Identification of a Binding Site For Blood Coagulation Factor Xa on the Heavy Chain of Factor Va. Amino Acid Residues 323−331 of Factor V Represent an Interactive Site for Activated Factor X[†,‡]

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ABSTRACT: We have recently shown that amino acid region 307−348 of factor Va heavy chain (42 amino acids, N42R) is critical for cofactor activity and may contain a binding site for factor Xa and/or prothrombin [(2001) *J. Biol. Chem. 276*, 18614−18623]. To ascertain the importance of this region for factor Va cofactor activity, we have synthesized eight overlapping peptides (10 amino acid each) spanning amino acid region 307−351 of the heavy chain of factor Va and tested them for inhibition of prothrombinase activity. The peptides were also tested for the inhibition of the binding of factor Va to membrane-bound active site fluorescent labeled Glu−Gly−Arg human factor Xa ([OG_{488}]-EGR-hXa). Factor Va binds specifically to membrane-bound [OG_{488}]-EGR-hXa (10nM) with half-maximum saturation reached at ∼6 nM. N42R was also found to interact with [OG_{488}]-EGR-hXa with half-maximal saturation observed at ∼230 nM peptide. N42R was found to inhibit prothrombinase activity with an IC_{50} of ∼250 nM. A nonapeptide containing amino acid region 323−331 of factor Va (AP_4') was found to be a potent inhibitor of prothrombinase. Kinetic analyses revealed that AP4′ is a noncompetitive inhibitor of prothrombinase with respect to prothrombin, with a K_i of 5.7 μM. Thus, the peptide interferes with the factor Va-factor Xa interaction. Displacement experiments revealed that the nonapeptide inhibits the direct interaction of factor Va with [OG_{488}]-EGR-hXa (IC_{50} ∼ 7.5 μM). The nonapeptide was also found to bind directly to [OG_{488}]-EGR-hXa and to increase the catalytic efficiency of factor Xa toward prothrombin in the absence of factor Va. In contrast, a peptadecapeptide from N42R encompassing amino acid region 337−351 of factor Va (P15H) had no effect on either prothrombinase activity or the ability of the cofactor to interact with [OG_{488}]-EGR-hXa. Our data demonstrate that amino acid sequence 323−331 of factor Va heavy chain contains a binding site for factor Xa.

The procoagulant enzymatic complex, prothrombinase, that is required for normal blood clotting, is composed of the enzyme factor Xa and the protein cofactor factor Va associated on a cell surface in the presence of divalent metal ions (*1*). Prothrombinase catalyzes two cleavages in prothrombin resulting in the formation of α-thrombin (*2*). Factor Xa alone can convert prothrombin to α-thrombin; however, factor Va is required for physiological prothrombinase activity. The incorporation of factor Va into prothrombinase and its interaction with factor Xa increase the catalytic efficiency of the enzyme by 5 orders of magnitude as compared to that of factor Xa alone (*2*).

Human plasma factor V circulates as a large single chain protein of M_r = 330 000 (*3*, *4*). The derived amino acid sequence from the isolated cDNA showed that human factor V is composed of triplicated "A" domains, duplicated "C" domains, and a connecting "B" region (*5*, *6*). Human factor V is cleaved by α-thrombin at Arg^{709}, Arg^{1018}, and Arg^{1545} to generate the active cofactor, factor Va. Factor Va is composed of a heavy chain (A1−A2 domains, M_r = 105 000, amino acid residues 1−709) and a light chain (A3−C1−C2 domains, M_r = 74 000, amino acid residues 1546−2196). The two chains are noncovalently associated via divalent metal ions (*7*). Factor Va is inactivated by activated protein C (APC) only in the presence of a membrane surface following cleavages at Arg^{506}, Arg^{306}, and Arg^{679}. Cleavage of the cofactor at Arg^{506} facilitates cleavages at Arg^{306} and Arg^{679} and subsequent inactivation of factor Va (*8*, *9*). These cleavages result in the release of the A2 domain of factor Va from the rest of the molecule (*10*). The acidic COOH-terminal portion of the cofactor is also released following cleavage of the cofactor at Arg^{679} (*11*−*13*). Factor Va is also inactivated by plasmin following dissociation of the A2

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domain of the cofactor after cleavage of the heavy chain at Lys^{309}, Lys^{310}, Arg^{313}, and Arg^{348} (*14*). Several cleavages at the COOH-terminal region of the heavy chain of the cofactor by plasmin, release the acidic COOH-terminal portion of the molecule (*14*). Plasmin cleavages of the cofactor and subsequent inactivation are accelerated in the presence of a membrane surface (*14*).

Factor Va and factor Xa interact stoichiometrically in the presence and absence of phospholipid. In the absence of phospholipid, the K_d for the factor Va-factor Xa interaction is 0.8 μM and is dependent upon the presence of Ca^{2+}(*15*). In the presence of a membrane surface and Ca^{2+}, the K_d of the factor Va-factor Xa association (within prothrombinase) is approximately 1 nM (*16*, *17*). Both chains of the cofactor are required for the interaction with factor Xa (*18*−*21*). Two sites on the light chain of the cofactor appear to be responsible for the interaction of factor Va with the membrane surface (*22*−*26*). The factor Va−membrane interaction is governed by a $K_d \sim 3$ nM and involves both hydrophobic and Ca^{2+}-independent electrostatic interactions (*22*, *25*, *26*). The factor Va−prothrombin interaction is promoted by the heavy chain of the molecule (*19*, *27*) with a K_d of approximately 1 μM and appears to be independent of the presence of Ca^{2+} (*27*). Binding sites of factor Va on the α-thrombin molecule that were localized on the heavy chain of the cofactor appear to involve anion-binding exosites I and II of the enzyme (*28*−*30*). Recently, it has been reported that a binding site for factor Va exists on the Gla domain of prothrombin (*31*). Thus, several binding sites for prothrombin may exist on factor Va heavy chain.

We have recently demonstrated that a synthetic peptide from the middle portion of the factor Va heavy chain (amino acid residues 307−348, N42R) inhibits factor Va clotting activity with an IC_{50} of 1.3 μM (*14*). We have concluded that the inhibitory potential of the peptide was most likely related to its ability to inhibit the interaction of the cofactor with factor Xa and/or prothrombin. The present study was undertaken to identify the minimum amino acid sequence within N42R that is responsible for the inhibitory potential of the peptide. We have also investigated the specific macromolecular interactions within prothrombinase that are inhibited by N42R-derived peptides resulting in impaired factor Va cofactor activity.

EXPERIMENTAL PROCEDURES

Materials and Reagents. N-[2-Hydroxyethyl]piperazine-N′-2-ethanesulfonic acid (Hepes), Trizma (Tris base), Q-Sepharose fast flow, Sepharose CL-4B, bovine serum albumin (BSA), diisopropyl-fluorophosphate (DFP), and factor V-deficient plasma were purchased from Sigma (St. Louis, Mo). Heparin-Sepharose was obtained from AmershamPharmacia Biotech Inc (Piscataway, NJ). L-α-Phosphatidylserine (PS) and L-α-phosphatidylcholine (PC) were purchased from Avanti Polar Lipids (Alabaster, AL). Normal reference plasma and hirudin were obtained from American Diagnostica Inc. (Greenwich, CT). The thromboplastin reagent used in the clotting assays was purchased from Organon Teknika Corp. (Durham, NC). Poly(ethylene glycol), $M_r = 8000$ (PEG), was obtained from J.T. Baker (Phillipsburg, NJ). The fluorescent thrombin inhibitor dansylarginine-N-(3-ethyl-1,5-pentanediyl)amide (DAPA), glutamylglycinylarginyl chloromethyl ketone active-site blocked human factor Xa (EGR-hXa), human α-thrombin, human APC, human factor Xa, human prothrombin, the monoclonal antibody αhFV#1 coupled to Sepharose, and human factor Xa labeled in the active site with Oregon Green 488 ([OG_{488}]-EGR-hXa) as previously described (*32*) were purchased from Haematologic Technologies Inc. (Essex Junction, VT). Overlapping peptides (AP1−AP8) as well as some of the other peptides used in this study (P15H, A9A (AYIEVEFEA, AP4′ scrambled), V9E (VEEAAIFYE, AP4′ reverse)) were synthesized in the analytical facility of Dr. Satya Yadav at the Cleveland Clinic Foundation (Cleveland, OH). Some of the peptides were also made in the analytical facility of Dr. Alex Kurosky (AP1−AP8, AP4′, University of Texas, Medical Branch, Galveston, TX). AP4′ is a nonapeptide containing the sequence of peptide AP4 minus tryptophane 322. The synthetic peptide representing amino acid residues 307−348 of factor Va heavy chain (N42R) was purchased from Peninsula Laboratories Europe Ltd. (Mereyside, England). The peptides were amidated at their COOH terminus. All peptides were purified to homogeneity using high-performance liquid chromatography (HPLC) with a reverse-phase C18 column, and the molecular masses of each peptide were verified by mass spectroscopy. The purity of all peptides was higher than 98%, as assessed from the HPLC chromatogram combined with the data obtained from the mass spectrometer. Amino acid analyses of some of the peptides were also performed, and the results obtained were used to calculate their molar concentration. Quantitative amino acid analysis was performed on an Applied Biosystems Model 420H amino acid analyzer in the laboratory of Dr. Alex Kurosky (University of Texas, Medical Branch at Galveston). The analyzer is a precolumn PTC analyzer. The instrument is online with a 130A Microbore HPLC and a model 900A data analysis module from Applied Biosystems. Hydrolysis was performed in 6 N HCl at 108 °C for 24 h under vacuum. Following amino acid composition, the recovered mass (in micrograms) was calculated. On the basis of the known molecular weight of each peptide and the known volume used, the concentration of the peptides in solution was calculated with an approximate error ranging between 10% and 15%. Phospholipid vesicles composed of 75% PC and 25% PS (referred to as PCPS throughout the manuscript) were prepared as previously described (*33*). The concentration of the phospholipid vesicles was determined by phosphorus assay as described earlier and is given as the concentration of inorganic phosphate (*34*).

Factor Va Preparation and Purification. Human factor V was purified as previously described using immunoaffintity chromatography with monoclonal antibody αhFV#1 coupled to Sepharose (*35*, *36*). In brief, 1.5 L of plasma was submitted to two PEG precipitations. The pellet of the second precipitation (10%) was dissolved in 20 mM Tris, 0.15 M NaCl, pH 7.4 (TBS), and applied to a 20 mL immunoaffintity column (αhFV#1). Following elution of the column, the solution containing factor V (approximately 50 mL) was extensively dialyzed against 20 mM Tris, 0.15 M NaCl, 5 mM $CaCl_2$ pH 7.4 (TBS, Ca^{2+}) and activated with α-thrombin (at a 1/50 enzyme substrate ratio, corresponding to approximately 8−10 nM enzyme) for 15 min at 37 °C. The reaction was stopped with 2 mM DFP followed by a 1 h incubation at 4 °C. The

mixture containing factor Va was loaded onto a heparin-Sepharose column (2 cm × 6 cm) equilibrated in TBS,Ca^{2+}. The flow through of the column containing the B region-derived fragments was collected and stored at −20 °C. Following washing of the column with TBS, Ca^{2+} factor Va was eluted with a buffer containing 0.4 M NaCl in 20 mM Tris, 5 mM $CaCl_2$ pH 7.4, and dialyzed against TBS,Ca^{2+}. Prior to use, factor Va was concentrated using Centricon (M_r = 30 000 cutt-off filter, Millipore Corp., Bedford MA). Factor Va was stored on ice at 4 °C, and its activity was stable for 2 weeks. The factor Va preparations could be frozen and thawed only once. Repeatedly freezing and thawing the preparations resulted in the loss of cofactor activity. Because of the instability of the active cofactor following prolonged storage, factor Va preparations were made weekly and in small quantities.

Assay Measuring Thrombin Formation. The formation of thrombin was analyzed using the fluorescent thrombin inhibitor DAPA. The buffer used in all cases was TBS, Ca^{2+}. All buffers were freshly made and filtered prior to use (using a 0.45 μM type HA filter, Millipore Corp., Bedford, MA). Fluorescence was measured with a Perkin-Elmer LS-50B Luminescence Spectrometer (Perkin-Elmer LLC, Norwalk CT) with λ_{ex} = 280 nm, λ_{em} = 550 nm, and a 500 nm long-pass filter in the emission beam (Schott KV-500). The data obtained were instantly analyzed using the software FL WinLab (Perkin-Elmer Corp, Norwalk CT). All peptides were weighed and dissolved in MQH_2O to 5 mg/ml. The peptides were readily soluble in water, with the exception of AP4 and AP4′, and no precipitate was visible. All peptide solutions were centrifuged 10 min at 14 000 rpm prior to use. AP4 and AP4′ were also made at 5 mg/mL however, because of the partial insolubility of the peptides, the solutions were centrifuged and the concentration of peptide in the supernatant was calculated by quantitative amino acid analysis. We have eliminated Trp^{322} from AP4′ in order to make it more soluble. However, even after this modification, AP4′ like its parent molecule, AP4, was still partially insoluble. Thus, following solubilization of the peptide in water to a theoretical concentration of 5 mg/mL and extensive mixing, the peptide solution was centrifuged (10 min at 14 000 rpm), and the supernatant used to test its effect on prothrombinase. Prior to all assays, the concentration of the peptide solution was calculated by quantitative amino acid composition analysis (and found to be 3.3 mg/mL). The presence of AP4′ in the supernatant was also verified by subjecting aliquots to mass spectrometry analysis and to NH_2-terminal amino acid sequencing. The correct sequence and molecular weight of the peptide (−Trp^{322}) was always observed. The mass spectrum of a preparation of AP4′ (dissolved in H_2O and centrifuged), obtained with a Quatro II Triple Quadrupole mass spectrometer (Micromass, England) following electrospray ionization in a 0.3% solution of formic acid, is shown in Figure 1. The calculated molecular weight of AP4′ is 1069. The spectrum shown in Figure 1 has two major peaks: one at 1070.5 and one at 535.9. Several other minor peaks that account for less than 2% of the total intensity are also visible at the bottom of the spectrum. The peak at 1070.5 represents the peptide (mass/charge) with one positive charge ($[M + H]^+$), i.e., [(1069 + 1=1070)]/1, whereas the peak at 535.9 represents AP4′ with two positive charges ($[M + 2H]^{2+}$), i.e., [(1069 + 2=1071)]/2 = 535.5. Overall, these data demonstrate that our preparations of AP4′ are highly pure and homogeneous.

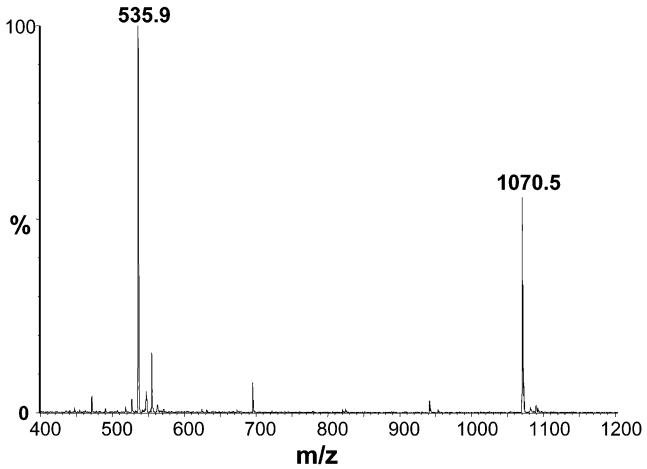

FIGURE 1: Mass spectrometry analysis of purified AP4′. AP4′ was purified by HPLC using a reverse phase C18 column. The peptide was dissolved in water and centrifuged (10 min, 14 000 rpm), and one aliquot of the supernatant was analyzed by mass spectrometry following electrospray ionization as described in the "Experimental Procedures" section. The data are represented as % intensity of the signal as a function of the mass of the peptide divided by the charge (*m*/*z*).

It is noteworthy that repeated freeze−thaw cycles of AP4′ resulted in the loss of its activity. NH_2 sequencing of a solution of AP4′ that showed impaired inhibitory activity, demonstrated hydrolysis of Glu^{323} from the rest of the peptide. For each new solution of AP4′, we needed to check the concentration of the peptide. Thus, for each series of experiments, AP4′ was prepared fresh and in a large stock solution of 3.5 mg/mL, its concentration was calculated following quantitative amino acid composition analysis, and frozen in small aliquots. Every aliquot was thawed and used once. Under these conditions the entire preparation retained its initial inhibitory activity for at least 1 month as long as the sample was thawed only once. In a typical experiment, a mixture containing prothrombin (350 nM), DAPA (700 nM), and phospholipid vesicles (10 μM) composed of 75% PC and 25% PS was incubated in the dark for 20 min. At selected time intervals, one aliquot of the mixture (1800 μl) was added to a cuvette, and the baseline was monitored for 15 s at room temperature. When the effect of the peptides on prothrombinase function was investigated, factor Xa was incubated with increasing concentrations of synthetic peptides. The mixtures were added to the cuvette containing prothrombin, DAPA, PCPS, and purified human factor Va (4 nM), and the fluorescence intensity due to the formation of thrombin and its complexation with DAPA was monitored with time (the reaction was started by adding factor Xa in the mixture (± peptide) to a final concentration of 10 nM). Under these conditions (4 nM factor Va and 10 nM factor Xa final concentrations in the mixture), the rate of thrombin formation is linearly related to the amount of active cofactor (factor Va) and to its proper interaction with factor Xa. The initial rate of the formation of thrombin (initial velocity in nM IIa·min^{-1}) was calculated as described (*8*, *37*). The concentration of peptide given in each graph in the results section represents the final concentration of peptide in the assay mixture. Control experiments demonstrate that the inhibitory peptides do not interfere with the ability of

α-thrombin to interact with DAPA during the course of the assay.

Fluorescence Anisotropy Measurements. Fluorescence anisotropy of [OG_{488}]-EGR-hXa was measured using a Perkin-Elmer LS-50B Luminescence Spectrometer in L-format essentially as described (*32*) in a buffer composed of 20 mM Hepes, 0.15 M NaCl, 5 mM $CaCl_2$, 0.01% PEG 8000, pH 7.4 [HBS, PEG(Ca^{2+}), "assay buffer"]. Anisotropy measurements were performed in a quartz cuvette under constant stirring (low) with $\lambda_{ex} = 490$ nm, $\lambda_{em} = 520$ nm with a long pass filter (Schott KV-520) in the emission beam. The assay buffer (HBS(Ca^{2+})) was made fresh prior to use and filtered, followed by the addition of an aliquot of a concentrated solution of PEG to a final concentration of 0.01%. All experiments were performed in the dark. The data obtained were instantly analyzed using the software FL WinLab (Perkin-Elmer Corp, Norwalk CT). Usually, reaction mixtures of 2 mL containing 10 nM [OG_{488}]-EGR-hXa, 10 μM PCPS were titrated with factor Va prepared in the assay buffer, for up to 25 nM (usually addition of 0.5 μL of a concentrated stock solution corresponded to 1 nM final concentration, so 25 nM factor Va corresponded to 12.5 μLs, which is less than 1% of the total volume in the cuvette, 2-mls). In all cases, at the end of each experiment the addition of factor Va did not exceed 1% of the total volume of the reaction in the cuvette. At each addition, anisotropy was measured for 60 s and averaged eight successive readings as the polarizer was automatically flipped from a vertical to a horizontal position. The Δr of the reaction 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). Displacement experiments were performed in a similar manner; [OG_{488}]-EGR-hXa was first titrated with up to 25 nM human factor Va. When the plateau was reached, successive additions of synthetic peptide were initiated. The anisotropy was measured, and the Δr at each addition of peptide was calculated by subtracting the observed anisotropy (average of eight readings) from the anisotropy of [OG_{488}]-EGR-hXa in the absence of factor Va (average of eight readings). For the titration of peptides to concentrations up to 100 μM, the additions did not exceed 4% of the total volume of the reaction (AP3 0.59 μL/μM, AP4′ 0.60 μL/μM, AP5 0.47 μL/μM, AP6 0.45 μL/μM, AP4′ reverse and AP4′ scrambled 0.61 μL/μM, and P15H 0.8 μL/μM). For reactions taken up to 300 μM peptide, the additions did not exceed 12% of the volume when using P15H and were between 6% and 10% of the volume of the reaction when using all other peptides. The concentration of peptide given in each graph is the final concentration of the peptide in the assay mixture.

Data Analysis. All the data from the fluorescence assay and the anisotropy measurements were analyzed and stored using the software FL WinLab (Perkin-Elmer Corp, Norwalk CT). The data were exported, further analyzed using Prizm (GraphPad, San Diego, CA), and plotted with the appropriate equations using DeltaGraph (DeltaPoint, Monterey, CA).

Gel Electrophoresis. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analyses were performed using 5−15% and 4−12% gradient gels accordingly to the method of Laemmli (*38*). 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.

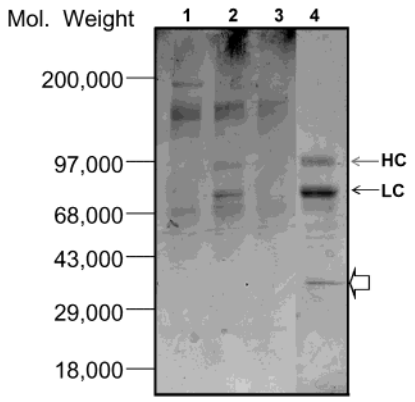

FIGURE 2: Purification of factor Va. Factor Va was purified as described in the "Experimental Procedures" section. Lane 1, partially activated factor V preparation eluted from the immunoaffinity column (sample from a pool of 50 mL total elution); lane 2, sample as in lane 1 but in the presence of 8 nM α-thrombin; lane 3, fragments contained in the flow through of the heparin-Sepharose column; lane 4, proteins contained in the elution of the heparin-Sepharose column. The position of the heavy chain (HC) and light chain (LC) of factor Va are identified at right. The position of the molecular weight markers is indicated at left. The open arrowhead represents a degradation fragment from factor Va.

Amino Acid Sequence Analysis and Amino Acid Composition. NH_2-terminal amino acid sequence analysis and quantitative amino acid composition analysis of the peptides were performed in the laboratory of Dr. Alex Kurosky (University of Texas, Medical Branch at Galveston). When necessary, the masses of the peptides were obtained by mass spectrometry using an ABI Voyager DE-STR matrix-assisted laser desorption time-of-flight mass spectrometer in the same laboratory. Quantitative amino acid composition of the peptide solutions was performed in the same laboratory and used to determine the concentration of each peptide solution.

RESULTS

Factor Va Purification. Cleavage and activation of human factor V by α-thrombin results in the formation of factor Va and the release of two heavily glycosylated fragments from the B region of the molecule (*3*, *4*, *39*). To identify binding sites for factor Xa on factor Va, a solution of pure factor Va without the B region-derived fragments is necessary to avoid misinterpretation of the data. Figure 2 shows a typical purification profile of the factor Va molecule used in all experiments detailed in the present manuscript. A diluted and partially purified and activated solution of factor V obtained following the elution of the anti-factor V immunoaffinity column (Figure 2, lane 1) was activated to completion with α-thrombin (Figure 2, lane 2). Following the inhibition of α-thrombin with DFP, the solution was loaded onto a heparin-Sepharose column, and the flow through of the column was collected and stored at −20 °C (Figure 2, lane 3). After extensive washing of the column, the factor Va molecule was eluted with 0.4 M NaCl (Figure 2, lane 4). The factor Va molecule appears homogeneous, with the exception of a band between the 29 000 and 43 000 markers that represents a degradation product from factor Va. This product always appears following activation of

A.

Hum	*NL***K***KI*	**TREQRR** *HM*	**KRWEYFIAAEEVIWDYAP***V*	**IPANMDK***K*	**YRS***Q***H**
Bov	*NP* **K***KL*	**TRDQRR** *HI*	**KRWEYFIAAEEVIWDYAP***I*	**IPANMDK***K*	**YRS***L***H**
Mous	*SP***K***TL*	**TREQRR** *YM*	**KRWEYFIAAEEVIWNYAP***V*	**IPANMDK***I*	**YRS***Q***H**
Porc	*KP*KKL	**TRDQRR** *HI*	**KRWEYFIAAEEVIWDYAP***I*	**IPANMDK***K*	**YRS***L***H**

* *

B.

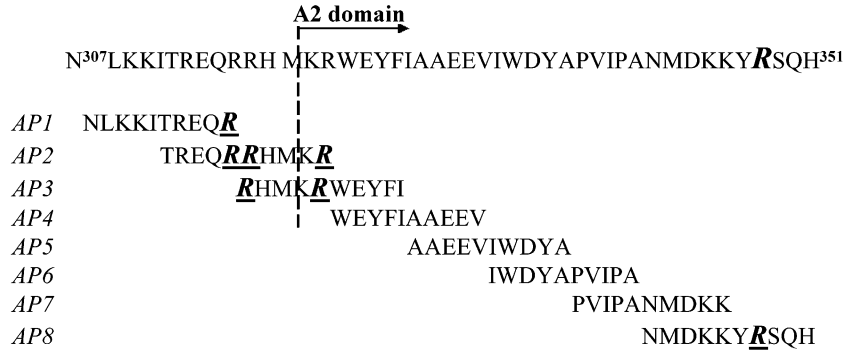

FIGURE 3: Peptides from the central portion of the heavy chain of factor Va (amino acid region 307−351). (A) Comparison of the amino acid region 307−351 of factor V between species (*5*, *40*−*42*). The two stars identify conserved substitutions between species. The amino acids in italic represent differences between species. (B) Overlapping peptides (10 residues each) from the middle portion of the heavy chain of human factor Va are shown (spanning amino acid residues 307−351, AP1−AP8). The arginines are identified (bold and underlined). The A2 domain of factor V starts at Lys^{320}. For the easy reading of the manuscript and the identification of the position of several important amino acids, Asn^{307} and His^{351} are identified as the beginning and the end respectively of the sequence of interest.

factor Va by thrombin. The factor Va molecule shown in Figure 2, lane 4, was freshly prepared and used in all experiments.

Inhibition of Prothrombinase Activity by Synthetic Peptides from the Region 307−*348 of Factor Va Heavy Chain.* We have previously shown that a 42-amino-acid peptide containing the region 307−348 of the cofactor (N42R) inhibits factor Va clotting activity with an IC_{50} of 1.3 μM (*14*). This region is likely to be important for cofactor activity since it was conserved throughout evolution and is 82% identical between the human (*5*), the bovine (*40*), the mouse (*41*), and the porcine (*42*) species (Figure 3A). To identify more specifically the amino acids residues responsible for the inhibitory effect of N42R on factor Va clotting activity, we have synthesized eight overlapping peptides spanning amino acid region 307−351 of the heavy chain of the cofactor (AP1−AP8, Figure 3B). Each synthetic peptide except the first and the last peptide has five amino acids in common with the preceding and the following peptide of the series (Figure 3B).

To evaluate the inhibitory potential of the peptides, we have designed an assay to test their potential to inhibit factor Va cofactor activity using purified reagents. In the assay, purified human factor Xa is first incubated with the synthetic peptides and added to a mixture containing PCPS, prothrombin, DAPA, and factor Va. Since N42R is a potent inhibitor of factor Va clotting activity (*14*), the inhibitory potential of N42R was first assessed to validate the prothrombinase assay (Figure 4A, filled squares). Under the conditions employed (i.e., 4 nM factor Va, and 10 nM factor Xa), N42R showed inhibition of prothrombinase, with an IC_{50} of approximately 230 nM. Complete inhibition of prothrombinase occurred at approximately 500 nM peptide (Figure 4A). These data demonstrate that the prothrombinase assay using purified reagents is sensitive to the inhibitory potential of N42R. As a consequence, this assay can be used to ascertain the inhibitory potential of all peptides shown in Figure 3B.

Under the conditions employed (factor Xa preincubated with the peptides at a fixed concentration prior to the addition to the assay mixture), four peptides were found to inhibit prothrombinase activity. Peptides AP3−AP6 inhibited prothrombinase activity when employed at 100 μM final concentration (Figure 4B). It is noteworthy that the pattern of inhibition points to a specific functional amino acid sequence in factor Va. Thus, since AP2 does not inhibit prothrombinase function and AP3 does, AP4 must contain the amino acid portion of AP3 that is responsible for inhibition of prothrombinase by the latter. Similarly, since AP7 does not inhibit prothrombinase function and AP6 does, AP5 must contain the amino acid sequence responsible for the inhibition of prothrombinase by AP6. Nevertheless, since AP6, which contains five amino acids that are not contained within AP4 (but are in AP5), has a lesser inhibitory potential than AP4 and AP5, the entire sequence of AP4 alone appears to be responsible for the inhibitory potential of N42R. Collectively our data also suggest that the amino acid region contained in AP1 and AP2 (residues 307−321) and AP7 and AP8 (337−351) do not appear to inhibit prothrombinase complex assembly and function.

In view of these data, we have synthesized four more peptides, P15H, AP4′, V9E, and A9A. P15H is a peptadecapeptide that spans amino acid region 337−351, represents the entire amino acid sequence of AP7 and AP8 and was readily soluble in water (see Figure 3B). AP4′ is a

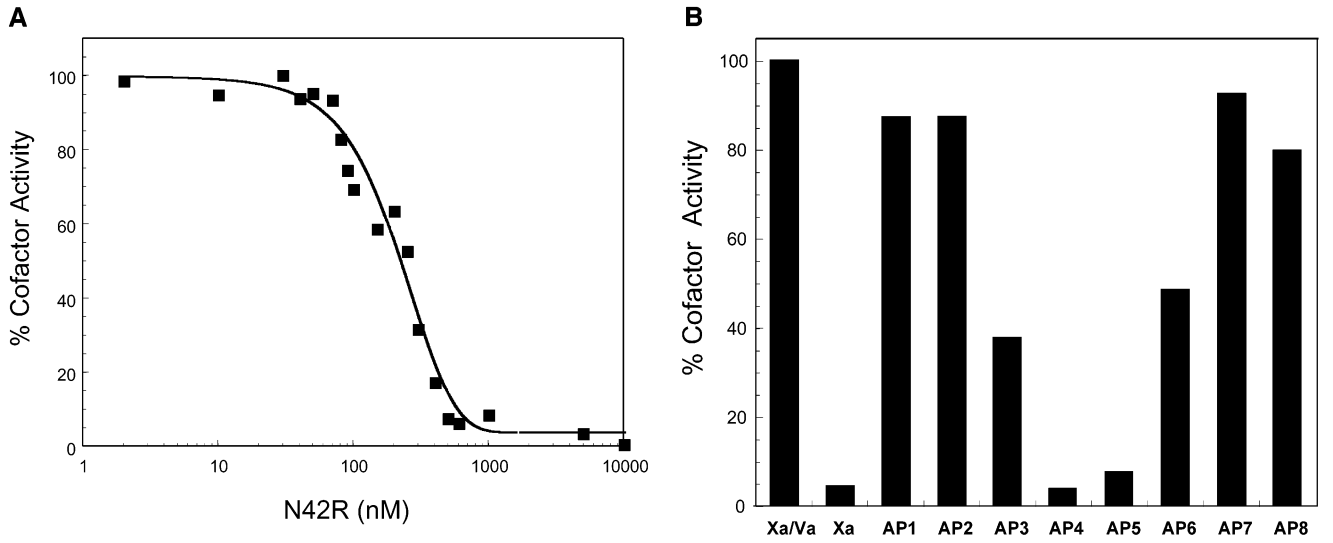

FIGURE 4: Inhibition of prothrombinase function. (A) Inhibitory potential of N42R. Increasing concentrations of N42R were incubated with factor Xa as detailed in the "Experimental Procedures" section. Factor Va cofactor activity was assayed as described in the "Experimental Procedures" section in a prothrombinase assay using purified reagents. The data are plotted as % factor Va cofactor activity as a function of increasing concentrations of N42R (filled squares). (B) Inhibition of prothrombinase by synthetic overlapping peptides from N42R. Peptides were incubated with factor Xa as described in the "Experimental Procedures" section at a fixed concentration. The solution was then transferred to a cuvette containing prothrombin (350 nM), DAPA (700 nM), and phospholipid vesicles (10 μM) composed of 75% PC and 25% PS. The final concentration of each peptide within the mixture was 100 μM (with factor Xa at 10 nM and factor Va at 4 nM). The percent of factor Va cofactor activity was calculated by comparing the activity of prothrombinase in the presence of a given peptide (at 100 μM) to the activity of prothrombinase determined in a control reaction in the absence of peptide and in the presence of factor Xa as detailed in the "Experimental Procedures" section. The amino acid sequence and identification of each peptide are given in Figure 3B.

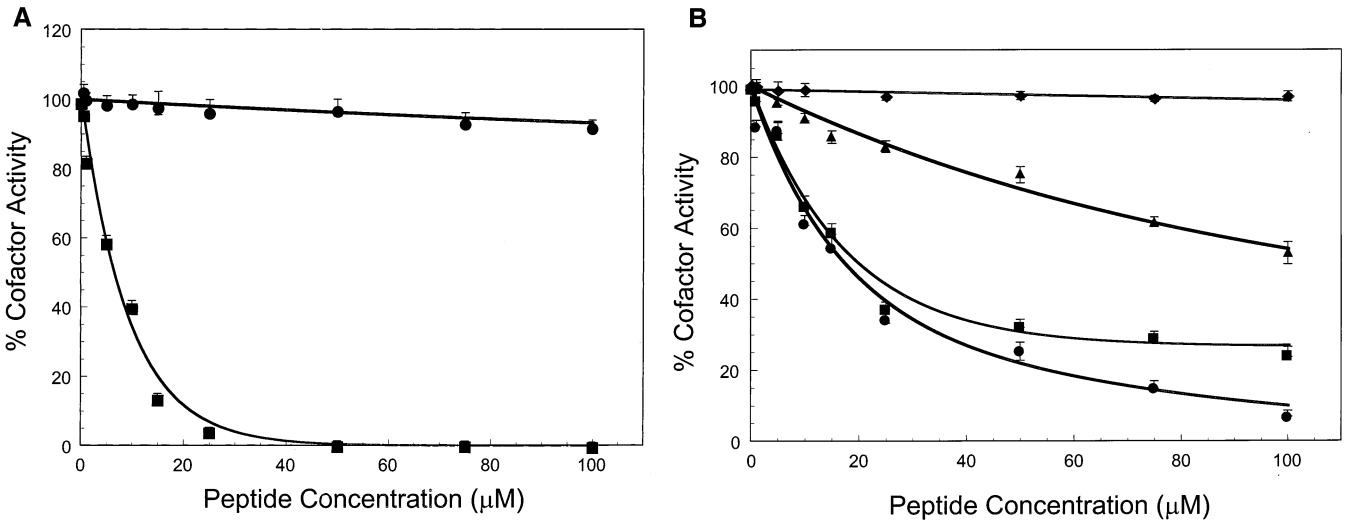

FIGURE 5: Inhibition of prothrombinase. (A) Increasing concentrations of AP4′ and P15H were preincubated with factor Xa and assayed for prothrombinase activity as described in the legend to Figure 4 and in the "Experimental Procedures" section. The final concentration of factor Xa in the mixture was 10 nM. AP4′ (filled squares) represents amino acid sequence 323−331 of human factor Va heavy chain; P15H (filled circles) represents amino acid sequence 337−351 of human factor Va heavy chain. (B) Titration of the inhibition of prothrombinase by synthetic peptides AP3 (filled squares), AP5 (filled circles), AP6 (filled triangles), and A9A (filled diamonds). The data represent the average of the results found in three independent experiments. The concentration of peptide given on the *x* axis represents its final concentration in the prothrombinase mixture.

nonapeptide containing the sequence of peptide AP4 minus tryptophane 322. We have also obtained two control peptides: a peptide with the reverse sequence of AP4′ (AP4′ reverse, VEEAAIFYE (V9E)) and a peptide containing a scrambled version of AP4′ (AP4′ scrambled, AYIEVEFEA (A9A)). The arginine-containing peptide P15H, which represents approximately one-third of N42R and contains an amino acid sequence entirely composed of the residues shown to have no effect of prothrombinase activity, as well as V9E and A9A were used as negative controls in our experiments in order to validate our results.

The effect of peptides AP4′ and P15H on prothrombinase activity is shown in Figure 5A. The data demonstrate that AP4′ inhibits prothrombinase activity with an IC_{50} of ~7 μM (Figure 5A, filled squares) while P15H does not have any significant effect on prothrombinase function even at concentrations as high as 100 μM (Figure 5A, filled circles). Complete inhibition of prothrombinase by AP4′ occurs at

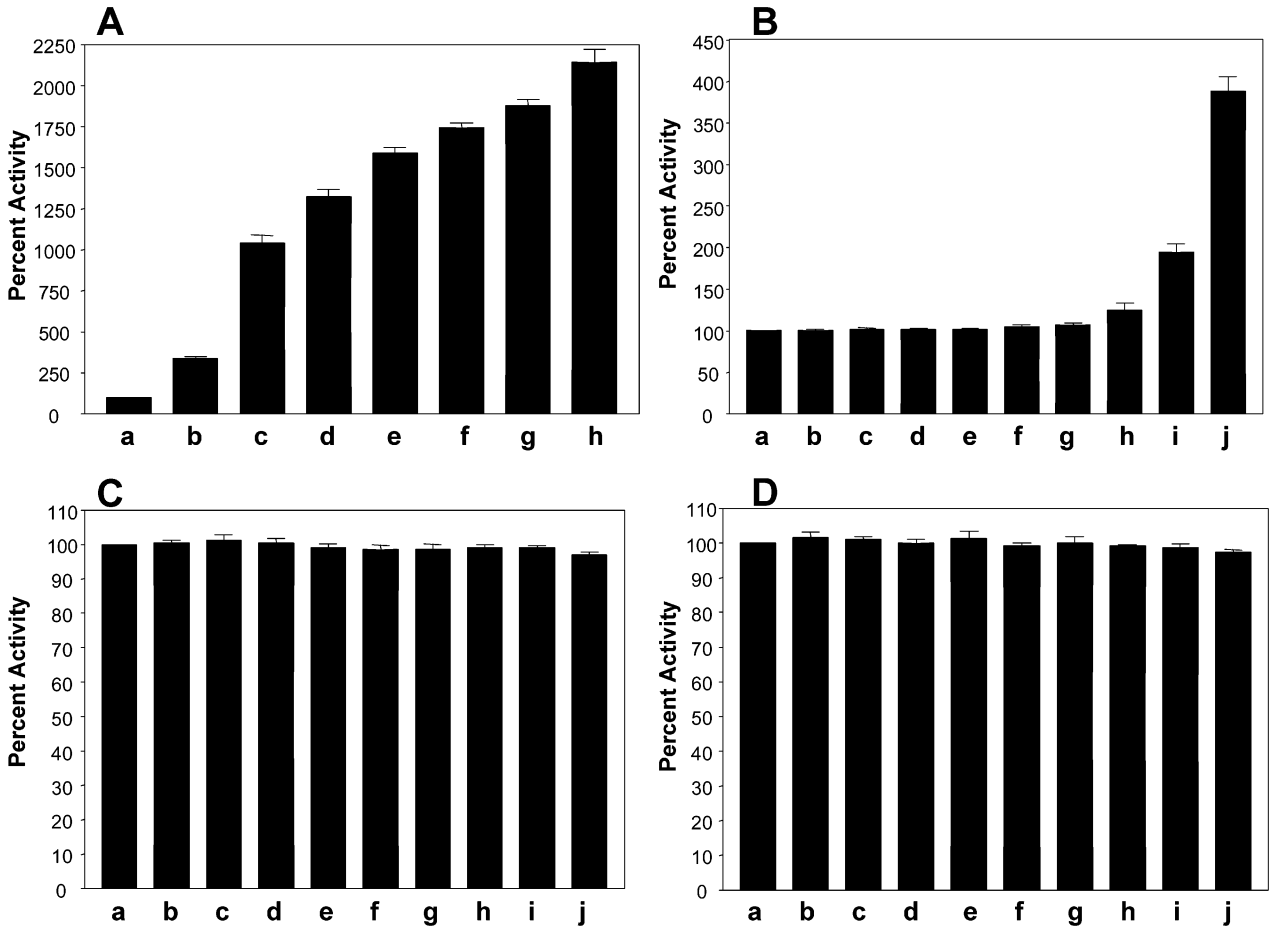

FIGURE 6: α-Thrombin generation in the absence of factor Va. Prothrombin activation experiments were performed as described in the legend to Figure 4 and in the "Experimental Procedures" section but in the absence of factor Va. The final concentration of factor Xa in the mixture was 10 nM. Results are reported as percent of the activity of the control. In this experiment the control corresponding to 100% activity was determined to be the activity of membrane-bound factor Xa alone (4.6 nM IIa/min). Panel A represents the activity of factor Xa alone in the presence of increasing concentrations of N42R; panel B depicts the activity of factor Xa in the presence of increasing concentrations of AP4′; panel C shows the activity of factor Xa in the presence of increasing concentrations of A9A; panel D depicts the activity of factor Xa in the presence of increasing concentrations of P15H. In panel A letters a−h represent 0 (factor Xa alone), 1, 5, 10, 15, 20, 25, and 50 μM N42R. In panels B−D letters a−j represent 0 (factor Xa alone), 1, 5, 10, 15, 20, 25, 50, 75, and 100 μM peptide. The data represent the average of the results found in three independent measurements.

approximately 50 μM (Figure 5A, filled squares). AP3, AP5, and AP6 were also analyzed for inhibition. Figure 5B shows that AP3 inhibits prothrombinase activity with an IC_{50} of ~20 μM, (filled squares) while AP5 also appears to be a potent inhibitor of prothrombinase with an IC_{50} of approximately 19 μM (Figure 5B, filled circles). In contrast, AP6 had a weak effect on prothrombinase function, with 40% inhibition reached at 100 μM (Figure 5B, filled triangles). The control peptide A9A, containing the scrambled version of AP4′, did not show any inhibition of prothrombinase activity even at concentrations as high as 100 μM (Figure 5B, filled diamonds). The data suggest that the entire amino acid sequence of AP4′, EYFIAEEV, which is also shared by AP3 and AP5, appears to be responsible for the inhibitory effect of these peptides on prothrombinase activity. In addition, while AP5 appears to be a potent inhibitor of the reaction, AP6 had only a marginal effect on prothrombinase activity and AP7 and AP8 had no effect (Figure 4B). The latter data as well as the data shown in Figure 4B suggest that the amino acid motif IWDYA may be also partially responsible for the inhibitory potential of prothrombinase by AP5 and AP6. It is noteworthy that the experiments shown in Figures 4 and 5 suggest that amino acid sequences 307−321 and 337−351, which together represent approximately 69% of the entire amino acid sequence of N42R, do not appear to be responsible for the inhibitory effect of N42R on prothrombinase function.

AP4′ was also tested for its effect on the factor Va-factor Xa interaction in solution, in the absence of PCPS vesicles. No effect of AP4′ on the factor Va-factor Xa association (no inhibition of the modest increase in the catalytic efficiency of factor Xa by factor Va in the absence of PCPS vesicles) was observed under these conditions even at concentrations as high as 100 μM peptide (with factor Va and factor Xa at 40 and 80 nM respectively, not shown). The effect of N42R, AP4′, A9A, and P15H on prothrombin activation by factor Xa was also tested in the absence of factor Va but in the presence of phospholipid (Figure 6). The data demonstrated that high concentrations of N42R (up to 50μM, Figure 6A) had a "cofactor" effect on factor Xa increasing its catalytic efficiency. Under the conditions employed (10 nM factor Xa, 10 μM PCPS vesicles, and 350 nM prothrombin), factor Xa alone had a catalytic activity of 4.6 nM IIa/min. In the presence of 50 μM N42R, factor Xa activity increased approximately 21-fold to 98.7 nM IIa/min (Figure 6A). Under similar experimental conditions, in the

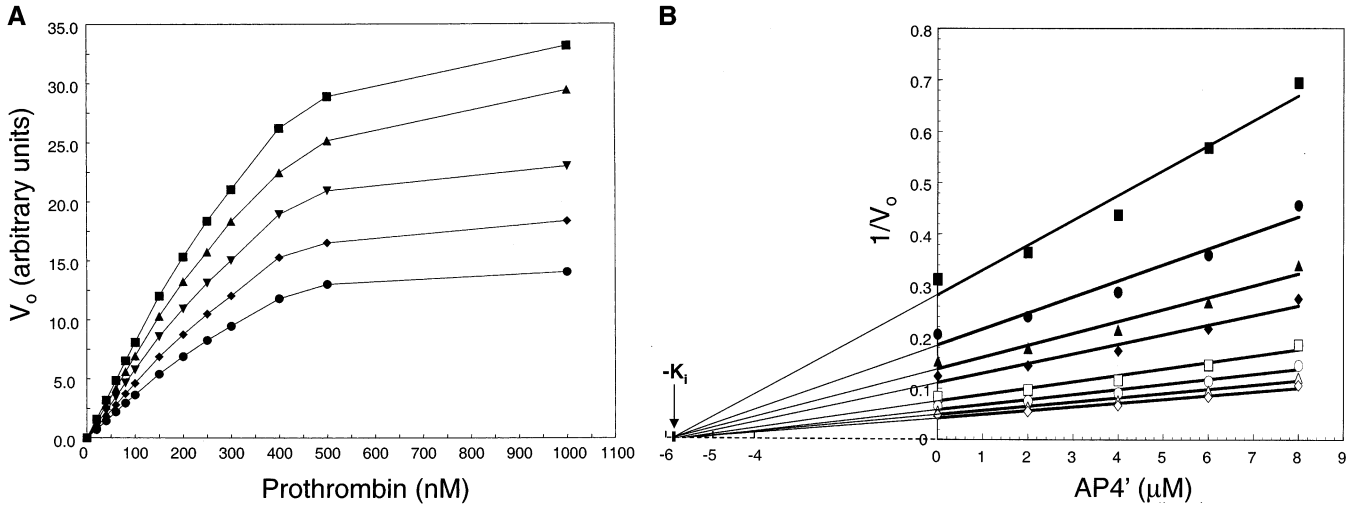

FIGURE 7: Kinetic analyses of prothrombin activation in the presence of AP4′. (A) Michaelis Menten plots. Prothrombin generation experiments were performed in the absence (filled squares) and presence of increasing concentrations of AP4′: 2 μM, filled triangles; 4 μM, filled inverted triangles; 6 μM, filled diamonds; 8 μM, filled circles, as described in the "Experimental Procedures" section using prothrombin concentrations varying from 40 nM−1 μM. Initial rates of thrombin formation are plotted as a function of the substrate concentration. (B) Analysis of the data using the Dixon plots. The data were analyzed and plotted as $1/V_o$ as a function of inhibitor concentration (AP4′, Dixon plots). For simple linear noncompetitive inhibition, a Dixon plot of $1/V_o$ as a function of increasing concentrations of inhibitor is linear at a fixed enzyme and substrate concentration (*43*). The apparent inhibition constant (K_i) reported in the text is the average value derived from the intercept of each of the eight graphs with the x axis at $1/V_o = 0$ (5.7 ± 0.06 μM). The lines drawn represent the best fit through the points with an R^2 varying from 0.958 (worst) to 0.97 (best). The concentrations of prothrombin used in all experiments are as follows: 40 nM (filled squares), 60 nM (filled circles), 80 nM (filled triangles), 100 nM (filled diamonds), 150 nM (open squares), 200 nM (open circles), 250 nM (open triangles), and 300 nM (open diamonds).

presence of 100 μM AP4′, the activity of factor Xa was increased by approximately 4-fold to 18 nM IIa/min (Figure 6B). For comparison, the activity of prothrombinase under similar experimental conditions (4nM factor Va with 10 nM factor Xa) was ~250 nM IIa/min. In contrast, A9A (Figure 6C) as well as P15H (Figure 6D) showed no "cofactor" effect for the factor Xa-mediated prothrombin activation. Overall the data suggest that N42R and AP4′, which inhibit factor Va cofactor activity within prothrombinase, most likely bind to factor Xa at a site capable of inducing an increase in the catalytic efficiency of the enzyme, thus mimicking the effect of factor Va, albeit less efficiently.

Mechanism of Inhibition. The mechanism of inhibition of prothrombinase by AP4′ was addressed by investigating the effect of the peptide on the kinetic parameters of prothrombinase assembly and function (K_m and V_{max}) in the presence of varying concentrations of inhibitor (Figure 7). Under the conditions employed and in the presence of increasing concentrations of peptide, the apparent K_m of prothrombinase remained unchanged (0.19 ± 0.02 μM), while the apparent V_{max} of the enzymatic reaction decreased (Figure 7A). Analysis of the kinetic data using the mathematical transformation associated with the Dixon plots demonstrated that AP4′ inhibited prothrombinase in a noncompetitive manner with respect to substrate (Figure 7B, all graphs at various prothrombin concentrations intercept the X axis at $1/V_o = 0$, which corresponds to $-K_i$;see ref *43*, p 135). The apparent inhibition constant (K_i) of prothrombinase by AP4′ extrapolated from the graph was 5.7 ± 0.06 μM (Figure 7B). These data suggest that AP4′ does not interfere with the binding of the substrate, prothrombin, to factor Va, but impairs optimum prothrombinase activity by inhibiting the interaction of the cofactor with factor Xa.

It is noteworthy that an alternative possibility for the inhibition of prothrombinase by AP4′ is that the amino acid sequence contained within this peptide interferes with the binding of factor Va to the membrane surface. One would predict that inhibition of this interaction by AP4′ would be noncompetitive with respect to substrate and would attenuate factor Xa activity since the dramatic increase in the catalytic activity of factor Xa in the presence of factor Va is completely dependent upon assembly of the cofactor−enzyme complex on a phospholipid surface. For this reason we have performed a control experiment in which we studied the inhibition of prothrombinase as a function of increasing concentrations of AP4′, in the presence of increasing concentrations of factor Va (Figure 8). Under the conditions employed (10 nM factor Xa, 10 μM PCPS vesicles) in the presence of 2 nM factor Va, AP4′ inhibited prothrombinase, with an IC_{50} of approximately 4.2 μM (Figure 8, filled squares). Complete inhibition occurred at 20 μM AP4′. A gradual increase in the factor Va concentration to 4, 6, and 8 nM resulted in the increase of the IC_{50}'s for the inhibition to 6.3, 8.8, and 13.6 μM, respectively (Figure 8, filled circles, filled triangles, and filled diamonds). Higher concentrations of peptide (>20 μM) were necessary to achieve complete inhibition of prothrombinase under these conditions. A further increase in the concentration of factor Va to 10 and 15 nM resulted in a weaker inhibitory effect of AP4′ on prothrombinase activity with IC_{50}'s of 19 and 24 μM, respectively (Figure 8, open squares and open circles). The data demonstrate that an increase in the concentration of factor Va within prothrombinase can overcame the inhibitory effect of AP4′ on cofactor function. These data demonstrate that binding of factor Va and AP4′ to factor Xa are mutually exclusive. Altogether the data demonstrate that AP4′ inhibits prothrombinase by specifically interfering with the binding of factor Va to factor Xa on the membrane surface, and thus inhibiting the incorporation of the cofactor into prothrombinase.

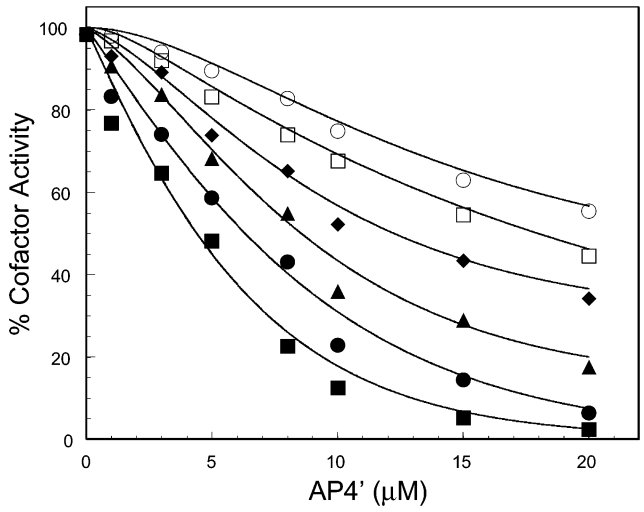

FIGURE 8: Overcoming the inhibition of prothrombinase by AP4′ by increasing the concentration of factor Va. Prothrombin activation experiments were performed as described in the legend to Figure 4 and in the "Experimental Procedures" section. In each experiment a different concentration of factor Va was used. The final concentration of factor Xa in the mixture was kept constant at 10 nM. The data represent the average of the results found in three independent measurements, with error bars varying from 0.15% (smallest) to 1.5% (largest). The lines drawn represent the best fit through the points. The concentrations of factor Va used in the experiments are as follows: 2 nM (filled squares), $R^2 = 0.987$; 4 nM (filled circles), $R^2 = 0.987$; 6 nM (filled triangles), $R^2 = 0.987$; 8 nM (filled diamonds), $R^2 = 0.991$; 10 nM (open squares), $R^2 = 0.999$; and 15 nM (open circles), $R^2 = 0.997$.

Inhibition of the Direct Binding of Active-Site Labeled Phospholipid-Bound Factor Xa to Factor Va. To ascertain that the peptides inhibit 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. Betz and Krishnaswamy used a factor Xa molecule of bovine origin labeled in the active site with the fluorescent probe Oregon Green 488 ([OG_{488}]-EGR-bXa]) to show that regions that are remote from the site of prothrombin cleavage determine substrate recognition by the prothrombinase complex (*32*). This derivative of factor Xa displayed a significant change in fluorescence anisotropy when incorporated into prothrombinase. We obtained [OG_{488}]-EGR-human factor Xa ([OG_{488}]-EGR-hXa]) and assayed this derivative for the change in the anisotropy when bound to purified human factor Va.

We observed a measurable increase in the fluorescent anisotropy of [OG_{488}]-EGR-hXa (10nM) when bound to phospholipid vesicles (PCPS, 10 μM) in the presence of increasing concentrations of factor Va (1 nM at a time for up to 25nM) added to the mixture (Figure 9A, filled circles). The sensitivity displayed was adequate and comparable to those used in previous kinetic studies (*16*, *32*). Titration of membrane-bound [OG_{488}]-EGR-hXa with increasing concentrations of human factor Va resulted in a saturable increase in the fluorescent anisotropy ($\Delta r \sim 0.042$). Half-maximum saturation was reached at approximately 6 nM. The data demonstrate direct interaction of factor Va to [OG_{488}]-EGR-hXa. The factor Va dependent increase in the anisotropy of [OG_{488}]-EGR-hXa was not observed in the presence of EDTA (Figure 9A, filled squares). These data are consistent with previous reports demonstrating that Ca^{2+} is required for the high affinity specific interaction between factor Va and factor Xa (*15*, *16*). Titration of reaction mixtures containing membrane-bound [OG_{488}]-EGR-hXa (10 nM) and human factor Va (25 nM) with increasing concentrations of inactivated nonfluorescent active-site labeled human factor Xa (EGR-hXa) resulted in a decrease of the anisotropy near to a value observed by [OG_{488}]-EGR-hXa alone (not shown). Thus, the interaction between [OG_{488}]-EGR-hXa and human factor Va is reversible, and this method can be used to ascertain the presence of a binding site for factor Xa on a candidate peptide, by direct displacement of the factor Va molecule that will result in a decrease in the anisotropy of [OG_{488}]-EGR-hXa. We have also assayed N42R for the direct interaction with [OG_{488}]-EGR-hXa (Figure 9B, filled circles). Titration of membrane-bound [OG_{488}]-EGR-hXa with increasing concentrations of N42R resulted in a saturable increase in the fluorescent anisotropy ($\Delta r \sim 0.045$). Half-maximum saturation was reached at approximately 230 nM. In the presence of EDTA, no significant change in the fluorescent anisotropy of [OG_{488}]-EGR-hXa was observed even in the presence of high concentrations of N42R (up to 800 nM, Figure 9B, filled squares). The data demonstrate direct binding of N42R to [OG_{488}]-EGR-hXa.

Titration of increasing concentrations of AP4′ into a preformed complex of membrane-bound [OG_{488}]-EGR-hXa-human factor Va (10 nM [OG_{488}]-EGR-hXa and 25 nM factor Va) resulted in the decrease of the factor Va-dependent anisotropy of [OG_{488}]-EGR-hXa (Figure 10A, filled circles) near to baseline levels in the presence of 20 μM peptide. The binding of human factor Va (25 nM) to [OG_{488}]-EGR-hXa (10 nM) was displaced by purified AP4′ with an IC_{50} of approximately 7.5 μM (Figure 10A, filled circles). In contrast, P15H did not induce a decrease in the fluorescent anisotropy of [OG_{488}]-EGR-hXa (Figure 10A, filled triangles) even at concentrations as high as 20μM demonstrating no effect of this region of factor Va (i.e., amino acid residues 337−351) on its interaction with factor Xa. These data confirm our results obtained with the functional prothrombinase assay. The specificity of the amino acid sequence of AP4′ for the displacement of factor Va from membrane-bound [OG_{488}]-EGR-hXa, was verified by using two control peptides: a peptide with the reverse sequence of AP4′ (AP4′ reverse, VEEAAIFYE (V9E), Figure 10A, open squares) and a peptide containing a scrambled version of AP4′ (AP4′ scrambled, AYIEVEFEA (A9A), Figure 10A, filled squares). As shown in Figure 10A, both peptides did not have any effect of factor Va binding to [OG_{488}]-EGR-hXa, at concentrations similar to that used by AP4′ to inhibit the factor Va dependent increase in the anisotropy of [OG_{488}]-EGR-hXa. The data strongly suggest that the amino acid sequence of AP4′ represents a specific binding site of factor Va for factor Xa.

Similar experiments using AP3, AP5, and AP6 at concentrations ranging from 1 to 30 μM each showed no effect on the anisotropy of [OG_{488}]-EGR-hXa when bound to human factor Va. However, high concentrations of AP3 and AP5 (up to 300 μM) showed a reproducible and significant decrease of 10% and 30%, respectively, in the anisotropy of [OG_{488}]-EGR-hXa bound to human factor Va (not shown). AP6 had no effect on the anisotropy of [OG_{488}]-EGR-hXa when bound to factor Va even at high concentrations of

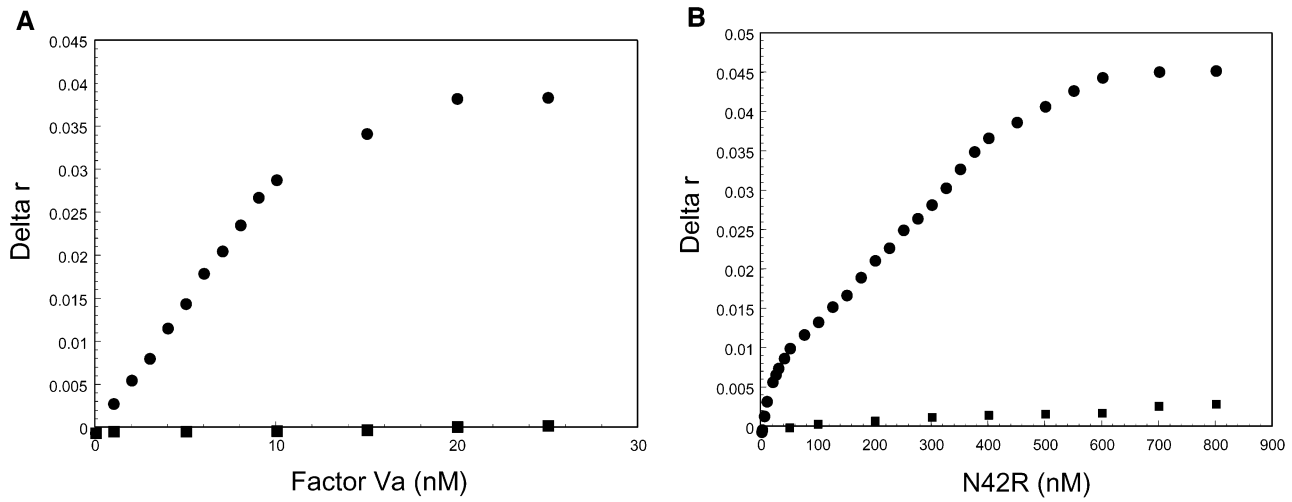

FIGURE 9: Fluorescence anisotropy measurements of prothrombinase assembly. (A) Prothrombinase was assembled with [OG_{488}]-EGR-hXa (10 nM), PCPS vesicles (10 μM) and increasing concentrations of human factor Va (filled circles). Titration of [OG_{488}]-EGR-hXa with factor Va in the presence of EDTA was also performed (filled squares). (B) [OG_{488}]-EGR-hXa (10 nM) in the presence of PCPS vesicles (10 μM) was titrated with increasing concentrations of N42R (filled circles). Similar experiments were also performed in the presence of EDTA (filled squares). Fluorescence anisotropy was measured at 25 °C, and the Δr was calculated as described (*32*) and detailed in the "Experimental Procedures" section. The data represent the average of the results found in three independent experiments for panel A, while the data in panel B represent all the data points from two independent experiments performed in triplicate.

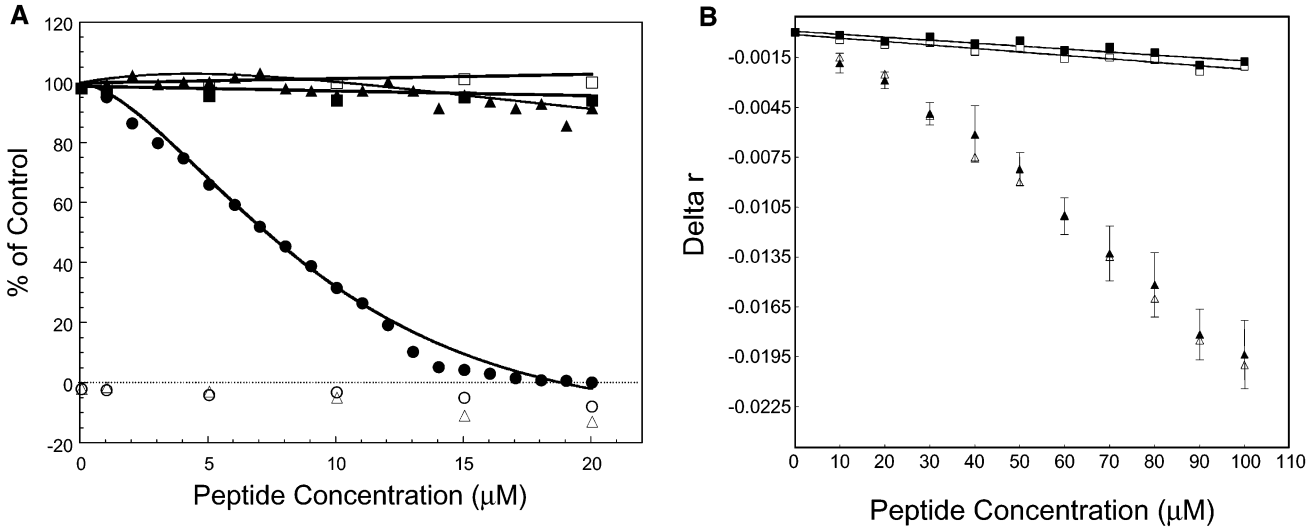

FIGURE 10: Contribution of AP4′ to the binding of factor Va to factor [OG_{488}]-EGR-hXa. (A) Inhibition of the factor Va-dependent fluorescence anisotropy increase of [OG_{488}]-EGR-hXa. Reaction mixtures containing a preformed complex composed of ([OG_{488}]-EGR-hXa at 10 nM and 10 μM PCPS) bound to factor Va (25 nM) were titrated with increasing concentrations of AP4′ (filled circles), P15H (filled triangles), AP4′ reverse (VEEAAIFYE, open squares), and AP4'scrambled (AYIEVEFEA, filled squares). Δr was calculated as described in the "Experimental Procedures" section and plotted as percent of the control value of the anisotropy in the absence of peptide. In separate experiments [OG_{488}]-EGR-hXa (10 nM) in the presence of PCPS vesicles (10 μM) but in the absence of factor Va was titrated with increasing concentrations of AP4′ (open circles) and P15H (open triangles). The data represent the average of the results found in three independent experiments using three different factor Va preparations. (B) Direct interaction of AP4′ with [OG_{488}]-EGR-hXa. Prothrombinase was assembled with [OG_{488}]-EGR-hXa (10 nM), PCPS vesicles (10 μM), and increasing concentrations of AP4′. The data shows two different titrations of [OG_{488}]-EGR-hXa with two different solutions of AP4′; one was performed in triplicate (filled triangles, with error bars) and the other one in duplicate (open triangles, average of two numbers). Titration of [OG_{488}]-EGR-hXa with AP4′ reverse (VEEAAIFYE, open squares) and AP4'scrambled (AYIEVEFEA, filled squares) was also performed. The data represent the average of the results found in three independent experiments using two different preparations of peptide.

peptide. A control experiment, measuring the change in the fluorescence anisotropy of [OG_{488}]-EGR-hXa when incubated with the synthetic peptides alone (AP4′ and P15H) in the absence of factor Va, at similar concentrations that were used for the displacement experiments, demonstrated no measurable changes in the fluorescent anisotropy of [OG_{488}]-EGR-hXa (Figure 10A, open circles and open triangles, for AP4′ and P15H, respectively). Thus, AP4′ alone had no detectable effect on the anisotropy of [OG_{488}]-EGR-hXa at concentrations, which resulted in the complete inhibition of the increase in the fluorescence anisotropy of [OG_{488}]-EGR-hXa mediated by factor Va. These data demonstrate that AP4′ competes with factor Va for the interaction with [OG_{488}]-EGR-hXa and suggest that AP4′ contains an interactive site for factor Xa. In separate experiments a significant and reproducible negative change (decrease) in the anisotropy of [OG_{488}]-EGR-hXa was observed when the molecule was incubated, with high concentrations of AP4′ alone, in the

absence of factor Va outside the range of concentrations shown in Figure 10A that inhibit the factor Va-dependent increase in the anisotropy of [OG_{488}]-EGR-hXa (30−100 μM, Figure 10B, filled triangles and open triangles). Half-maximal saturation was reached at approximately 65 μM. Further, AP4′ reverse and AP4′ scrambled did not produce a change in the fluorescent anisotropy of [OG_{488}]-EGR-hXa under similar experimental conditions (Figure 10B open squares and filled squares respectively). No significant changes in the fluorescent anisotropy of [OG_{488}]-EGR-hXa were observed when the fluorescent molecule alone was incubated with P15H even at concentrations as high as 300 μM (not shown). These data are in agreement with previous observations (*44*) and suggest a direct interaction between AP4′ and [OG_{488}]-EGR-hXa. These data also confirm our findings shown in Figure 6B demonstrating direct interaction of AP4′ with unlabeled human factor Xa, inducing an increase in the catalytic activity of the enzyme. Overall the data demonstrate the specificity of the amino acid sequence contained within AP4′, representing the region 323−331 of the heavy chain of factor Va, for its direct interaction with [OG_{488}]-EGR-hXa. However, it is important to note that N42R and AP4′ not only contain a binding site for factor Xa, but can also induce a factor Va-like effect on the enzyme.

DISCUSSION

Our data demonstrate that a binding site for factor Xa is located on the heavy chain of factor Va between amino acid residues 323−331. More specifically, the data suggest that amino acid sequence Glu^{323}−Val^{331} competes with factor Va for the high affinity binding to factor Xa on the membrane surface (Figure 11). Our data also suggest that amino acid residues 307−321 (AP1 and AP2) and 337−351 (P15H) of the cofactor do not have any major effect on prothrombinase complex assembly and function (Figure 11).

We have previously shown that the heavy chain of factor Va contains a binding site for factor Xa and/or prothrombin (*14*, *21*) and that region 307−348 of the heavy chain of the cofactor (N42R) is crucial for factor Va cofactor activity (Figure 11, ref. *14*). In the present study, we have narrowed down the inhibitory potential of N42R to a nonapeptide (AP4′, EYFIAAEEV, amino acid residues 323−331, Figure 11) and we have shown that the inhibitory potential of N42R is due to the ability of the nonapeptide to interfere with the high affinity interaction between factor Va and membrane-bound factor Xa. Our data also show that while AP3, AP4′, and AP5 inhibit prothrombinase activity, only AP4′ inhibits the direct interaction of the cofactor with [OG_{488}]-EGR-hXa. Further, AP4′ was found to interact directly with the fluorescent derivative of the enzyme and AP4′ alone, at high concentrations, had a "cofactor" effect on factor Xa increasing the catalytic efficiency of the enzyme by approximately 4-fold when compared to factor Xa alone. Altogether, these data strongly suggest that the entire amino acid sequence that is contained within AP4′ is a specific motif that represents a binding site of factor Va for factor Xa. In addition, it appears that AP4′ contains several amino acid(s) that are also responsible for the effector function of factor Va. A similar approach to the one described herein resulted in the identification of an octapeptide from the A2 domain of factor VIIIa that inhibited intrinsic tenase assembly and function (*45*). The peptide was also found to inhibit the factor

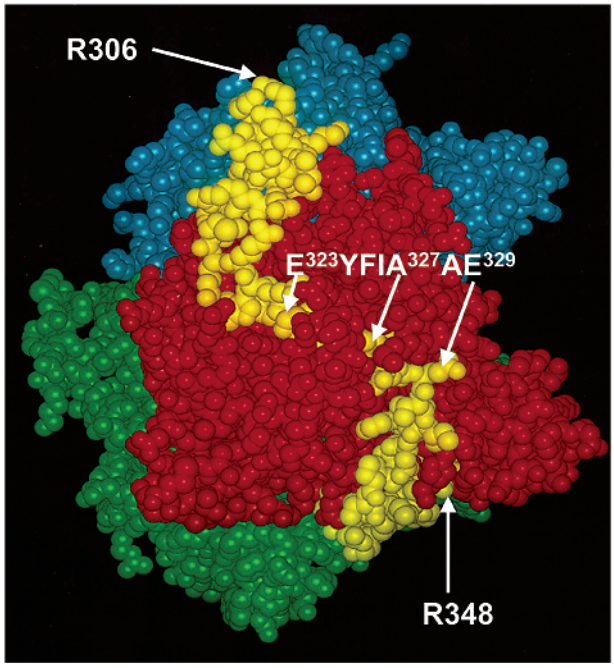

FIGURE 11: Structural analysis of the region 307−348. Structural analysis of the region 307−348 of factor Va (in yellow) was performed by using as a template the known X-ray structure of ceruloplasmin (*50*, *52*). The A domains of factor Va are represented in color as follows: A1, blue, A2 red, A3 green. Amino acid sequence $Y^{324}FIAA^{328}$ (in yellow, indicated by the white arrows) was found to be located inside the A2 domain of the cofactor (*14*, *50*, *52*). Exposure of these amino acids on the surface of the cofactor is hindered by two distal acidic amino acids (i.e., Asp^{373} and Glu^{374}) which are part of a hydrophilic loop of the A2 domain of factor Va (*51*). The position of amino acid residues Arg^{306} and Arg^{348} is indicated for orientation purposes.

VIIIa dependent-anisotropy increase of active site labeled fluorescein factor IXa (*46*, *47*). As a consequence, the factor VIII-derived octapeptide containing amino acid sequence 558−565 was reported to contain an interactive site for factor IXa (*46*). Our data are also comparable with studies showing inhibition of binding of active-site blocked factor IXa to the light chain of factor VIII by a pentadecapeptide containing a specific amino acid sequence that derives from factor VIII light chain (*48*).

The reasons for the dramatic enhancement of the catalytic efficiency of factor Xa when bound to factor Va are not yet fully understood. It is well established that activation of factor V by α-thrombin enables proper interaction of the cofactor with factor Xa and prothrombin. It is possible that factor Va positions the enzyme in a correct orientation that is necessary for optimum catalysis of prothrombin by factor Xa. Data using a recombinant human factor V molecule mutated at all proteolytic sites reported to be required for activation of the procofactor, showed that activation of the procofactor by α-thrombin resulted in exposure of binding site(s) for factor Xa and the increase in its affinity for the enzyme when the two molecules interacted on the membrane surface (*49*). The K_d of the cofactor for factor Xa in the absence of a membrane surface is 0.8 μM (*15*). It was suggested that factor Va and factor Xa first bind to the membrane surface and then slide toward each other (*16*). The dissociation rate constants of factor Va and factor Xa from the membrane surface are much lower than their respective association rate constants (*17*). Further, the

interaction of factor Va with the membrane surface and factor Xa increases the affinity of the enzyme for the phospholipid bilayer (*16*). The data accumulated thus far indicate that the binding of factor Va and/or factor Xa to the lipid bilayer most likely results in conformational changes in one or both proteins and this contribute to aspects of the factor Va-factor Xa interaction. Upon binding of both proteins to lipid, the K_d of the interaction between factor Va and factor Xa decreases by approximately 1000-fold to 1 nM (*16*), suggesting that more specific points of interaction between the two proteins are exposed following their binding to the membrane surface, resulting in the tighter interaction. Because of the physiological concentration of the two proteins in plasma, the only physiologically relevant K_d for the association between the two proteins is the K_d observed when the two proteins are bound to a membrane surface. In other words, because of their plasma concentrations, the two molecules interact in vivo only when bound to a membrane surface. Thus, upon binding of the light chain of the cofactor to a membrane surface, the newly exposed sequences on the heavy and light chains of factor Va may be the reason for its increase in the affinity for factor Xa. In contrast, while cleavage of factor Va by APC at Arg^{506} results in a 10-fold decrease in the affinity of the molecule for factor Xa, subsequent cleavage at Arg^{306} of the cofactor, and elimination of the A2 domain from the rest of the molecule produces a cofactor molecule that no longer interacts with factor Xa (*10*). Thus, the positive and negative regulatory processes associated with factor V activation and its inactivation by APC are central processes required for normal hemostasis and control of thrombosis.

Structural analyses of N42R using the known X-ray crystal structure of ceruloplasmin as a template (*14*, *50*) revealed that amino acids 324−328 are located inside the A2 domain (Figure 11, in yellow) and are not completely exposed on the surface of the molecule. The X-ray structure of ceruloplasmin as well as the hypothetical model of the A domains of factor V based on the coordinates of the latter, represent the structure of the two molecules in solution (not bound to a membrane surface) and to date are the only available models of this region of the cofactor (*50*−*52*). As a consequence of this hypothetical model of the cofactor, several amino acids that were found in the present study to be critical for the interaction between the two proteins are not completely exposed on the surface of the molecule when the cofactor is in solution (not bound to a membrane surface). According to the published model of factor Va in solution, the access to Tyr^{324} and Phe^{325} is hindered by two distal amino acids (i.e., Asp^{373} and Glu^{374}) located within a hydrophilic loop of the A2 domain of the cofactor (Figure 11, *49*, *51*). This may be of physiological relevance and appears to be necessary to avoid needless interaction of the cofactor with factor Xa that will ultimately result in unnecessary prothrombin activation when both proteins are not bound to a procoagulant membrane surface.

Most of the data related to factor Va function published thus far and especially the data relating to the mechanism of inactivation of the cofactor by APC and plasmin provide evidence for a conformational transition of the cofactor when bound to a membrane surface (*8*, *9*, *14*). All cleavages of the heavy chain that are reported to inactivate the cofactor are slow in the absence of a membrane surface (*9*, *14*), suggesting that the specific amino acids are not appropriately exposed on the surface of the cofactor for efficient proteolysis. The amino acid region 306−330 that is part of a flexible loop structure is located within a distorted helical segment of the A2 domain of the heavy chain of factor Va (Figure 11) (*50*, *52*). Upon binding to a membrane surface, the region 306−330 most likely undergoes a conformational transition facilitating cleavage and inactivation of the cofactor by APC and plasmin at Arg^{306}, Lys^{309}, Lys^{310}, and Arg^{313} (*9*, *14*). Cleavage of the cofactor at Arg^{348} by plasmin is also accelerated when factor Va is bound to lipids (*14*). Thus, upon binding to a membrane surface, a conformational transition of factor Va occurs, resulting in the exposure of the necessary amino acids which are critical for the cofactor's down regulation. Further, while the affinity of factor Va for factor Xa increases by 3 orders of magnitude in the presence of a membrane surface, under similar conditions, the catalytic efficiency of the enzyme is increased by 5 orders of magnitude (*2*, *16*). Thus, it is probable that amino acid region 323−331 of factor Va becomes available for the interaction with factor Xa upon binding of the cofactor to a lipid bilayer. As a consequence, this region of the cofactor may act as a control switch turning on and off the activity of prothrombinase (i.e., regulating the high affinity interaction with factor Xa). It is noteworthy that a change in protein conformation, which is followed by exposure of critical amino acids upon activation, is very common among regulatory proteins that include integrins and G proteins. For example, using a similar procedure as the one described herein a binding site for the complement iC3b fragment was localized to a small linear peptide contained within the integrin complement receptor type 3 (CR3) A-domain (*53*). The peptide was also found to inhibit iC3b binding to the A-domain alone as well as to CR3. Data published subsequently to these findings and based on a model built from the crystal structure of the A domain from the α chain of integrin CR3 suggested that the inhibitory peptides and particularly the amino acids of interest (i.e. Phe^{275} and Phe^{302}) were buried inside the structure of the A domain of the integrin (*54*). At first, the two sets of data appeared to be in contradiction; however, recent results have demonstrated that integrins switch from a low to a high affinity binding state following a change in conformation, in response to cell activation signals, exposing high-affinity binding sites for ligands that are buried inside the structure of the nonactive form of the molecules (*55*−*58*). Thus, in the open (active) form, the two phenylalanines (Phe^{275} and Phe^{302}) are solvent exposed due to conformational changes involving several domains of the integrin. A similar change in structural conformation with major rearrangements upon activation and hydrolysis of GTP was also observed in the signal-transducing G-proteins (*59*, *60*). Thus, since factor Va is at least part of the physiological receptor and catalytic effector for factor Xa on the platelet surface (*61*, *62*), and since all procoagulant reactions occur on a membrane/cell surface (*1*, *63*), it is logical to assume that the portion of factor Va heavy chain conferring its high affinity for factor Xa (amino acid residues 323−331) is partially hidden within the core of the active cofactor in the absence of a procoagulant cell surface and is not appropriately oriented for optimum interaction with factor Xa. This region of the heavy chain of factor Va is rearranged and the appropriate amino acids exposed on the surface following a conformational

transition occurring when factor Va interacts with an appropriate (procoagulant) membrane surface at the place of vascular injury. Our data underline the importance of this region of the molecule for expression of cofactor activity. As a consequence, factor Va switches from a low affinity state (in solution) to a high affinity state for factor Xa upon binding to a membrane surface, exposing more amino acids residues able to specifically interact with the enzyme.

In conclusion, our data identifies a physiologically relevant binding site for factor Xa within amino acid residues 323−331 of factor Va that is responsible for the high affinity interaction between the two proteins. This amino acid sequence is crucial for the interaction of the cofactor with factor Xa on the membrane surface, appears to be at least partially responsible for the receptor and effector properties of factor Va, and thus seems to regulate prothrombinase assembly and function.

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