FVa, prothrombin, and phospholipid vesicles (20% phosphatidylserine, 80% phosphatidylcholine) were prepared as described (18–20). Human FXa was purchased from Enzyme Research Labs (South Bend, IN). Diisopropylphosphoryl (DIP)-FXa (≥ 99% inactivated) was prepared by incubation of FXa at 1 mg/ml with 2 mM diisopropyl fluorophosphate (Sigma) on ice for 2 h followed by prolonged dialysis at 4 °C against Tris-buffered saline (0.05 M Tris-HCl, 0.1 M NaCl, 0.02% NaN_3, pH 7.4). Human 1,5-dansyl-Glu-Gly-Arg-factor Xa (DEGR-Xa) was purchased from Hematologic Technologies, Inc. (Essex Junction, VT). FVII/VIIa and rabbit anti-FVII were purchased from Celsus (Cincinnati OH), and monoclonal antibody against prothrombin was from Biodesign (Kennebunk, ME).

Prothrombinase Assay—Prothrombinase assays were performed at room temperature as described elsewhere (19) and employed 20 pM FVa, 1 nM FXa, 25 μM or 50 μM phospholipid vesicles, 5 mM $CaCl_2$, and 0.3 μM prothrombin unless otherwise indicated in buffer containing 0.05 M Hepes, 0.1 M NaCl, 5 mM $CaCl_2$, 0.1 mM $MnCl_2$, 0.02% NaN_3, and 0.5% bovine serum albumin. The rate of prothrombin activation was assessed using the chromogenic substrate H-D-cyclohexylglycyl-L-α-

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‡ To whom correspondence should be addressed: Dept. of Molecular and Experimental Medicine, SBR-5, Scripps Research Inst., 10550 N. Torrey Pines Rd., La Jolla, CA 92037. Tel.: 619-784-8220; Fax: 619-784-2243; E-mail: jgriffin@scripps.edu.

[1] The abbreviations used are: FVa, factor Va; FV, factor V; FXa, factor Xa; APC, activated protein C; DIP, diisopropylphosphoryl; dansyl, 5-dimethylaminonaphthalene-1-sulfonyl; DEGR-Xa, 1,5-dansyl-Glu-Gly-Arg-factor Xa.

TABLE I
Sequences of peptides representing factor V heavy chain amino acid sequences

Peptide	Residues	Sequence
VP271	271–285	TVGPEGKWIISSLTP
VP281	281–295	SSLTPKHLQAGMQAY
VP291	291–305	GMQAYIDIKNCPKKT
VP301	301–315	CPKKTR^{306}NLKKITREQ
VP311	311–325	ITREQRRHMKRWEYF
VP321	321–335	RWEYFIAAEEVIWDY
VP331	331–345	VIWDYAPVIPANMDK

aminobutyryl-L-arginine-*p*-nitroanilide (final concentration, 0.2 mM) (American Bioproducts, Parsippany, NJ) in an EL312 microplate reader using Kineti-calc software (Biotek, Winooski, VT). It should be noted that this amidolytic assay cannot distinguish formation of α-thrombin from meizo-thrombin.

Fluorescence Titrations—Fluorescence titrations were performed using an SLM Aminco Bowman Series 2 Luminescence Spectrometer (Spectronic Instruments, Inc., Rochester, NY) following the procedures of Krishnaswamy *et al.* (21) with some modifications. For these experiments the excitation wavelength was 340 nm (band pass, 4 nm) and the emission wavelength was 545 nm (band pass, 16 nm). A 408-nm-long pass filter (KV-408) was used in the emission path to minimize scattered light artifacts. All buffers were filtered with 0.2-μm filters, and protein solutions were centrifuged to remove particulate matter. The sample compartment was maintained at 25 °C with a circulating water bath. Microliter additions of a 1 mM stock solution of peptide or buffer alone were added to a square 5-mm path length cuvette containing 300 μl of reaction mixture of DEGR-Xa at 200 nM in 50 mM Hepes, pH 7.4, 0.15 M NaCl, 5 mM $CaCl_2$, and fluorescence intensity measurements were made 1 min after each addition. Three 5-s readings were made and averaged to determine the final value. Three titrations were done to allow for correction of fluorescence intensity values because of light scattering or any other artifacts. Titration A involved additions of peptide to DEGR-Xa. Titration B involved additions of control buffer to DEGR-Xa. Titration C involved additions of peptide to buffer alone. The corrected fluorescence change was then calculated according to the expression

$$\frac{F}{F_o} = \frac{F_A - F_C}{F_B - F'_C} \quad \text{(Eq. 1)}$$

where F_A, F_B, and F_C are the fluorescent intensities from the above titration mixtures and F'_C is the intensity recorded for control buffer alone in the absence of added peptide. The net fluorescence intensity change (F/F_o) was converted to percent, and nonlinear least squares regression was used to fit the data to the single ligand binding equation

$$\Delta F = \frac{\Delta F_{max}[P]}{K_d + [P]} \quad \text{(Eq. 2)}$$

where [P] is the peptide concentration. The K_d and ΔF_{max} were derived from data fitted using this equation.

Plate Binding Assays—Binding assays were performed as described (19). Peptides at 20 μM were coated on the wells of Xenobind microtiter plates (Xenopore, Saddle Brook, NJ) according to manufacturer's instructions and then blocked with 3% hydrolyzed fish skin gelatin (Sigma) in Tris-buffered saline. After washing the plate with Tris-buffered saline, various concentrations of DIP-FXa or FXa in binding buffer consisting of 0.05 M Tris, 0.2 M NaCl, 5 mM $CaCl_2$, 0.1 mM $MnCl_2$, 0.02% NaN_3, and 0.5% porcine skin gelatin (Sigma) were incubated in plate wells for 1 h at room temperature. Following washings, bound DIP-Xa was detected using a monoclonal antibody to FX (purified IgG from Biodesign), which was quantitated with biotin-secondary antibody, streptavidin-alkaline phosphatase, and phosphatase substrate as described (19). Detection of bound factor VII/VIIa and prothrombin was similarly made using appropriate antibodies. Detection of bound FXa was made using a chromogenic substrate *N*-α-benzyloxycarbonyl-D-arginyl-L-glycyl-L-arginine-*p*-nitroanilide (Chromogenix, Franklin, OH). The absorbance values observed for duplicate noncoated wells lacking peptides served as nonspecific controls for binding and were subtracted from absorbance values for corresponding duplicate peptide-coated wells. Nonspecific binding ranged from 5 to 30% of total observed binding in various experiments.

RESULTS

To clarify potential functional roles of the region around the APC cleavage site at Arg^{306} in the FVa heavy chain, seven overlapping 15-mer synthetic peptides representing FVa sequences from residues 271–345 (Table I) were tested for their ability to inhibit prothrombinase assays in the presence and absence of FVa (Fig. 1). At 100 μM, peptide VP311 strongly inhibited prothrombinase activity in the presence of FVa, whereas peptide VP321 had a moderate inhibitory effect on prothrombinase activity. In the absence of FVa, VP311 did not inhibit prothrombin activation; however, it modestly and reproducibly enhanced prothrombinase activity by 50% (Fig. 1). To define prothrombinase inhibition by peptides, various concentrations of peptides were preincubated with FXa, FVa, or prothrombin, followed by addition of other prothrombinase components for activity assays (Fig. 2). VP311 inhibited prothrombinase activity only in the presence of FVa (Fig. 2*B*). In the absence of FVa, VP311 at 100–200 μM reproducibly modestly enhanced prothrombinase activity by approximately 50% (Figs. 2*B* and 3*B*). Peptide VP321 showed only moderate inhibition in the presence of FVa, whereas at 200 μM it also modestly enhanced prothrombinase activity in the absence of FVa (Fig. 2*C*). Peptide VP301, which contains Arg^{306} and peptide VP331, like VP271, VP281, and VP291 (Fig. 1 and data not shown), had no effect on prothrombinase activity under any preincubation conditions (Fig. 2, *A* and *D*).

A control peptide with the reverse sequence of amino acids of VP311, designated VP311reverse, was synthesized and tested in parallel with VP311 for inhibition of prothrombinase. Fig. 3*A* shows that under conditions where VP311 inhibited prothrombinase by up to 90%, peptide VP311reverse inhibited prothrombinase only slightly. In the absence of FVa (Fig. 3*B*) where VP311 at 100–200 μM stimulated prothrombinase activity by 80%, VP311reverse in contrast slightly inhibited prothrombinase activity just as it did in the presence of FVa. Moreover, the inhibition of prothrombinase by VP311 cannot be simply due to a net high positive charge effect or an effect due to adjacent basic residues because VP301, which also contains a high net positive charge and two sets of adjacent basic residues, did not inhibit prothrombinase activity (Fig. 1). These results suggest that residues 311–335 in the FVa heavy chain may contribute to FXa-FVa and/or prothrombin-FVa interactions.

A series of Lineweaver-Burk plots for prothrombinase activity at varying prothrombin concentrations is seen in Fig. 4 for various concentrations of VP311. Peptide VP311 inhibited prothrombinase activity with a pattern of noncompetitive inhibition, and the apparent K_i under these experimental conditions was 140 μM. This suggests that the effect of VP311 is not explained by competition for binding of the substrate, prothrombin, to FVa.

The specific binding of peptide VP311 to FXa was measured to test the hypothesis that the sequence of VP311 represents a FXa-binding site in FVa. Because we found that the addition of VP311 to DEGR-Xa quenched the dansyl fluorescence of the labeled protein, binding of VP311 to the protein in solution was monitored by fluorescence intensity changes of the dansyl group in DEGR-Xa (Fig. 5). The apparent K_d of peptide VP311 for DEGR-Xa was determined, based on the average value from

three experiments, to be 71 ± 9 μM with a ΔF_{max} of −39%. This agrees reasonably well with the concentration of peptide VP311 required for 50% inhibition of the prothrombinase assays, *i.e.* 40–140 μM (Figs. 2*B*, 3*A*, and 4). The VP311-dependent decrease in dansyl fluorescence of DEGR-FXa (Fig. 5) was specific because the control peptide VP311reverse at 0–100 μM did not cause a significant change (<4%) in dansyl fluorescence (data not shown). Moreover, peptide VP301 that has a high positive charge because of its Arg/Lys content and that contains Arg^{306}, which presents the peptide bond cleaved during inactivation of FVa by activated protein C, did not cause a significant change in the fluorescence of DEGR-Xa. These data support the hypothesis that FVa residues 311–335 provide a binding site for FXa.

An additional approach was used to assess the binding of VP311 to FXa in which FXa was bound to peptides immobilized on microtiter plates. In this type of solid phase binding assay

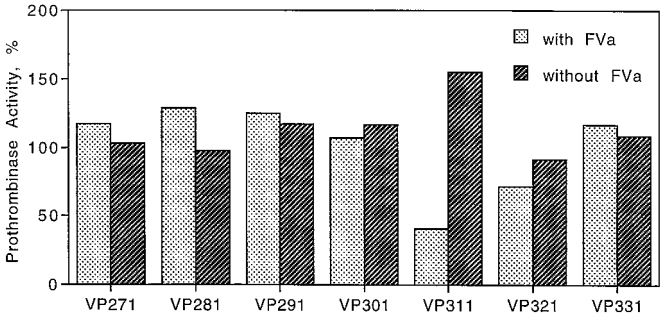

FIG. 1. **Inhibition of prothrombinase by synthetic overlapping 15-mer peptides representing residues 271–345 of factor V.** Peptides at a final concentration of 100 μM were preincubated with 1 nM (final concentration) FXa for 30 min at room temperature. Phospholipid vesicles, FVa, and prothrombin were added as described under "Experimental Procedures." Aliquots from the reaction mixture were removed every 30 s into amidolytic assay buffer containing 10 mM EDTA to stop the prothrombinase reaction. The rate of appearance of thrombin amidolytic activity was determined. *Stipled* and *striped bars* show prothrombinase activity in the presence or absence of FVa, respectively. The percentage of activity was calculated by defining the activity of control reaction mixtures in the absence of peptide as 100%. Peptide abbreviations and sequences are given in Table I.

that involves a small surface-bound peptide, apparent K_d values may be significantly lower than apparent K_d values determined in fluid phase binding assays, possibly because proteins have an abnormally low off-rate constant once bound near a surface that is multivalent because it is coated with ligand and possibly because the hydrophobic surface itself may contribute to protein binding. Furthermore, immobilized peptides have reduced degrees of freedom. Thus, apparent binding constants determined by solid phase assays cannot be considered to be real binding constants and cannot be compared with fluid phase real binding constants. Nevertheless, these types of assays can be useful to compare relative binding affinities for similar ligands. DIP-FXa and FXa bound tightly to immobilized VP311 (Fig. 6). Apparent K_d values calculated by Scatchard analysis using Enzfitter software averaged 10 nM (n = 4 experiments) for DIP-FXa and 46 nM (n = 2 experiments) for FXa. This demonstrated that both normal FXa and FXa with a modified active site bound to VP311. As controls, factor VII/VIIa showed no binding to VP311 and prothrombin showed only weak binding (apparent K_d > 400 nM) (Fig. 6). Moreover, FXa did not bind to the immobilized basic peptide VP301. These results further support the hypothesis that FVa residues 311–335 provide a FXa-binding site.

DISCUSSION

Synthetic peptides that inhibit multicomponent enzyme complexes can provide useful information about protein-protein interactions. To identify potential roles in the prothrombinase complex of FVa heavy chain residues near the APC cleavage site at Arg^{306}, seven 15-mer peptides representing FVa residues 271–345 were studied, and peptide VP311 (residues 311–325) was found to inhibit prothrombinase activity but only in the presence of FVa. The sequence of peptide VP311 represents a major part of the connecting region between the A1 (residues 1–301) and A2 (residues 320–656) domains of the heavy chain (residues 1–709) of FVa (6, 22). Inhibition of prothrombinase activity by VP311 only in the presence of FVa suggests that this connecting region of FVa containing residues 311–325 might contribute to FXa-FVa and/or prothrombin-FVa interactions. Alternatively or additionally, VP311 could inhibit

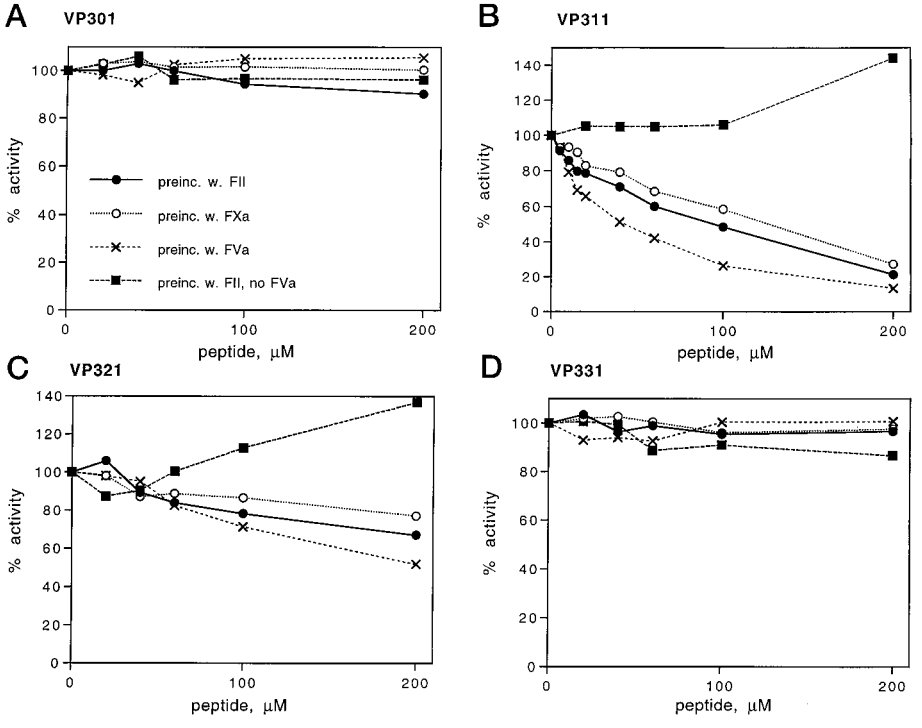

FIG. 2. **Inhibition of prothrombinase by synthetic 15-mer peptides representing residues 301–345 of factor V.** Peptides at various concentrations (0–200 μM) were preincubated with (*preinc. w.*) 0.3 μM prothrombin (*FII*) ( ), 1 nM FXa (○), 20 pM FVa (×), or 0.3 μM prothrombin without subsequent FVa addition (■) for 15 min at room temperature. Then the other prothrombinase components were added to initiate thrombin formation, except where the absence of FVa is indicated. The synthetic peptides (Table I) were VP301 (*A*), VP311 (*B*), VP321 (*C*), and VP331 (*D*). The percentage of prothrombinase activity (rate of thrombin formation) without peptide was defined as 100%. Each point is the average of duplicate determinations.

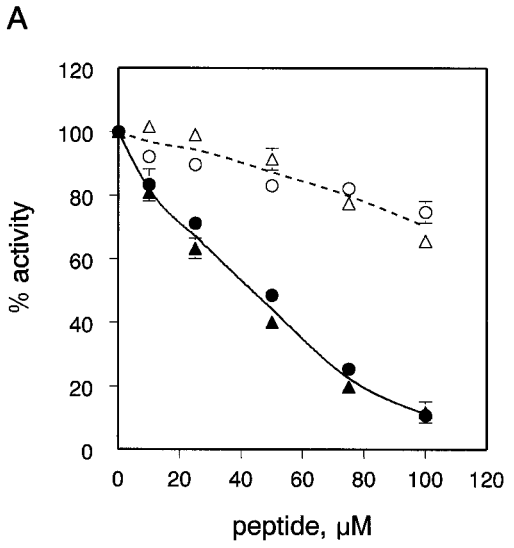

FIG. 3. **Effect of peptides VP311 and VP311reverse on prothrombinase in the presence and absence of factor Va.** Peptide VP311 (*closed symbols* and *solid line*) or a control peptide, VP311 reverse, that contained the reverse amino acid sequence (*open symbols* and *dashed line*) were preincubated at concentrations indicated for 30 s with FXa (*circles*) or with FVa (*triangles*) and phospholipids for 30 s prior to addition of other prothrombinase components to initiate thrombin formation. Prothrombinase assays were performed as described under "Experimental Procedures" except that the buffer contained 0.05 M NaCl. In *panel B*, FVa was omitted. *Symbols* represent the mean of two (*panel A*) or three (*panel B*) separate experiments.

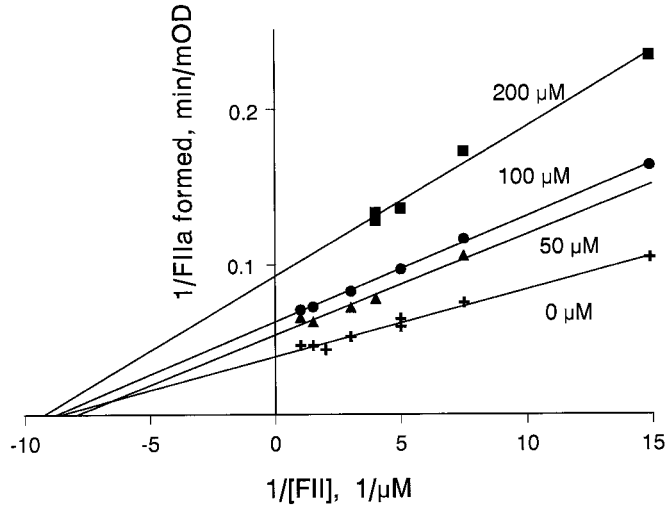

FIG. 4. **Lineweaver-Burk plots for prothrombin activation at varying concentrations of VP311.** FXa (final concentration, 1 nM) was preincubated with 0, 50, 100, or 200 μM of VP311 in duplicate for 15 min. FVa and phospholipids were then added, and various concentrations of prothrombin (0.07 to 0.67 μM) were added to initiate prothrombin activation that was quantitated as described under "Experimental Procedures."

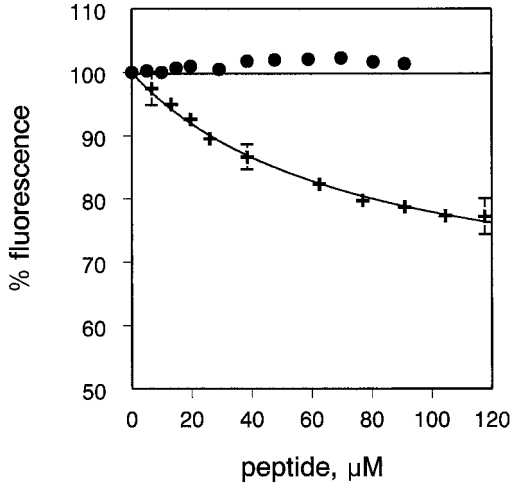

FIG. 5. **Binding of Peptide VP311 to DEGR-Xa.** Peptide VP311 (+) or VP301 ( ) was titrated into a 200 nM solution of DEGR-Xa in HBS containing 5 mM $CaCl_2$. The dansyl fluorescence intensity of DEGR-Xa was monitored as described under "Experimental Procedures." The percentage of fluorescence is relative to the original fluorescence intensity of DEGR-Xa without peptide additions defined as 100%. The line for VP311 was generated according to a single ligand binding equation using nonlinear regression, with K_d = 71 μM and ΔF_{max} = −39%.

prothrombinase activity by disrupting important FVa intramolecular interactions. Kinetic data showed prothrombinase inhibition by VP311 to be noncompetitive with respect to prothrombin, suggesting that VP311 is not simply competing for prothrombin binding to the prothrombinase complex.

Human FVa heavy chain has 84% overall homology with bovine FVa heavy chain, whereas peptide VP311 has 12 of 15 residues identical in human and bovine FV. Protein regions with a high percentage of homology between different species are often functionally important. Peptide VP311 also has a sequence motif that is present in peptides representing sequences in APC and human group II secretory phospholipase A_2 that have been implicated in prothrombinase inhibition. This motif (KR*XX*KR) is present in the inhibitory peptide 142–155, including residues 146–151 of APC (KRMEKK), which was shown to inhibit FXa coagulant activity in the presence of FVa (23). A similar motif is present in phospholipase A_2 from residues 52–57 (KRLEKR). A peptide from residues 51–74 of phospholipase A_2 was found to bind specifically to FXa (24). This motif in peptide VP311 involving residues 315–320 (RRHMKR) may be responsible for binding to a specific FVa-binding exosite on FXa. Based on these three peptides, each of which inhibit prothrombinase only in the presence of FVa, the putative FXa-binding motif is (K/R)R*XY*K(R/K) where there may be a preference for E at residue *Y* and for a bulky hydrophobic or neutral side chain at residue *X*. The residues preceding the basic hexapeptide motif in the proteins include Trp, Tyr, and Gln and may indicate a requirement for a large side chain capable of H-bonding.

To test the hypothesis that VP311 disrupts FXa-FVa interactions by binding to FXa, both solution phase and solid phase

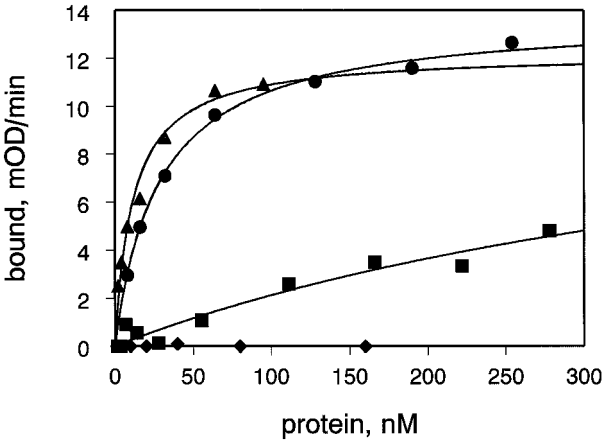

FIG. 6. **Binding of DIP-FXa and FXa to immobilized peptide VP311.** Various concentrations of DIP-FXa (▲), FXa ( ), factor VII/VIIa (♦), or prothrombin (■) were incubated in microtiter plate wells that had been coated with peptide VP311, and then bound proteins were detected as described under "Experimental Procedures."

binding studies were performed. In solution, peptide VP311 bound to DEGR-Xa with a K_d of 71 μM, whereas peptides VP311reverse and VP301 did not significantly bind to DEGR-Xa. The K_d of 71 μM based on fluorescence titrations is similar to the VP311 concentration 40–140 μM required for 50% inhibition of prothrombinase activity. The interactions of bovine factor Va and bovine DEGR-Xa have been studied (25, 26). Upon binding to DEGR-Xa factor Va causes an increase in the fluorescence intensity of the dansyl reporter group in DEGR-Xa. In the presence of phospholipid vesicles the calculated K_d of bovine factor Va for DEGR-Xa was 1 nM. Unlike these results peptide VP311 caused a quenching of dansyl fluorescence intensity rather than an increase. It is not entirely clear why the direction of the effect would be opposite of that for factor Va. However, because fluorescence intensity is dependent on a variety of factors, including protein conformation and solvent exposure, it should not be unexpected that two molecules of such drastically different size might have different effects on the fluorescent intensity of the dansyl group.

Although protein binding studies using immobilized peptides do not yield real equilibrium binding constant values and cannot be compared with fluid phase binding constants, such studies can provide useful qualitative descriptions of binding and may allow comparisons of relative affinities for different ligands or peptides. Binding assays using immobilized peptides showed that DIP-FXa and FXa do bind to VP311 with relatively high affinity, whereas two homologous vitamin K-dependent proteins, factor VII and prothrombin, do not bind to immobilized VP311 with comparable measurable affinity. Thus, the fluid phase and the solid phase binding studies combined with the prothrombinase inhibition data support the hypothesis that FVa residues 311–325 contain a binding site for FXa.

In the absence of FVa, peptide VP311 at 200 μM reproducibly mildly enhanced rather than inhibited FXa activity, possibly mimicking in some way the cofactor effect of FVa on FXa. This effect in the absence of FVa is consistent with the concept that the sequence of VP311 binds to FXa. The control peptide, VP311reverse, did not stimulate FXa activity, showing specificity for the normal 311–325 sequence. In parallel to the ability of VP311 to stimulate FXa activity in the absence of FVa, it was recently reported that a peptide corresponding to FVIII residues 698–712 enhances FIXa activity in the absence of FVIIIa, whereas the same FVIII peptide inhibits FIXa activity in the presence of FVIIIa (27). Thus, each respective peptide may represent a protease-binding site on the respective cofactors, and binding of each peptide may induce a conformational change in its respective coagulation protease, producing a mild enhancement of the protease activity that is much less effective than that of the intact cofactor.

FV and FVIII possess a common domain structure, A1-A2-B-A3-C1-C2 (6, 22, 28). There is approximately 40% amino acid sequence identity between FV and FVIII in the amino-terminal heavy chain regions (A1-A2), and the three A domains of FV and FVIII show a minimum of 30% identity with any other A domain (28). In addition, schematic models of the structures of FVa and FVIIIa based on electron micrographs show certain similarities (29–31). The three A domains of FV and FVIII resemble the three A domains of human ceruloplasmin whose three-dimensional structure was solved using x-ray crystallography (32). Ceruloplasmin is a six-domain structure comprising a heterotrimer of heterodimers, each dimer containing two β-barrel structures homologous to plastocyanin (32, 33). A homology model of the three A domains of FVIII based on this ceruloplasmin structure has recently been published (34), and another FVIII homology model based on nitrite reductase has appeared (35). The FVIII homology models propose that the A1-A2-A3 domains of FVIIIa form a trimer of heterodimers, with each domain containing two similar but distinct β-barrel plastocyanin-like structures (34). Based on the homologies of FV, FVIII, and ceruloplasmin, some reasonable though speculative insights about FVa structure may be drawn from inspection of the FVIII homology model and the ceruloplasmin x-ray crystallographic structure. The APC cleavage site at Arg^{306} in the FVa heavy chain is in the solvent-exposed sequence (residues 302–319) connecting the A1 and A2 domains, and VP311 contains much of this sequence that is easily accessible to FXa and/or APC. Binding of FXa to this connecting region could block the accessibility of Arg^{306} to APC, thereby causing the known protective effect of FXa against FVa cleavage by APC (36–40). Furthermore, the APC cleavage site at Arg^{506} in FVa is situated between the two plastocyanin-like β-barrels of the A2 domain and is exposed to solvent, homologous to Arg^{562} in FVIIIa (35).

In the prothrombinase complex, FVa and FXa interact stoichiometrically and FVa has an extended binding site for FXa with contributions from both the heavy and light chains (2, 41–43). Included in this extended binding interaction are residues 311–325, as shown here, and residues 493–506, which were previously shown to interact with FXa (44, 45). In the human ceruloplasmin x-ray crystallographic structure the sequences homologous to residues 493–506 and 311–325 of FVa are adjacent on the protein surface and are generally within 9–20 Å of one another (32). Inspection of the FVIIIa homology model structure of Pemberton *et al.* (34) indicates that the peptides homologous to these two sequences of FVa are directly adjacent to one another on the surface of the "bottom" of the protein. The distance in the FVIIIa model between the α-carbons of FVIII residues 562 and 385 (corresponding to FV residues 506 and 325) is 15.1 Å, and the α-carbons of FVIII residues 561 and 382 (corresponding to FV residues 505 and 322) are 9.2 Å apart. Because cleavage at Arg^{306} in FV or FVa causes loss of most or all FVa activity, whereas cleavage at only Arg^{506} ablates approximately 30% of FVa activity (10, 14), the structural integrity of the region around Arg^{306} is apparently more important than that of Arg^{506} for the structure and function of FVa. The Arg^{306} cleavage may be lethal because of loss of the FXa-binding site, destabilization of the trimeric A1-A2-A3 structure of FVa because of loss of the covalent link between the A1 and A2 domains potentially with dissociation of the A2 domain (16), or an overlapping combination of these effects.

In conclusion, our data suggest that residues 311–325 in FVa

provide a FXa-binding site that may be essential for prothrombinase activity. Cleavage of FVa at Arg^{306} by APC severs the covalent linkage between the A1 and A2 domains and likely alters FVa tertiary structure, especially of the FXa-binding site involving residues 311–325, such that FXa binding is ablated or greatly diminished and FVa is irreversibly inactivated.

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