Thrombin activated factor Va (factor V$_{\text{IIa}}$, residues 1–709 and 1546–2196) has an apparent dissociation constant ($K_{\text{d,app}}$) for factor Xa within prothrombinase of $\sim0.5$ nM. A protease (NN) purified from the venom of the snake Naja nigricollis nigricollis, cleaves human factor V at Asp$^597$, Asp$^696$, and Asp$^{1545}$. To produce a molecule (factor V$_{\text{NN}}$) that is comprised of a $M_r$ 100,000 heavy chain (amino acid residues 1–689) and a $M_r$ 80,000 light chain (amino acid residues 1509/1514–2196), factor V$_{\text{NN}}$ has a $K_{\text{d,app}}$ for factor Xa of 4 nM and reduced clotting activity. Cleavage of factor V$_{\text{IIa}}$ by NN at Asp$^{97}$ results in a cofactor that loses $\sim60$–$80\%$ of its clotting activity. An enzyme from Russell’s viper venom (RVV) cleaves human factor V at Arg$^{1018}$ and Arg$^{1545}$ to produce a $M_r$ 150,000 heavy chain and $M_r$ 74,000 light chain (factor V$_{\text{RVV}}$, residues 1–1018 and 1546–2196). The RVV species has affinity for factor Xa and clotting activity similar to the thrombin-activated factor Va. Cleavage of factor V$_{\text{NN}}$ at Arg$^{1018}$ by a-thrombin (factor V$_{\text{NNth}}$) or RVV (factor V$_{\text{NNRV}}$) leads to enhanced affinity of the cofactor (residues 1–709, factor Xa ($K_{\text{d,app}}$ $\sim0.5$ nM). A synthetic peptide containing the last 13 residues from the heavy chain of factor Va (amino acid sequence 697–709, D13R) was found to be a competitive inhibitor of prothrombinase with respect to prothrombin. The peptide was also found to specifically interact with thrombin-agarose. These data demonstrate that 1) cleavage at Arg$^{1545}$ and formation of the light chain of factor V$_{\text{IIa}}$ is essential for high affinity binding and function of factor Xa within prothrombinase and 2) a binding site for prothrombin is contributed by amino acid residues 697–709 of the heavy chain of the cofactor.

The prothrombinase complex responsible for the generation of a-thrombin in the hemostatic process is composed of factor Va and factor Xa associated on a phospholipid membrane in the presence of Ca$^{2+}$ (1, 2). Although factor Xa alone can convert prothrombin to a-thrombin, the prothrombinase complex has a catalytic efficiency five orders of magnitude greater than factor Xa acting alone (3). Plasma factor V circulates as a large single chain protein of $M_r$ 330,000 (4–6). The cDNA sequences for human, murine, porcine, and bovine factor V have been reported previously (7–11). The factor V molecule is composed of triplicated “A” domains, duplicated “C” domains, and a “B” region. Human factor V is cleaved by a-thrombin at Arg$^{1018}$, Arg$^{1545}$, and Arg$^{696}$ and generates the active cofactor factor Va, which is composed of a heavy chain (A1–A2 domains, $M_r$ 105,000, amino acid residues 1–709) non-covalently associated with the light chain (A3–C1–C2 domains, $M_r$ 74,000, amino acid residues 1546–2196). The interaction between the two chains is promoted by divalent cations (12, 13).

Activation of factor V by a-thrombin is required for the interaction of the cofactor with factor Xa and prothrombin. Factor Va and factor Xa interact stoichiometrically in the absence of phospholipids with a $K_f$ of $0.8 \mu$m, and the interaction is dependent upon the presence of Ca$^{2+}$ (14). The $K_f$ for the factor Va-prothrombin interaction is $1 \mu$m and appears to be independent of the presence of Ca$^{2+}$ (15). In the presence of a membrane surface and Ca$^{2+}$, the $K_f$ of the factor Va-factor Xa association is $\sim1$ nM (3, 16). Both chains of the cofactor are required for the interaction with factor Xa (17–20). The factor Va-prothrombin interaction is promoted by the heavy chain of the molecule (15, 18). The factor Va-membrane interaction, governed by a $K_f$ value of $\sim3$ nM, occurs at diffusionaly limited rates, involves both hydrophobic and Ca$^{2+}$-dependent electrostatic interactions, and results in penetration of a portion of the light chain into the membrane bilayer (21–25). Two sites on the light chain of the cofactor appear to be responsible for the interactions of factor Va with the membrane surface. The factor Va-membrane complex is a “true receptor” for factor Xa on the platelet surface (26, 27).

Although proteolytic cleavage of factor Va by APC$^1$ at Arg$^{1018}$/Arg$^{696}$ results in a 10-fold decrease in the affinity of the molecule for factor Xa, the same cleavages result in the elimination of its interaction with prothrombin (15, 18, 28, 29). It has been shown that prothrombin and thrombin have two separate electrostatic exosites (anion binding exosite I, ABE-I, and anion binding exosite II, ABE-II) that are responsible for the majority

$^1$ The abbreviations used are: APC, activated-protein C; NN, protease from the venom of N. nigricollis nigricollis; PS, 1–α-phosphatidyleholine; PC, 1–ω-phosphatidylcholine; PCPS, small unilamellar phospholipid vesicles composed of 75% PC and 25% PS (w/w); DAPA, dansylarginine-N’-3-ethyl-1,5-pentanediylamide; EGR-hXa glutamylglycinylarginyl chloromethyl ketone active-site blocked human factor Xa; [OG$_{4\text{ac}}$]-EGR-hXa, human factor Xa labeled in the active site with Oregon Green 488; PVDVF, polyvinylidene difluoride; LC/MS, liquid chromatography/mass Spectrometry, D13R, synthetic peptide containing the last 13 amino acids from the factor Va heavy chain; factor V$_{\text{NNth}}$, factor V cleaved by the protease from N. nigricollis nigricollis; factor V$_{\text{NNRV}}$, factor V cleaved by a-thrombin; factor V$_{\text{NNANC}}$, factor V$_{\text{NNRV}}$, factor V cleaved by the RVV-V activator; factor V$_{\text{NNth}}$, factor V cleaved by the protease from N. naja nigricollis then cleaved by thrombin; factor V$_{\text{NNANC}}$, factor V cleaved by the protease from N. nigricollis nigricollis then treated with RVV-V activator; ABE-I, anion binding exosite I; ABE-II, anion binding exosite II; RVV-V, Russell’s viper venom factor V activator; HPLC, high-performance liquid chromatography.
of the functions of the molecules (30–39). Whereas ABE-I has been involved in the binding to thrombomodulin (40), fibrinogen (41), PAR1 (42), the COOH-terminal hirudin peptides (43), and heparin cofactor II (44) among others, ABE-II was found to be involved in the interaction with protease nexin (45) and antithrombin III (44). Data from separate laboratories have demonstrated that both exosites bind factors V and VIII (33, 34, 35). Interestingly, proexosite I of prothrombin, which is present at a low affinity state on the molecule, and its affinity for ligators decreases by ~100-fold following activation and formation of thrombin (33, 39), was found to be directly involved in the productive interaction with factor Va within prothrombinase (32, 33).

Thrombin, factor Xa, and the RVV-factor V activator can produce factor Va by discrete proteolytic cleavages (for details see reviews (1, 2)). It has also been reported that a protease from the venom of Naja naja oxiana produces a factor Va-like molecule lacking a major portion of the carboxyl terminal domain of the heavy chain, with diminished cofactor activity (46). The reasons for this diminished cofactor activity, which is mostly observed at low factor Xa concentrations, have yet to be elucidated. The present study was undertaken to understand the role of the last 13 amino acids from the COOH-terminal portion of the heavy chain of the cofactor. Thus, our present study adds to the knowledge regarding the structure/function relationships required for an active factor Va molecule.

**EXPERIMENTAL PROCEDURES**

**Materials and Reagents—**Hepes, Sepharose CL-4B, 1-palmitoyl-2-oleoylphosphatidylserine (PS) from bovine brain, and 1-palmitoyl-2-oleoylphosphatidylcholine (PC) from hen egg were purchased from Sigma (St. Louis, MO). The thromboplastin reagent used in these experiments was purchased from Organon Teknika Corp. (Durham, NC). Dansylarginine-N-(3-ethyl-1,5-pentanediyl)amide (DAPA), glutamylglycinylarginyl chloromethyl ketone (EGR), prothrombin-Sepharose, thrombin coupled to agarose through the active site as described (30), glycine-Ala-thrombin (1 unit/ml, 37 °C) was incubated at 37 °C for 10 min. In all cases, 20 nM hirudin was added following incubation at 37 °C when the fixed concentration of factor V was known.

**Cleavage of Factor V by NN Protease and Inactivation by APC—**

\[ \text{[Va]} \text{F} \text{ [Xa]} \text{F} \text{ [Xa]} \text{V} \] (Eq. 1)

Determination of the Apparent Binding Constants for Factor Xa—

To determine the apparent dissociation constant \( K_{\text{app}} \) of factor Xa for factor Va in the presence of factor V, a titration with factor Xa was performed using concentrations ranging from 100 pM to 10 nM. The \( K_{\text{app}} \) for the factor Va-factor Xa interaction is given by Equation 1,

\[ K_{\text{app}} = \frac{[\text{Va}][\text{Xa}]}{[\text{VaXa}]} \] (Eq. 1)

where \([\text{Va}]\) and \([\text{Xa}]\) are the concentrations of free factor Va and factor Xa, respectively, \([\text{VaXa}]\) is the concentration of factor Va–factor Xa complex, and \( K_{\text{app}} \) is the apparent dissociation constant.

**Generation of Various Factor V Species—**The various factor V species were generated by incubation of single chain human plasma factor V with the purified enzymes. Phospholipid vesicles slightly increase the rate of cleavage of factor V by NN, whereas PCPS vesicles diminish the rate of activation of factor V by α-thrombin (58). Thus, the experiments studying the effect of α-thrombin on factor V was conducted in the presence of PCPS vesicles. The nomenclature for the proteolyzed factor V products is as follows: factor VNN, human factor V incubated with α-thrombin for 5 min; factor VXN, human factor V (220 nM) incubated with human factor V in 20 nM NaCl for 37 °C for 3 h; factor VX; factor VXN incubated with α-thrombin (1 unit/ml) at 37 °C for 10 min. In all cases, 20 nM hirudin was added following proteolytic treatment. To generate factor V, human factor V was incubated at 37 °C with 4.2 nM RVV-V activator for 2 h. In some experiments following full activation to factor V, 2.1 nM APC and 50 μM PCPS vesicles were added and the reaction was allowed to proceed for 1 h. Aliquots were taken at the time points indicated in the figure legends and analyzed by SDS-PAGE.

**Measurements of Rates of Thrombin Formation using DAPA—**

The initial rate of thrombin formation for each factor Xa concentration was determined using a procedure based on the measurement of the change in the fluorescence intensity of DAPA present in the reaction mixture. The use of DAPA in the reaction mixture prevents feedback reactions catalyzed by the newly formed thrombin.

**Determination of the Apparent Binding Constants for Factor Xa—**

To determine the apparent dissociation constant \( K_{\text{app}} \) of factor Xa for factor Va in the presence of factor V, a titration with factor Xa was performed using concentrations ranging from 100 pM to 10 nM. The \( K_{\text{app}} \) for the factor Va-factor Xa interaction is given by Equation 1,

\[ K_{\text{app}} = \frac{[\text{Va}][\text{Xa}]}{[\text{VaXa}]} \] (Eq. 1)

where \([\text{Va}]\) and \([\text{Xa}]\) are the concentrations of free factor Va and factor Xa, respectively, \([\text{VaXa}]\) is the concentration of factor Va–factor Xa complex, and \( K_{\text{app}} \) is the apparent dissociation constant.

**Assuming that m = moles of factor Xa bound/mole of factor Va at saturation; throughout this study n = 1; the stoichiometry of the factor Va-factor Xa interaction was fixed at 1.**

\[ V_{\text{cis}} = V_{1} + (\text{VX}_{\text{bound}} \cdot V_{1}) \]

where \( V_{\text{cis}} \) is the rate at \( \text{Xa} = 0 \) and \( V_{1} \) is the rate at infinite factor Xa concentrations (i.e. when the fixed concentration of factor Va is saturated with factor Xa). Prior to each experiment, the maximum amount of complex formed under the above conditions was verified by replacing \([\text{Va}]\) and \([\text{Xa}]\) in Equation 1 by,
FIG. 1. Cleavage of factor V by the enzyme contained in the snake venom of *N. nigrigollis* nigrigollis. Factor V was incubated with the NN protease as described under “Experimental Procedures.” At selected time intervals aliquots of the mixture were withdrawn and assayed for cofactor activity in a clotting assay using factor V-deficient plasma (the activity of each sample is shown at the bottom of the figure in units/mg). Fragments were visualized following Coomassie Blue staining on a 4–12% (linear gradient) SDS-PAGE.

Lane 1, factor V; lane 11, factor V; lane 12, factor Va control; lanes 2–11, factor V at 1, 3, 5, 10, 20, 30, 60, 90, 120, and 180 min following the addition of the enzyme; lane 13, factor Va control activated with a-thrombin. Molecular weight markers are shown in lane 13.

\[
[\text{Val}_1] = [\text{Val}_2] - [\text{Va}x]_T \quad \text{(Eq. 3)}
\]

\[
[\text{Xa}] = [\text{Xa}]_T - [\text{Va}x] \quad \text{(Eq. 4)}
\]

where \([\text{Val}_1]\) and \([\text{Xa}]_T\) are the total concentrations of factor Va and factor Xa.

**Inhibition of Thrombin Formation**—The formation of thrombin was also analyzed using the fluorescent thrombin inhibitor DAPA as described (48) using a PerkinElmer Life Sciences LS-50B luminescence spectrometer (PerkinElmer Life Sciences LLC, Norwalk, CT) with a C18 column from the salt components using an HPLC system (HP 1100, HPLC). The eluate of the column was ascertained by the optical density. Position in the elution of the factor Va was incubated with the peptide (at concentrations ranging from 0.5 mM to 100 mM) for 10 min and added to the assay. The initial rate of thrombin formation (nM per min) was calculated as described (48). The concentration of peptide given in each figure is the final concentration of the peptide in the assay mixture. All data were initially analyzed and stored using the software FL WinLab (PerkinElmer Life Sciences) and further analyzed and plotted with the software Prizm (GraphPad, San Diego, CA).

**Fluorescence Anisotropy Measurements**—Fluorescence anisotropy of [OGlu]EGR-Hxa was measured using a PerkinElmer Life Sciences LS-50B luminescence spectrometer in L-format as recently described (48). Anisotropy measurements were performed in a quartz cuvette under constant stirring (low) with \(\lambda_{ex} = 490 \text{ nm}, \lambda_{em} = 520 \text{ nm} \) with a long pass filter (Schott KV-520) in the emission beam. At each addition, anisotropy was measured for 20 s and eight successive readings were averaged. In all cases, the total addition of peptide did not exceed 10% of the volume of the reaction. All data were initially analyzed and stored using the software FL WinLab (PerkinElmer Life Sciences) and further analyzed and plotted with the software Prizm (GraphPad). Some of the data were also plotted using DeltaGraph (DeltaPoint, Monterey, CA).

**Direct Binding of the Peptides to Thrombin**—Thrombin immobilized to agarose through the active site was used (30). Peptide solutions of D13R and P15H (48) were dissolved in water to a given concentration. In control experiments it was determined that the maximum amount of peptide retained by the thrombin-agarose column was approximately 400 µg. The peptides were then diluted in 20 mM Heps, 0.1 mM NaCl, pH 7.4, in a manner so that ~400 µg was contained in each of the starting solutions. The two peptides used in this study contained aromatic amino acid residues; thus, their presence in the flowthrough or the eluate of the column was ascertained by the optical density. Position identification of bound material was verified by LC/MS as detailed below in the analytical facility of Dr. David Anderson at Cleveland State University.

**Mass Spectrometry Instruments and Conditions**—Eluate was separated from the salt components using an HPLC system (HP 1100, HPLC gradient system, Agilent Technologies, Palo Alto, CA) with a C18 column (1 mm × 15 cm, GraceVydac, Hesperia, CA) and buffers A (0.3% acetic acid in water) and B (0.3% acetic acid in acetonitrile), and the eluted material was monitored using a Micromass Quattro II ESI-Triple quadrupole mass spectrometer (Waters, Milford, MA). The data were collected using a Compaq Professional Workstation (AP200, Hewlett-Packard, Palo Alto, CA) and analyzed by MassLynx version 3.3 (Waters).

**Gel Electrophoresis—SDS-PAGE analyses** were performed using 5–15% and 4–12% gradient gels according to the method of Laemmli (62). Proteins were visualized after staining with Coomassie Brilliant Blue in 50% methanol, 10% acetic acid followed by destaining by diffusion using a solution of 50% methanol and 10% acetic acid.

**Amino Acid Sequence Analyses**—Human factor V (630 nM) was incubated with PCPS vesicles (200 µM) and the purified NN protease for 3 h at 37°C. The reaction was stopped by the addition of 2% SDS, 2% β-mercaptoethanol, and the mixture was heated for 5 min at 90°C. Approximately 40 µg of total factor V digest was analyzed on a 8–18% linear gradient SDS-PAGE gel. Similar experiments were performed with the RVV-cleaved cofactor. Following electrophoretic separation, proteins were transferred to a PVDF membrane, and the NH2-terminal sequences were determined using automatic Edman degradation on an Applied Biosystems 475A protein sequencing system as described (60) in the laboratory of Dr. Alex Kurosky (University of Texas, Medical Branch at Galveston).

**RESULTS**

**Limited Proteolysis of Factor V by N. nigrigollis nigrigollis (NN) Protease**—Factor V prior to the activation by α-thrombin typically displays 13- to 15-fold lower clotting activity than the active cofactor factor Va obtained following activation by α-thrombin (50). The apparent activity of single chain factor V is a consequence of a limited amount of cleavage, which produces the active cofactor during the initial phase of the assay (63, 64). In experiments using purified factor Xa, prothrombin, and phospholipid in the presence of the fluorescent thrombin inhibitor DAPA, which prevents feedback activation of single chain factor V, measurements of factor V activity give results that are 400-fold lower than for thrombin-activated factor V (3). Thus, the single-stage clotting assay overestimates factor V cofactor activity, because the clotting end-point occurs when ~1% of the total circulating procofactor is activated and relies upon the adventitious activation of the procofactor by the initial thrombin produced during the assay. For isolated human factor V the single stage assay yields a value of 54 units/mg (Fig. 1, lane 1), which increases 15-fold to 740 units/mg upon activation with α-thrombin (factor Vx, Fig. 1, lane 12). Treatment of factor V with the NN protease results in the progressive development of a species (factor VNN, Fig. 1, lanes 2–11) with a specific activity lower than factor V (21 units/mg, Fig. 1, lane 11). NN cleaved factor V (factor VNN, lane 11) exhibits a
Role of Factor V Heavy and Light Chains

The reduced activity of factor V NN in the clotting assay (121 ± 21 nM, with parallel analysis by SDS-PAGE. After 10-min incubation with NN protease (2.1 nM), clotting assay, the cofactor activity of factor VIIa (210 nM, 750 units/mg) was followed during treatment with NN protease (2.1 nM), with similar clotting activity to factor VIIa (12, 66, 67). We have reported that APC-inactivated membrane-bound factor V (cleaved at Arg306, Arg506, Arg679, and Lys994) is a species with only a light chain, equivalent (by electrophoretic migration) and with similar clotting activity to factor VIIa (12, 66, 67). According to the amino acid sequence derived from the cDNA of the human cofactor (8) these sequences are identified to represent cleavage by NN at Asp1509 and Asp1514. The arrowhead at the right of the sequence shown for fragment d depicts Arg709, which is a specific cleavage site by α-thrombin for activation of human factor V (8).

FIG. 2. Identification of the proteolytic cleavages of the factor V molecule by the enzyme contained in the snake venom of N. nigricollis nigricollis. Human factor V was incubated with PCPS vesicles and the purified NN protease as described under "Experimental Procedures." After electrophoresis, the proteins were transferred to a PVDF membrane. A, one of the PVDF membranes that was used for the NH2-terminal sequence analysis stained with Coomassie Blue and represents the fragments derived from human factor V following cleavage by NN. The letters a–d depict fragments of human factor V. The arrowhead underneath fragment d denotes a fragment that is derived from the B region of factor V and does not stain with Coomassie Blue. The NH2-terminal sequence of this fragment could not be determined positively. B, NH2-terminal sequence of the fragments a–d shown in A. The numbers in parentheses indicate picomoles of amino acid at the given cycle. Fragment b was found to contain two amino acid sequences. According to the amino acid sequence derived from the cDNA of the human cofactor (8) these sequences are identified to represent cleavage by NN at Asp1509 and Asp1514. The arrowhead at the right of the sequence shown for fragment d depicts Arg709, which is a specific cleavage site by α-thrombin for activation of human factor V (8).
possesses cofactor activity in the plasma clotting assay similar to the α-thrombin-activated cofactor, factor Vα (approaching 700 units/mg, Fig. 4A). The activation of factor V by the RVV-V activator is a slower process than α-thrombin activation at similar catalyst and substrate concentrations. In addition to the light chain (Mₙ 74,000), factor V₁₅₀ displays a Mₙ 150,000 heavy chain (Fig. 4B). NH₂-terminal amino acid sequence analyses of the fragments obtained following cleavage of factor V by the RVV-V activator (not shown) confirm our previous findings with the APC-treated factor V (65) consistent with the conclusion that RVV-V activator cleaves the procofactor at Arg₁⁰¹⁸ and Arg₁⁵⁴⁵.

The subunit compositions for each factor V molecule and their clotting activities are illustrated in Fig. 5, and their characteristics are reported in Table I. Lane 1 shows thrombin-activated factor V, followed by factor V₁₅₀, which possesses a normal light chain and a heavy chain that is longer than that of factor V₁₅₀. Treatment of factor V with NN produces factor V₁₅₀ (Fig. 5) with a shorter heavy chain and longer light chain than factor V₁₅₀. Factor V₁₅₀ possesses minimum clotting activity. Factor V₁₅₀ can be further cleaved by α-thrombin at Arg₁⁰¹⁸ to reduce the light chain to normal size (factor V₁₅₀). Coincidentally, the clotting activity of the molecule increases to ~50% of that of factor V₁₅₀. Cleavage of factor V₁₅₀ with α-thrombin produces a species indistinguishable from factor V₁₅₀ (factor V₁₅₀), whereas factor V₁₅₀ appears with a normal light chain and truncated heavy chain (identical to factor V₁₅₀).

Functional Activity of the Factor V Species Derived from NN and Thrombin—Cleavage of factor V by NN produces a molecule exhibiting impaired cofactor activity either because of impaired factor Xa receptor capability, diminished catalytic effector properties, or both. To compare the receptor capacities of the factor V₁₅₀, factor V₁₅₀, factor V₁₅₀, factor V₁₅₀, and factor V₁₅₀, thrombin generation was measured in the DAPA assay as a function of added factor Xa (Fig. 6). The use of DAPA in the reaction also inhibited feedback cleavage by thrombin of the factor V₁₅₀ and factor V₁₅₀ species. The curves in Fig. 6 represent best fits to Equation 2 (see “Experimental Procedures”), and each yields a K_d,app describing on a functional basis the affinity between factor Xa and the factor V species (16, 61). These dissociation constants and corresponding activities in the clotting assay are listed in Table I. Fits were obtained from similar data compiled for the other factor V...
FIG. 5. Various factor Va species. Single chain, human factor V was subjected to proteolysis by thrombin, N. nigricollis nigricollis, and/or RVV snake venom as indicated under “Experimental Procedures.” Activity was determined by clotting assay and is expressed in units/mg at the bottom. Species are as follows: Lane 1, factor VHH (single chain human factor V was incubated with α-thrombin); lane 2, factor VHV; lane 3, factor VHV; lane 4, factor VHV, (factor VHV incubated with α-thrombin); lane 5, factor VHV, (factor VHV incubated with α-thrombin); lane 6, factor VHV, (factor VHV incubated with RVV-V activator).

TABLE I
Properties of the different forms of factor Va

| Factor Va species | Subunit composition, HC/LC × (10⁻⁷) | Clotting activity | Factor Xa binding, Kdapp | Velocity of saturating concentrations of factor Xa |
|------------------|--------------------------------------|------------------|--------------------------|---------------------------------------------|
|                  | % of control |                     |                           |                                            |
| V                | <5%                | 100                |                           |                                            |
| VHV              | 150/74            | 102                | 0.29 ± 0.085             | 804 ± 56                                    |
| VHV              | 100/80            | 7                  | 4.17 ± 0.67              | 838 ± 48                                    |
| VHV              | 150/74            | 102                | 0.41 ± 0.11              | 742 ± 38                                    |
| VHV              | 100/74            | 48                 | 0.5 ± 0.086              | 866 ± 39                                    |
| VHV              | 100/74            | 48                 | 0.57 ± 0.17              | 733 ± 48                                    |

* The subunit composition of each species is given in M, × 10⁻⁷: HC = heavy chain; LC = light chain.
* Activity was determined in a clotting assay using factor V-depleted plasma as described under “Experimental Procedures” (50). The numbers show the clotting activities of the factor Va species depicted in Fig. 5; the maximum activity (100% = 749 units/mg) was arbitrarily assigned to the α-thrombin-activated species (Fig. 5, lane 1).
* The Kd for each factor Va species was calculated using the quartic equation (Equation 2) described under “Experimental Procedures” with a stoichiometry fixed at 1 (n = 1).
* The velocity is given in nm Ila/min. The prothrombinase assuming that at saturating concentrations of factor Xa all factor Va (0.5 nm) will be saturated with factor Xa, and, thus, the maximum enzyme concentration of active enzyme is 0.5 nm prothrombinase.
* The species VHV, or VHV, and VHV, or VHV, have similar properties and subunit composition. Thus, the data relating to the properties and subunit composition of only one species of the pairs are reported.

species, and the results of these are also given in Table I. Compared with factor VHV (Fig. 6A, filled circles), factor VHV (Fig. 6A, open circles) exhibits both reduced factor Xa affinity and diminished clotting activity at limiting concentrations of factor Xa (Table I). The Kd(app) for factor VHV, with human factor Xa was ~4 nm (with a 1:1 stoichiometry). The effector efficiency of VHV was not affected at saturating concentrations of factor Xa. Upon treatment with α-thrombin or RVV, factor VHV improves its affinity for factor Xa and its ability to serve optimally as receptor for factor Xa within prothrombinase (Fig. 6A, open squares); however, only partial recovery of the clotting activity was observed (Table I). Similar results were observed when factor VHH was treated with NN (Kd(app) for factor VHVNN of ~0.5 nm). Because both factor VHV and factor VHV, possess a fully formed light chain, resulting from cleavage at Arg1545 (Fig. 6B, lanes 1 and 3), it appears that cleavage at Arg1545 is not required for full expression of factor Xa factor activity, although cleavage is necessary for full expression of factor Va factor activity at limiting amounts of factor Xa, as those available in the clotting assay. Thus, although cleavage at Arg1545 is not a prerequisite for expression of maximum factor Va factor activity, it is required for optimal factor Xa binding. Overall, the data also indicate that elimination of 13 amino acids from the COOH-terminal portion of the heavy chain of factor VHV, results in partial loss in cofactor activity with no diminution in the factor Xa binding capability.

Inhibition of Prothrombinase Activity by a Synthetic Peptide Containing the 13 Amino Acids from the COOH-terminal Portion of the Heavy Chain of Factor Va (D13R)—To ascertain the importance of the region 697–709 for cofactor activity, we obtained a 13-amino acid peptide encompassing this region (D13R). This peptide was tested for inhibition on factor Va factor activity in a prothrombinase assay using purified reagents. In the prothrombinase assay purified human factor Va was preincubated with the synthetic peptide and added to a cuvette containing PCPS, prothrombin, DAPA, and factor Xa. D13R showed complete inhibition of factor Va cofactor activity (Fig. 7A, filled squares) with an IC₅₀ of ~12 µM. Complete inhibition occurred at ~100 µM peptide. The residual 6–7% prothrombinase activity observed at this peptide concentration...
is due to the activity of factor Xa alone. Under similar experimental condition a control pentadecapeptide from the middle portion of factor Va (P15H (48)) did not show any inhibition of prothrombinase function (Fig. 7A, filled triangles). The pentadecapeptide has 7 amino acids in common with D13R (in a different order), contains a tyrosine and an aspartic acid, derives from the factor Va sequence, and overall possess a similar charge as D13R. P15H is thus an adequate control for D13R.

Overall data demonstrate that D13R inhibits prothrombinase by impairing the interaction of the cofactor with one of the components of prothrombinase. It is noteworthy that D13R was previously found to be non-inhibitory in a clotting assay using factor V-deficient plasma (68). These discrepancies may result from the way the assays are conducted, and from the definition of factor Va activity per se in each assay. In the prothrombinase assay, the peptide is preincubated with factor Va and the end-point of the assay is measured by the rate of thrombin formation as measured by its complexation with DAPA. In the clotting assay, the end-point of the assay is the formation of a fibrin clot. We must thus conclude that the nature of an assay is important for the determination of the inhibitory potential of a peptide.

To verify if the peptide inhibits prothrombinase activity by impairing the direct interaction of the cofactor with factor Xa, we have employed an assay using a fluorescent derivative of the enzyme as recently described (48): [OG488]-EGR-hXa. Titration of increasing concentrations of D13R into a preformed complex of membrane-bound [OG488]-EGR-hXa-human factor Va (10 nM [OG488]-EGR-hXa and 25 nM factor Va) did not result in the decrease of the factor Va-dependent anisotropy of [OG488]-EGR-hXa even in the presence of 100 μM D13R (Fig. 7B, filled squares). Under similar experimental conditions P15H didn’t have any effect on the factor Va-[OG488]-EGR-hXa interaction, whereas a previously described nonapeptide from the heavy chain of factor Va (AP4) showed complete inhibition of the binary interaction with an IC50 of ~8 μM (Fig. 7B, filled triangles) (48). These data are consistent with the data of Fig. 6, which indicated that deletion of 13 amino acids from the COOH-terminal part of the heavy chain of factor Va does not interfere with the binding of factor Xa to the cofactor (Fig. 6, factor VaNN/He). Altogether these data suggest that D13R inhibits prothrombinase, because it interferes with other interactions in prothrombinase.

The mechanism of D13R inhibition of prothrombinase was addressed by investigating the effect of the peptide on the kinetic parameters of prothrombinase (Kd and Vmax) in the presence of varying concentrations of inhibitor (2–5 μM, Fig. 7C). Under the conditions employed and in the presence of increasing concentrations of peptide, the saturation curves were sigmoidal (Fig. 7C). The curves tended toward a similar asymptote, which is the Vmax of prothrombinase (525 ± 17 nM IIa/min), whereas the apparent Kd of the enzymatic reaction increased with increasing concentration of inhibitor (Fig. 7C, 2 μM (filled triangles), 3 μM (filled circles), 4 μM (filled diamonds), and 5 μM (filled inverted triangles)). These data represent an unusual competitive inhibition mechanism were only free substrate (prothrombin) can produce thrombin in the presence of prothrombinase (69). According to this model D13R binds prothrombin in competition with the binding of prothrombin to prothrombinase (factor Va-factor Xa) (69). This unusual competitive mechanism was previously described for the inhibition of prothrombin activation by the sulfated version of Hir54–65 peptide, which inhibited prothrombinase with a Ki of ~3 μM (33). The inhibition constant (K) of prothrombinase by D13R calculated from the IC50 (Fig. 7A) using the Km obtained from Fig. 7C (0.4 μM ± 0.03, filled squares) was found to be 6.4 μM. These values are in complete agreement and strongly suggest that the hirudin-like peptide D13R is an unusual competitive inhibitor of prothrombinase, because it inhibits prothrombin activation by interfering with the interaction of the substrate (prothrombin) with the enzyme (prothrombinase) rather than interfering with the active site of the enzyme like a classical competitive-type inhibitor (69).
FIG. 7. Function of a peptide containing amino acid residues 697–709 (D13R). A, prothrombinase function. Increasing concentrations of D13R were incubated with factor Va. Factor Va cofactor activity was assayed in the presence of increasing concentrations of D13R as described under “Experimental Procedures” in a prothrombinase assay using purified reagents (filled squares). The control peptide P15H previously shown to have no effect on factor Va cofactor activity is shown by the filled triangles (48). The data with D13R represent the average value found in three independent experiments, whereas the results with P15H represent the average value found in two independent experiments. B, prothrombinase assembly. Reaction mixtures containing a [OG_{488}]-EGR-hXa at 10 nM and 10 μM PCPS were titrated with factor Va to saturation (25 nM (48)). The preformed complexes were titrated with increasing concentrations of AP4 (filled triangles), P15H (filled inverse triangles), and D13R (filled squares). Delta r was obtained by subtracting the value of the anisotropy of [OG_{488}]-EGR-hXa alone (average of eight readings) in the absence of factor Va from the anisotropy observed in the presence of a given concentration of factor Va (average of eight readings) as described (48). The graph represents two different things: Three titrations of membrane-bound [OG_{488}]-EGR-hXa with factor Va (filled circles, in nM), followed by the displacement of factor Va from [OG_{488}]-EGR-hXa by the synthetic peptides (in μM). C, kinetic analyses of prothrombinase inhibition by D13R. The data are plotted as V₀ (initial velocity, in arbitrary units) as a function of increasing prothrombin concentration in the presence of increasing concentrations of inhibitory peptide (D13R). The lines drawn represent the best fit through the points with an R² of at least 0.99. The concentrations of D13R used in the experiments are as follows: control no peptide (filled squares), 2 μM peptide (filled triangles), 3 μM peptide (filled circles), 4 μM peptide (filled diamonds), and 5 μM (filled inverted triangles). The data represent the average of the results found in three independent experiments. The apparent inhibition constant (Kᵢ) reported in the text is the value derived from the formula: IC_{50} = K_{ᵢ}(1 + S/K_m) (69), where K_m is the Michaelis-Menten constant of the reaction in the absence of inhibitor.
Fig. 8. Direct interaction of the hirudin-like peptide from the COOH terminus of factor Va heavy chain with thrombin-agarose. Chromatography of control and acidic peptides was performed on a thrombin-agarose column as described (30). Each run represents 400 µg of peptide. This amount of peptide was determined to be saturable for the thrombin-agarose column used. Elution was performed with 2 M NaCl and was started at the point indicated by the arrow. The presence of the peptides in the corresponding fractions was monitored by absorbance at 280 nm. Identification of D13R in the elution fractions was performed by LC/MS as described under “Experimental Procedures.” Results show P15H (filled squares), D13R (first run, filled circles), and D13R (second run, filled triangles).

the data presented thus far suggest that a binding site for prothrombin is located within D13R, which represents the last 13 amino acids from the heavy chain of factor Va. To ascertain that the peptide interacts directly with prothrombin and/or thrombin, we have used a thrombin-Sepharose column and a thrombin-agarose column. Attempts to study the binding of D13R to thrombin-Sepharose failed, because D13R was consistently found in the flowthrough of the column. Two facts may account for this result: 1) the peptide has low affinity for prothrombin-Sepharose and our experimental conditions did not allow for the binary interaction to occur and/or 2) ABE-I of prothrombin, which has been reported to interact with hirudin-like peptides and to interfere with prothrombin activation (33), may have been blocked during the coupling procedure. However, although it has been previously demonstrated that factor Va interacts with prothrombin and thrombin via ABE-I, it has been also shown that the affinity of ABE-I for its ligands is increased following activation and thrombin formation (33, 39).

We have thus used a thrombin-agarose column where thrombin was coupled to the agarose beads via the active site as described (30). Because D13R and the control peptide P15H both contain tyrosine residues, we can measure their optical density, thus we can identify the presence of the peptides in the collected fractions. The thrombin-agarose column was loaded with the peptides and washed extensively. Subsequently, elution was performed with 2 M NaCl. The results of these experiments are shown in Fig. 8. The control peptide, P15H (filled squares), which has 7 amino acids identical to D13R, was detected in the flowthrough of the thrombin-agarose column. In contrast, D13R bound to the thrombin-agarose column and eluted with the high salt buffer (Fig. 8, arrow, filled circles, and filled triangles). The identity of D13R in the elution fractions from both runs was verified by LC/MS (not shown). The data demonstrate that D13R contains a binding site for thrombin. A direct comparison, of the amino acid sequence of D13R with the amino acid sequence of several hirudin-like peptides that interact with thrombin, suggests that the hirudin-like motif DYQ (30–33, 70–72) contained within D13R may be responsible for its interaction with the thrombin-agarose column.

Taken together the data indicate that prothrombinase assembly can occur following cleavages at Asp<sup>697</sup>, Asp<sup>1509</sup>, and Asp<sup>1514</sup>, whereas optimum function requires cleavage at Arg<sup>1545</sup> and generation of the light chain of factor Va. This cleavage appears to be required for optimum binding of factor Xa, which in turn is a prerequisite for subsequent expression of efficient catalytic effector function by the cofactor at low physiological concentrations of factor Xa. Thus, although deletion of residues 697–709 in the heavy chain of factor Va species did not affect factor Xa binding, the clotting function of factor Va was significantly affected by truncation at the COOH terminus. Thus, the COOH-terminal portion of the heavy chain of factor Va appears to contribute to the prothrombin binding site of prothrombinase and is required for expression of optimum clotting activity.

DISCUSSION

Fig. 9 is a schematic representation of the factor V products derived following NN (Fig. 9A), RVV-V activator (Fig. 9B), and α-thrombin (Fig. 9C) treatment. NN cleaves factor V at Asp<sup>697</sup> and Asp<sup>1509</sup>/1514 to produce a molecule that has less clotting activity than unactivated factor V (factor V<sub>NN</sub>, Fig. 9A). The present work demonstrates that formation of the light chain of factor Vα (following cleavage at Arg<sup>1545</sup> of factor V) is a common feature of the cleaved products, which exhibit optimum association for factor Xa in the assembly of prothrombinase (Fig. 9). Thus, factor V<sub>IIa/NN</sub>, factor V<sub>RVV/NN</sub>, and factor V<sub>IIa</sub> display similar affinities for factor Xa (K<sub>d</sub> of 0.5, 0.57, and 0.3, respectively, Table I). Each of these three forms of factor V has a fully formed light chain (1546–2196) and differs only in the COOH terminus of the heavy chain. With an extended light chain, as in factor V<sub>NN</sub> (where the NH<sub>2</sub> terminus has the additional residues 1509–1514–1545), a weaker factor V<sub>NN</sub>-factor Xa interaction was observed (K<sub>d</sub> of 4.1 nm). Thrombin cleavage at Arg<sup>1545</sup> restored the affinity of factor V<sub>NN</sub> for factor Xa to levels found for factor V<sub>IIa</sub> (K<sub>d</sub> of 0.5 nm, Fig. 9D). The current study indicates that formation of the light chain (1546–2196) is required to capture factor Xa on the lipid surface and that the addition of as few as 30 additional residues in the light chain reduces the cofactor and clotting activity by an order of magnitude or more. This correlates with observations made during tissue factor-initiated coagulation, in which the cleavage of factor V at Arg<sup>1545</sup> coincides with explosive activation of prothrombin (73, 74). It is noteworthy that a similar situation is observed for factor VIII where cleavage at Arg<sup>1689</sup> by α-thrombin in the light chain of the cofactor is required for activation of the cofactor and optimum activity within the intrinsic tenase complex (75).

Although the species factors V<sub>IIa/NN</sub>, V<sub>RVV/NN</sub>, and V<sub>IIa</sub> ex-
Hibited similar affinities for factor Xa, factors V_{IIa/NN} and V_{RVV/NN} had lower and more variable activity (20–40%) in clotting assays using factor V-deficient plasma (Fig. 9D). However, for prothrombin activation at high (i.e., saturating) concentrations of factor Xa, the catalytic efficiency of the factor V_{IIa/NN}-factor Xa complex was as high as that observed for the factor V_{IIa}-factor Xa complex. Furthermore, we have found that the missing amino acid sequence from the heavy chain of factor V_{NN} represents a binding site for prothrombin and that the clotting activity of factor V_{IIa/NN} is considerably impaired by removal of residues 607–709 from the COOH-terminal region of the heavy chain. Thus, this region of the molecule is crucial for cofactor activity under low factor Xa concentrations (physiological conditions).

Bakker et al. (46), using a protease purified from the venom of the snake N. naja oxiana, prepared a species of factor Va missing an even larger portion (residues 683–709) of the heavy chain. These investigators concluded that the region 683–709 is
required for the binding of factor Xa and/or prothrombin to factor Va. Thus, whereas amino acid region 697–709 contains a binding site for prothrombin and appears to be required for prothrombin interaction and expression of factor Va clotting activity under conditions of limiting factor Xa concentrations (physiological conditions), it is also possible that the additional stretch from amino acid residues 683–696 may be also required for factor Xa and/or prothrombin binding. Thus, this highly acidic COOH-terminal segment of the heavy chain, which has also been found to be phosphorylated by a platelet-derived casein kinase II (76, 77), appears to play a critical regulatory role for factor Va cofactor function under limiting concentrations of factor Xa.

We have demonstrated an interaction between D13R, a hirudin-like peptide, and thrombin-agarose. We have also shown that D13R is a competitive inhibitor of prothrombinase function. We can thus conclude that D13R contains at least a portion of the binding site of factor Va for prothrombin. Several studies have suggested a contribution from the heavy chain of factor Va to the interaction of prothrombin with prothrombinase. Our findings are in complete agreement with the data from Dharmawardana and Bock (30) and Anderson et al. (33) demonstrating a productive interaction between factor Va and ABE-I of thrombin and prothrombin and suggesting that this interaction involves hirudin-like motifs from the COOH terminus of the factor Va heavy chain (33). The only hirudin-like motif of D13R is the sequence Asp-Tyr-Gln (70). Thus, our findings together with those on record provide for a new binding site for thrombin and prothrombin on the COOH terminus of the heavy chain of factor Va to which the amino acid residues Asp797–Gln699 contribute.

Factor V requires proteolytic processing for expression of full cofactor activity (3, 4–6, 12, 66, 67). The activation of factor V by α-thrombin, factor Xa (78, 79), RVV-factor V activator (80, 81), plasmin (68, 82), cathepsin G (83), human neutrophil elastase (83), and other enzymes has been the subject of investigation the past decade and has produced some controversy. Early data using bovine factor V suggested that generation of the heavy chain of the cofactor following cleavage at Arg713 by α-thrombin (the equivalent of Arg709 in the human molecule) is sufficient for expression of maximum cofactor activity in an assay measuring α-thrombin generation and employing purified reagents and saturating concentrations of factor Xa (4). Using a similar assay and human plasma factor V, it was reported that cleavage of human plasma factor V at Arg1018 by factor Xa and generation of a heavy chain portion of the cofactor (amino acid residues 1–1018) and of a M1220,000 fragment (amino acid residues 1019–2196, factor VaX,1018) was sufficient to promote maximum cofactor activity (79). No increase in cofactor activity was found when factor Xa was treated with α-thrombin (factor Vα1018), and no differences in activities were observed between Vαx and factor VaX. In contrast, using a clotting assay (5) or an assay that measures α-thrombin formation and employs limiting factor Xa concentrations (67), it was reported that cleavage of factor V at Arg554 and generation of the light chain alone was sufficient for optimum factor Xa activity within prothrombinase. The data presented here suggest that at saturating concentrations of factor Xa, cleavage at Arg554 is sufficient for optimum factor Va cofactor expression, whereas under conditions of limiting factor Xa, cleavages at both Arg545 and Arg554 are required to promote maximum cofactor activity. Hence, results obtained in a clotting assay or using factor Xa concentrations below the Kc for the factor Xa-factor Va bimolecular interaction within prothrombinase cannot and should not be compared with the data obtained when using saturating amounts of factor Xa to study the effect of various factor Va species on prothrombinase assembly and function. Thus, differences between different groups using the “active species of factor Va” are most likely related to the assay conditions used rather than to specific proteolytic cleavages of the factor V molecule.

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