The Role of the Membrane in the Inactivation of Factor Va by Plasmin

AMINO ACID REGION 307–348 OF FACTOR V PLAYS A CRITICAL ROLE IN FACTOR Va COFACTOR FUNCTION

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The mechanism of inactivation of bovine factor Va by plasmin was studied in the presence and absence of phospholipid vesicles (PCPS vesicles). Following 60-min incubation with plasmin (4 nm) membrane-bound factor Va (400 nm) is completely inactive, whereas in the absence of phospholipid vesicles following a 1-h incubation period, the cofactor retains 90% of its initial cofactor activity. Amino acid sequencing of the fragments deriving from cleavage of factor Va by plasmin demonstrated that while both chains of factor Va are cleaved by plasmin, only cleavage of the heavy chain correlates with inactivation of the cofactor. In the presence of a membrane surface the heavy chain of the bovine cofactor is first cleaved at Arg348 to generate a fragment of membrane surface the heavy chain of the bovine cofactor with inactivation of the cofactor. In the presence of a lipid bilayer with factor Va assembled on a membrane surface in the presence of Ca$^{2+}$ ions (1). Formation of the prothrombinase complex increases the catalytic efficiency of prothrombin activation by 5 orders of magnitude as compared with factor Xa alone (2).

Plasmin was studied in the presence and absence of phospholipid vesicles (PCPS vesicles). Following 60-min incubation with plasmin at 4 nm, complete inactivation of factor Va was observed (1). The active form of the protein, factor Va, contains a heavy chain (Mr 94,000), which is derived from the NH$_2$-terminal part of the molecule (amino acids 1–713), and a light chain (Mr 42,000) (Fig. 1). These cleavages result in the release of the COOH terminus of the molecule and the production of a Mr 40,000 fragment containing the NH$_2$-terminal portion of the factor Va molecule. Factor Va was treated with plasmin in the absence of phospholipid vesicles followed by the addition of PCPS vesicles and activated protein C (APC). A rapid inactivation of the cofactor was observed as a result of cleavage of the Mr 47,000 fragment at Arg306 by APC and appearance of a Mr 39,000 fragment. These data suggest a critical role of the amino acid sequence 307–348 of factor Va. A 42-amino acid peptide encompassing the region 307–348 of human factor Va (N42R) was found to be a good inhibitor of factor Va clotting activity with an IC$_{50}$ of 1.3 mM. These data suggest that plasmin is a potent inactivator of factor Va and that region 307–348 of the cofactor plays a critical role in cofactor function and may be responsible for the interaction of the cofactor with factor Xa and/or prothrombin.

Coagulation involves a multitude of proteins that respond to a vascular injury by forming the procoagulant enzyme α-thrombin. Prothrombin is activated to α-thrombin by the prothrombinase complex, which is composed of the serine protease factor Xa, and the protein cofactor factor Va assembled on a membrane surface in the presence of Ca$^{2+}$ ions (1). Formation of the prothrombinase complex increases the catalytic efficiency of prothrombin activation by 5 orders of magnitude as compared with factor Xa alone (2).

Factor V circulates in plasma as a single chain procofactor (Mr, 330,000). The cDNA sequence for bovine factor V and the deduced amino acid sequence has been determined previously (3). The active form of the protein, factor Va, contains a heavy chain (Mr, 94,000), which is derived from the NH$_2$-terminal part of the procofactor (residues 1–713), and a light chain (Mr, 74,000), which corresponds to the COOH-terminal part of the molecule (amino acids 1537–2183 (9)) (see Fig. 1). The two chains are non-covalently associated via divalent metal ions (4). The inactivation of both bovine and human factor Va by APC occurs in the presence of a lipid bilayer or platelet surface and is well documented (5, 6). APC inactivates bovine factor Va by cleavage at Arg$^{306}$, Arg$^{306}$, and Arg$^{306}$ (5) (Fig. 1).

Blood coagulation and fibrinolysis, although having completely opposite influences on the hemostatic process, communicate and regulate each other via several proteins. Important regulatory roles are exercised by thrombin on fibrin clot formation and fibrin clot solubilization. Although the former role of thrombin is well established, the latter effect is exercised on thrombolyis via thrombin activatable fibrinolyis inhibitor, which is both activated and subsequently inactivated by α-thrombin (7, 8). The effect of plasmin, the end product of fibrinolysis, on several coagulation proteins (i.e. factors IX and X) has been recently reported (9, 10). It has also been shown that plasmin is a potent inactivator of factor Va, and previous studies have demonstrated that bovine factor Va is inactivated by plasmin in the absence and presence of a lipid bilayer following cleavages of both the heavy and light chains (11). The effect of plasmin on the human procofactor has been also reported previously (12). However, no details concerning the specific proteolytic cleavage sites on factor V/Va have yet been

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‡ The abbreviations used are: APC, activated protein C; PS, 1-palmitoyl-2-oleoyl-phosphatidylserine; PCPS, phosphatidylcholine phosphatidylserine; DAPA, dansylarginine-(3-ethyl-1,5-pentanediyl)amide; PAGE, polyacrylamide gel electrophoresis; PVDF, polyvinylidene difluoride; DIC, differentiated intravascular coagulation.

1 The numbering in this study refers to bovine coagulation factor V (see Fig. 1).
Inactivation of Membrane-bound Factor Va Plasmin

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**Figure 1.** Schematic representation of the proteolytic cleavages activating (upper) or inactivating (lower) bovine factor Va. The factor Va heavy chain (residues 1–713) is composed of two A domains associated through a connecting region (amino acid residues 305–319). The light chain of the cofactor (amino acid residues 1537–2183) includes one A and two C domains. The position of the activating cleavage sites as well as the position of the factor Xa cleavage are shown on top. The inactivating cleavage sites by APC are shown on the bottom. Positions of the disulfide bridges and free cysteines are also indicated.

**Experimental Procedures**

**Materials and Reagents—**N-(2-Hydroxyethyl)piperazine-N′-2-ethanesulfonic acid (Hepes), Q-Sepharose fast flow, Sepharose CL-4B, cyanogen bromide (CNBr), bovine serum albumin, 1-palmitoyl-2-oleoyl-phosphatidyl serine (PS), and 1-palmitoyl-2-oleoyl phosphatidyl choline (PC) were purchased from Sigma Chemical Co. (St. Louis, MO). N-Phenylalanlyglycylarginyl-chloromethylketone was purchased from Calbiochem (San Diego, CA). The fluorescent thrombin inhibitor dansylarginine-N-(3-ethyl-1,5-pentanediamine) (DAPA) and bovine APC were obtained as previously described (13, 14) and were gifts of Dr. Paul Haley (Hematologic Technologies Inc., Essex Junction, VT). Bovine prothrombin, thrombin, and factor Xa were obtained according to previously described methods (15, 16). The synthetic peptides used in this study (i.e., D13R and N42R, were purchased from Peninsula Laboratories Europe Ltd., Merseyside, England). Bovine factor Va was obtained as previously described (17, 18). Phospholipid vesicles composed of 75% PC and 25% PS, were prepared as previously described (19). The concentration of each mixture was determined by phosphorous assay (20).

**Assay Measuring Thrombin Formation—**The formation of thrombin was analyzed using the fluorescent thrombin inhibitor DAPA. The buffer used in all cases was composed of 20 mM Hepes, 0.15 M NaCl, 5 mM CaCl2, pH 7.4 (HBS/Ca2+)). In a typical experiment, a mixture containing prothrombin (1.4 μM), DAPA (3 μM) and phospholipid vesicles (20 μM), composed of 75% PC and 25% PS, was incubated in the dark for 20 min. At selected time intervals an aliquot of the mixture (1.85 μl) was added to a cuvette containing 10 μl factor Xa, and the baseline was monitored for 15 s at room temperature using a PerkinElmer Life Sciences MFP-44A fluorescence spectrophotometer with λex = 280 nm, λem = 550 nm and a 500-nm long pass filter in the emission beam.

**Factor Va Plasmin Cleavage Studies—**Bovine factor Va (400 nM) was incubated with APC (4 nM) in the absence and presence of PCPS (50 μM) at 37 °C. At selected time intervals aliquots of the mixture (10 μl) were added to the cuvette containing prothrombin, DAPA, PCPS, and factor Xa, and the fluorescence intensity due to the formation of thrombin and its complexation with DAPA was monitored with time (the final concentration of factor Va in the mixture was 1 nM). Under these conditions the rate of thrombin formation is linearly related to the amount of active cofactor (factor Va). The initial rate of the formation of thrombin (nanomolar Ia min−1 nm−2) was calculated as described previously (5, 13). At the same time intervals another aliquot of the mixture (140 μl) was mixed with 2% SDS, 2% β-mercaptoethanol heated for 5 min at 90 °C and stored at −20 °C prior to analysis by SDS-PAGE.

**Clotting Assay—**Factor Va cofactor activity is measured at selected time intervals by clotting assay using factor V-deficient plasma (immunodepleted) is mixed with an equal volume of the sample to be analyzed. To start the assay 100 μl of the PT reagent (Simplastin Excel) is added while rocking the tube at 37 °C. The assay end point is determined by visualization of the fibrin clot. A standard curve using normal plasma (dilution of normal plasma from 1:4 to 1:1024) is established before each experiment. The standard curves are linear when plotted as a log of clotting time versus log units/ml. Control experiments using an enzyme solution alone (plasmin, in the absence of factor V) in the clotting assay, at the same concentrations that are used to cleave factor V, demonstrate no interference with the clotting process (≥ normal plasma). This control is important, because it will show if trace amounts of the enzymes introduced into plasma during the clotting assay (or in the prothrombinase assay) will (or will not) interfere with the results obtained during the period of time that the assay is performed. Factor Va molecules in the presence or absence of plasmin are assayed directly in such a clotting assay.

**Inhibition of Factor V/Va Clotting Activity by Synthetic Peptides—**Inhibition studies of factor V/Va clotting activity by N42R and D13R were performed as follows: Factor V was diluted to a concentration (1–10 nM) that will give a clotting time of ~25 s. This value was converted to units/ml and was arbitrarily determined to be 100% activity. The same amount of factor V was incubated with serial dilutions of synthetic peptide. The mixture was assayed for clotting activity. The results were plotted as a percentage of control of factor V activity as a function of synthetic peptide concentration. The same experimental protocol was performed with factor Va with the following exception: factor V (50 nM) was treated with 1 nM α-thrombin for 10 min following by the addition of hirudin (2 nM). Factor Va was then diluted in a Ca2+-containing buffer to a concentration (0.1–1 μM) corresponding to a clotting time of ~20 s. Factor Va clotting activity was then assayed in the presence of increasing concentrations of synthetic peptide and plotted as above as a percentage of control of factor Va clotting activity as a function of synthetic peptide concentration.

**Synthetic Peptide Cleavage by Plasmin—**N42R (348 or 34.8 μM) was incubated with plasmin (350 nM) for 24 h at 37 °C. Following digestion, the reaction was stopped by freezing. The masses of the resulting peptides were determined by automatic Edman degradation on a 475A protein sequencing system (Applied Biosystems, Foster City, CA). The NH2-terminal sequences of the resulting peptides were determined by mass spectrometry using an ABI Voyager DE-STR matrix-assisted laser desorption time-of-flight mass spectrometer in the laboratory of Dr. Alex Kurosky (University of Texas, Medical Branch at Galveston, Galveston, TX). NH2-terminal sequence analysis of the resulting peptides was also performed in the same bioanalytical facility.

**Gel Electrophoresis—**SDS-PAGE analysis were performed using 5–15% and 8–18% gradient gels according to the method of Laemmli (21). Proteins are visualized by 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.

**Electroblotting and Amino Acid Sequencing—**An 8–18% gradient SDS-PAGE gel was used to analyze the proteolytic fragments resulting from the digestion of factor Va by APC in the absence or presence of PCPS vesicles. Usually the digestion was performed for 3 h at 37 °C using 50 μg of bovine factor Va at 1/20 enzyme/substrate ratio (molar ratio). The samples were analyzed, reduced, and transferred to a polyvinylidene difluoride (PVDF) membrane using a modification of a previously described method (5, 22). The NH2-terminal amino acid sequences of the peptides were determined by automatic Edman degradation on a 475A protein sequencing system (Applied Biosystems, Branch at Galveston, Galveston, TX).
slow loss in factor Va cofactor activity (Fig. 2, filled circles).

A close examination of the two gels depicted in Fig. 3 suggest that only the disappearance of the $M_r 47,000$ fragment (shown by a star in Fig. 3A, lane 5, and identified as A$^i$ at the right of lane 14) is associated with the dramatic decrease in factor Va cofactor activity when the latter is incubated with plasmin and PCPS vesicles (Fig. 3A, lanes 5–10) compared with the same reaction in the absence of phospholipid vesicles (Fig. 3B, lanes 5–13, A$^i$). A comparison between our data and previous results analyzing factor Va inactivation by plasmin (11) suggest that the fragments of $M_r 48,000, 46,000$, and 30,000 derive from the light chain of the cofactor. These products are also observed following APC cleavage of the light chain of the cofactor from either human or bovine origin (5, 6). The fragments of $M_r 47,000, 42,000$, and 40,000 appear to be derived from the heavy chain of the cofactor. Altogether these data demonstrate that: 1) the light chain of bovine factor Va is cleaved by plasmin at Arg$^{1752}$ to generate a $M_r 46,000/48,000$ doublet and a $M_r 30,000$ fragment representing the COOH- and NH$_2$-terminal portions of the light chain, respectively; 2) disappearance of the fragment of $M_r 47,000$ and appearance of a $M_r 40,000$ fragment correlates with loss of factor Va cofactor activity observed in the presence of PCPS and plasmin (Fig. 3A, lanes 5–10); 3) the slow cleavage of the fragment of $M_r 47,000$ by plasmin in the absence of a lipid surface results in the slow disappearance of the cofactor activity (Fig. 3B, lanes 7–13).

Membrane-dependent Cleavage and Inactivation of Factor Va by Plasmin—To ascertain whether cleavage of the $M_r 47,000$ fragment correlates with inactivation of the cofactor and to understand the effect of the membrane surface on the efficiency of plasmin to cleave and inactivate factor Va, two solutions of factor Va were prepared. These solutions were incubated with plasmin for 3 h in the absence of a membrane surface. As expected, following a 3-h incubation with plasmin, factor Va loses ~30–40% of its initial cofactor activity (Figs. 4 and 5). The expected proteolytic fragments were observed during this period of time (Figs. 6 and 7, lanes 1–5). PCPS vesicles and APC were then added to the plasmin-treated cofactor in one solution. In the other solution only PCPS vesicles were added. The arrows in Figs. 4 and 6 indicate the point of addition of PCPS vesicles and APC. In Figs. 5 and 7 the arrows indicate the point of addition of PCPS alone. The addition of a PCPS membrane surface and APC resulted in the immediate loss of the cofactor activity (Fig. 4, arrow-filled circles). In contrast, only a 25% loss in activity was observed when PCPS vesicles alone were added to the plasmin-treated cofactor (Fig. 5, arrow-filled circles). This initial drop of activity was followed by a slow decrease in cofactor activity with 20% cofactor activity remaining after a 4-h incubation period (Fig. 5).

Analyses of the cleavage pattern of the plasmin-treated cofactor by APC/PCPS revealed that the fragments of $M_r 47,000$ and 42,000 were cleaved (Fig. 6, lane 6). Cleavage of the fragment of $M_r 47,000$ was rapid whereas the cleavage of the fragment of $M_r 42,000$ was slower. Coincident with these cleavages is appearance of fragments of $M_r 40,000, 24,000$, and a $M_r 18,000/20,000$ doublet. The arrowhead at the bottom of the figure identifies fragments of factor Va that appear following the addition of APC/PCPS to the plasmin-treated cofactor.

No striking differences in the cleavage pattern of the plasmin-treated cofactor were observed following the addition of PCPS vesicles alone to the plasmin-treated cofactor (Fig. 7) other than the slow cleavage of the $M_r 47,000$ fragment (Fig. 7, lanes 6–13). These data indicate that complete inactivation of the plasmin-treated cofactor by APC/PCPS is most likely correlated with cleavage of the $M_r 47,000$ fragment at Arg$^{306}$. This cleavage is rapid when the membrane-bound cofactor is incu-
bated with plasmin. In marked contrast to the APC/PCPS mixture, the addition of PCPS vesicles alone to the plasmin treated-cofactor has no accelerating effect on the Mr 47,000 fragment cleavage. These data suggest an inactivating cleavage site by plasmin on the cofactor different than Arg 306.

Identification of the Factor Va-derived Fragments—All fragments deriving from proteolytic cleavage of factor Va by plasmin in the presence or absence of PCPS vesicles were analyzed for NH2-terminal sequence and compared with the amino acid sequence derived from the cDNA of the bovine molecule (3). The fragments depicted in Fig. 8 show factor Va incubated with plasmin in the absence of a membrane surface. Two NH2-terminal sequences were found for the Mr 48,000 fragment (A) and for the Mr 46,000 (B) fragment indicating that plasmin cleaves the light chain of the cofactor at two positions, Arg 1752 and Arg 1753, generating fragments that differ by one amino acid at their NH2 termini. This proteolytic “wobble” of the bovine light chain cleavage was previously observed when using bovine APC (5) and demonstrates that bovine factor Va light chain has a very sensitive region exposing two cleavage sites at either Arg1752 or Arg1753.

The Mr 47,000 fragment (d′), which is first fragment generated following plasmin cleavage (Fig. 3A, lane 1, and Fig. 3B, lane 3) has a sequence that matches the NH2-terminal sequence of factor V. The Mr 42,000 fragment (c) has an NH2-terminal sequence that matches with a portion of factor Va heavy chain starting at amino acid residue 349. The Mr 40,000 fragment (d) has a sequence matching the amino terminus of the heavy chain while the Mr 30,000 fragment (e) is the NH2-terminal of the bovine factor Va light chain. Thus, the heavy chain of the cofactor is first cleaved at Arg1753 to produce a Mr 47,000 fragment (d′) spanning amino acid residues 1–348, which contains the A1 domain, the connecting basic region and portion of the A2 domain of factor Va, and a complementary fragment (c) representing the rest of the heavy chain of the cofactor. The NH2-terminal fragment (d′) of the heavy chain is subsequently cleaved at the COOH-terminal portion to generate a Mr 40,000 fragment (d) with an NH2-terminal sequence corresponding to the NH2-terminal sequence of bovine factor V. This cleavage appears to be slow in the absence of a membrane surface. It is noteworthy that for the sequencing reaction from PVDF membranes the gels were loaded with ~30 µg of total protein per lane. Thus, we were able to sequence any fragments that would derive from secondary proteolytic action of plasmin.

Fig. 3. Analysis of factor Va inactivation by plasmin. The samples assayed for activity in Fig. 2 were also analyzed on a 8–18% SDS-PAGE. A, proteolysis of membrane-bound factor Va by plasmin. Lane 1, factor Va control, no plasmin; lanes 2–13, factor Va and plasmin at 1, 3, 5, 7, 10, 15, 20, 30, 45, 60, 120, and 180 min; lane 14, membrane-bound factor Va, no plasmin after incubation at 37 °C for 180 min. The position of the molecular weight markers is indicated on the left. B, proteolysis of factor Va by plasmin in the absence of PCPS (same time points as A). Molecular weight markers are indicated at the left of A. The star in lane 5A identifies the fragment that disappearance correlates with inactivation. The open arrowheads identify light chain-derived fragments. The schematic at the bottom identifies the origin of the fragments. For the easy reading of the manuscript, some of the fragments are identified by the first amino acid at their NH2 termini (position in the bovine factor V sequence as superscript). Secondary cleavages by plasmin, that are of no consequence on cofactor activity are identified by the dashed arrows at the bottom of the graph. Detailed description of the results obtained for NH2-terminal sequencing of all fragments is provided in Figs. 8–10. Fragments are as follows: Mr 47,000 (A1), Mr 40,000 (A1), Mr 42,000 (S349), Mr 48,000 (A1753), Mr 46,000 (A1753), and Mr 30,000 (S1537).
The two fragments of Mr 20,000 and 24,000 that stain very poorly with Coomassie (bottom of Fig. 8) were sequenced, and both fragments had an NH2-terminal sequence matching a stretch starting at amino acid residue 506 of the bovine factor V molecule. Thus, plasmin cleaves bovine factor Va heavy chain in the absence of a membrane surface very slowly at Arg505. Several other smaller fragments were sequenced, and all gave the same NH2-terminal sequence corresponding to a sequence of factor V starting at amino acid residue 506. However, the concentration of all these fragments was very low, because only few amino acids could be determined with certainty because of low yields.

To understand the effect of lipids on the plasmin-mediated inactivation of factor Va, all fragments derived from the digestion of the membrane-bound cofactor by plasmin following a 60-min incubation period were sequenced, and the results are depicted in Fig. 9. All fragments found in the absence of a membrane-surface are produced in the presence of PCPS vesicles. The only new cleavage site observed is on the light chain at Lys1643. This cleavage was previously described to occur only at Arg306. Factor Va (400 nM) was incubated with plasmin (4 nM) at 37 °C. At selected time intervals aliquot of the mixture was assessed for cofactor activity as described under “Experimental Procedures.” At the same time intervals, aliquots were mixed with 2% SDS, 2% β-mercaptoethanol, heated for 5 min at 90 °C, and analyzed by SDS-PAGE (Fig. 6). Following 3-h incubation, PCPS vesicles (40 μM final concentration) and APC (20 nM) were added to the mixture and incubation was allowed to proceed for 120 additional minutes. Results are expressed as percent of initial cofactor activity as a function of time before and after addition of APC/PCPS vesicles. The arrow indicates the moment of the addition of APC/PCPS (185 min).

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**Fig. 4.** Inactivation of factor Va correlates with cleavage of the Mr 47,000 fragment of the heavy chain at Arg306. Factor Va (400 nM) was incubated with plasmin (4 nM) at 37 °C. At selected time intervals, aliquots of the mixture were assessed for cofactor activity as described under “Experimental Procedures.” At the same time intervals, aliquots were also mixed with 2% SDS, 2% β-mercaptoethanol, heated for 5 min at 90 °C, and analyzed by SDS-PAGE (Fig. 6). Following 3-h incubation, PCPS vesicles (40 μM final concentration) and APC (20 nM) were added to the mixture and incubation was allowed to proceed for 120 additional minutes. Results are expressed as percent of initial cofactor activity as a function of time before and after addition of APC/PCPS vesicles. The arrow indicates the moment of the addition of APC/PCPS (185 min).

**Fig. 5.** Inactivation of factor Va by plasmin (± a membrane surface). Factor Va (400 nM) was incubated with plasmin (4 nM) at 37 °C. At selected time intervals, aliquots of the mixture were assessed for cofactor activity as described under “Experimental Procedures.” At the same time intervals, aliquots were also mixed with 2% SDS, 2% β-mercaptoethanol, heated for 5 min at 90 °C, and analyzed by SDS-PAGE (Fig. 6). Following 3-h incubation, PCPS vesicles (40 μM final concentration) and APC (20 nM) were added to the mixture, and incubation was allowed to proceed for 120 additional minutes. Results are expressed as percent of initial cofactor activity as a function of time before and after addition of the PCPS vesicles. The arrow indicates the moment of the addition of PCPS (185 min).

**Fig. 6.** Analysis of the factor Va inactivation by the APC/PCPS mixture of the plasmin-treated cofactor. SDS-PAGE analysis (8–18% linear gradient gel) stained with Coomassie Blue of the samples shown in Fig. 4. Lane 1, factor Va control, no plasmin; lanes 2–5, factor Va and plasmin in the absence of PCPS vesicles at 15 min, 30 min, 1 h, and 3 h. Following 185-min incubation at 37 °C with plasmin, PCPS vesicles and APC were added to the mixture. Lanes 6–13 depict aliquots withdrawn from the solution at 1 min, 3 min, 5 min, 10 min, 15 min, 30 min, 60 min, and 2 h after the addition of APC/PCPS. The position of the molecular weight markers is indicated at left. The arrowhead identifies fragments that appear following the addition of the APC/PCPS mixture. The doublet of Mr 39,000/40,000 results from cleavage of the Mr 47,000 by plasmin and APC.

**Fig. 7.** Analysis of the factor Va inactivation following the addition of a membrane surface to the plasmin-treated cofactor. SDS-PAGE analysis (8–18% linear gradient gel) stained with Coomassie Blue of the samples shown in Fig. 5. Lane 1, factor Va control, no plasmin; lanes 2–5, factor Va and plasmin in the absence of PCPS vesicles at 15 min, 30 min, 1 h, and 3 h. Following 185-min incubation at 37 °C with plasmin, PCPS vesicles were added to the mixture. Lanes 6–13 depict aliquots withdrawn from the solution at 1 min, 3 min, 5 min, 10 min, 15 min, 30 min, 60 min, and 2 h after the addition of PCPS. Lane 14, factor Va in the absence of plasmin following incubation at 37 °C for 5 h. The position of the molecular weight markers is indicated at left.
on the bovine cofactor when the membrane-bound light chain of factor Va was incubated with trypsin (23). The concentrations of low molecular weight fragments was sufficient to allow for sequencing of 10–15 amino acids with a picomolar or subpicomolar level. Thus, production of all these fragments is accelerated in the presence of a membrane surface.

Previous data has demonstrated that cleavage at Arg348 by factor Xa has no effect on cofactor activity (24). Factor Xa has only one cleavage site on the heavy chain (i.e., Arg348) whereas plasmin has at least two major cleavage sites. Collectively these data demonstrate that cleavage of the M, 47,000 fragment (amino acid residues 1–348) at the COOH terminus is most likely responsible for the complete loss of factor Va cofactor activity. Thus, the minimal loss in cofactor activity observed when the cofactor is incubated with plasmin alone (Fig. 2, filled circles −10% after a 1-h incubation period and −30% after a 3-h incubation period) is due to slow cleavage of the M, 47,000 at the COOH-terminal portion and slow generation of a M, 40,000 fragment (Fig. 3B, lanes 9–13). The present data also demonstrate that cleavage at Arg505 is substantially accelerated in the presence of PCPS vesicles. A phospholipid-associated rate increase of the cleavage sites was previously reported for cleavage of both bovine and human factor Va by APC, which is a membrane binding protease (5, 6, 25). In contrast, plasmin does not bind to PCPS membranes, suggesting that the rate increase for all cleavages is a property of the substrate binding to membranes.

To test whether cleavage of the M, 47,000 fragment is completely responsible for the total loss in cofactor activity, factor Va was incubated with plasmin under identical conditions as in Fig. 8, followed by the addition of the APC/PCPS mixture. Previous data have demonstrated that APC cleaves the heavy chain of factor Va in the presence of PCPS vesicles at Arg306, which is located on the M, 47,000 fragment. The results, shown in Fig. 10, demonstrate that the loss in activity of the cofactor following plasmin addition is the result of cleavage of the M, 47,000 fragment at Arg506 and appearance of a M, 39,000 fragment (c). These data also demonstrate that the inactivating cleavage site at the COOH-terminal portion of the M, 47,000 fragment by plasmin is close to Arg506, because cleavage by APC and plasmin generates similar but not identical fragments (Fig. 6).

Close examination of Fig. 6 demonstrates a doublet of M, 39,000/40,000. The products seen in Fig. 6 are the consequence of two proteases, APC and plasmin. NH2-terminal sequencing demonstrates that both fragments of the doublet have the same NH2-terminal sequence, which matches the NH2-terminal sequence of factor V. Most likely the more intense band of the doublet (the lower band) is produced by cleavage of the M, 47,000 fragment at Arg506 by APC, whereas the upper band, which appears to be less concentrated, results from cleavage of the M, 47,000 fragment by plasmin at a position distal to Arg506. This region of the cofactor contains multiple lysines and arginines (3). Most likely plasmin cleaves the M, 47,000 fragment in the region 307–325.

Collectively, these data suggest that the inactivating cleavage by plasmin at the COOH terminus of the M, 47,000 fragment is efficient only on the membrane-bound cofactor and immediately follows cleavage at Arg348. In contrast, complete cleavage of the cofactor at Arg348 in solution followed by its binding to a membrane surface results in a diminished rate of
cleavage within the region 307–325. Thus, the factor Va structure is influenced by binding to the membrane surface. Plasmin, unlike APC, does not interact with PCPS vesicles, therefore, cleavage of the cofactor by plasmin at the COOH terminus of the M, 47,000 fragment is probably impaired because the enzyme cannot efficiently attack the membrane-bound cofactor.

Inhibition of Factor Va Clotting Activity by a Synthetic Peptide Containing Amino Acid Residues 307–348 of Factor V—Structural analysis of the region 307–348 of factor Va, by using the known x-ray structure of ceruloplasmin as a template (26), revealed that this region is located on the surface of the molecule and that only amino acids 324–328 are buried inside the A2 domain of factor V (Fig. 11, in black). The region 307–348 is 80% conserved in the human, bovine, and mouse sequences, suggesting its importance throughout evolution (Fig. 12) (3, 27, 28). To ascertain the importance of this region for factor Va cofactor activity, we obtained a 42-amino acid peptide encompassing the region 307–348 of human factor Va (N42R, Fig. 13, filled squares). The inhibitory potential of the peptide was tested for inhibition of factor V clotting activity. The peptide is a potent inhibitor of factor Va clotting activity with an IC50 of ~1.3 μM (Fig. 13, filled squares, inset). Recently, Bakker et al. (29), using a protease purified from the venom of the snake Naja naja oxiana, prepared a species of factor Va missing the COOH-terminal portion of the heavy chain (residues 683–709). These investigators concluded that the region 683–709 is required for the binding of factor Xa and prothrombin to factor Va. In our hands, a peptide from this region, representing the last 13 amino acids of the COOH terminus of the heavy chain of human factor Va (D13R, amino acid residues 686–709) did not inhibit factor Va clotting activity even at concentrations as high as 250 μM (Fig. 13, filled circles). However, the apparent discrepancy between the latter study and our studies may be due to differences in assays used to test factor Va activity and function. Overall, our data strongly suggest that region 307–348 is important for factor Va cofactor activity. Our data also indicate that although plasmin cleavage at Arg348 has no effect on factor Va cofactor activity, plasmin cleavage within amino acid region 307–325 inactivates factor Va.

Identification of the Plasmin Cleavage Site within Amino Acid Region 307–348—To identify the plasmin cleavage site within this critical region of factor Va, N42R (348 μM) was incubated with plasmin (350 nM). Proteolytic processing of

\[
\text{Ham: NLKKI TREQRR \text{IY} KRWEEFIAEVEIWY YAPY IPANMDK} \text{K} \text{Y} \\
\text{Rev: NK}K\text{I TREQRR \text{IY} KRWEEFIAEVEIWY YAPY IPANMDK} \text{K} \text{Y} \\
\text{Meas: SPK}K\text{I TREQRR \text{IY} KRWEEFIAEVEIWY YAPY IPANMDK} \text{K} \text{Y}
\]

FIG. 12. Comparison of the amino acid region 307–348 of factor V between species. The asterisk indicates a conserved substitution. The region 324–328 (boxed) was found to be located inside the molecule.

N42R by plasmin was verified by mass spectrometry. Amino acid sequence of the peptides revealed the existence of four sequences (Table I). Sequence I corresponds to the amino acid sequence of the undigested peptide, whereas sequences II–IV correspond to sequences of factor V starting at amino acid residues Lys310, Lys311, and Gin314, respectively (Table I). These data demonstrate cleavage of factor V by plasmin at Lys309, Lys310, and Arg313. Although the picomole yield of fragments II and III was high, the yields of amino acids making up peptides I and IV were low, demonstrating that plasmin cleaves factor Va primarily at amino acid residues Lys309 and Lys310. The low picomole yield for peptide IV suggests that cleavage by plasmin at Arg312 is slow. Thus, a proteolytic wobble with functional significance is observed on the heavy chain of the cofactor at Lys309 and Lys310. A similar proteolytic wobble was observed on the light chain of bovine factor Va when using APC (5) or plasmin (present study). Nonetheless, the later wobble is of no physiological consequence for factor Va cofactor activity (Ref. 5, and present study). Thus, following binding to a membrane surface, factor Va heavy chains expose specific sites that are sensitive to circulating plasma anticoagulants (Arg306 for APC, and Lys309, Lys310, and Arg313 for plasmin).

DISCUSSION

Our data demonstrate that plasmin inactivates membrane-bound factor Va following cleavage of the heavy chain of the cofactor at Lys309, Lys310, and Arg313. These cleavages result in the dissociation of the A2 domain of factor Va from the rest of the molecule (Fig. 14). Previous data have demonstrated that factor Xa cleavage at Arg348 has no effect on cofactor activity (24) and that incubation of factor Va with factor Xa and or prothrombin results in the protection of the cofactor from inactivation by plasmin (11). Our data also demonstrate that a 42-amino acid residue peptide spanning the region 307–348 is a good inhibitor of factor VVa clotting activity. Together, these data suggest that amino acid region 307–348 of factor Va is involved in the interaction of the cofactor with factor Xa and/or prothrombin. This region may also be involved in chain-chain interaction.

Using monoclonal antibodies to the light and heavy chains of bovine factor Va, we have demonstrated that the A1 (Mr ~40,000 fragment) and the A3 (Mr 30,000 fragment) domains of factor Va are involved in the interaction with factor Xa (30). Although the binding to each chain separately was weak, inhibition of either of the binding sites separately by a specific monoclonal antibody to factor Va completely abolished factor Xa–factor Va interaction. Thus, it is possible to completely abolish the factor Xa interaction with factor Va by inhibiting only one of its interactions with the cofactor. Our present data indicate that the fragment previously identified to contain the epitope of the monoclonal antibody comprises amino acid residues 1–309. Structural modeling of the amino acid region 307–348 using as a template the published structure of ceruloplasmin (26) (Fig. 11, in black) suggests that this portion of factor Va heavy chain is almost entirely exposed to the solvent. This region is 80% identical between the human, bovine, and mouse species, verifying its importance throughout evolution.

FIG. 11. Structural analysis of the region 307–355. Structural analysis of the region 307–355 of factor Va (in black) was performed by using as a template the known x-ray structure of ceruloplasmin (26). Amino acids 324–328 were found to be buried inside the A2 domain (see Fig. 12 for the identification of these four amino acids).
over, two of the plasmin cleavage sites within this region, i.e. Lys309 and Arg313, are conserved in all three species, demonstrating the importance of these residues throughout evolution for the regulation of the activity of the cofactor. A peptide containing this amino acid portion of the molecule was found to inhibit factor Va clotting activity with an IC50 of 1.3 \mu M. We have recently demonstrated that cleavage of factor Va heavy chain by APC at Arg306 results in the dissociation of the A2 domain of the cofactor from the rest of the molecule and subsequent inactivation (31). Our present data narrows down a smaller domain that is important for factor Va cofactor activity (i.e. amino acid region 314–348). Collectively the data suggest that amino acid region 1–309 of factor Va (A1 domain) is most likely involved in chain-chain interaction, whereas amino acid region 314–348 is involved in factor Va cofactor function and possesses a binding site for factor Xa and/or prothrombin (Fig. 14).

Cleavage of factor Va by APC at Arg306 is lipid-dependent, and it occurs with a significant rate when factor Va and APC are bound to a membrane surface (5, 6). Cleavages of factor Va by plasmin at Lys309, Lys310, and Arg313 are also accelerated in the presence of a membrane surface. However, plasmin does not interact with the phospholipid vesicles. Thus, it must be

### TABLE I

| Cycle no. | Sequence I pmol | Sequence II pmol | Sequence III pmol | Sequence IV pmol |
|-----------|-----------------|------------------|------------------|------------------|
| 1.        | N 11.5          | K 39.7           | I 17.9           | E 7.8            |
| 2.        | L 33.2          | I 29.1           | T 10.2           | Q 3.7            |
| 3.        | K 33.2          | T 16.3           | R 24.7           | R 24.7           |
| 4.        | K 29.7          | R 48.9           | E 2.6            | R 48.9           |
| 5.        | I 23.6          | E 13.0           | Q 8.8            | H 2.9            |
| 6.        | T 12.9          | Q 13.6           | R 29.3           | M 6.6            |
| 7.        | R 88.3          | R 88.3           | R 88.3           | K 6.5            |
| 8.        | E 5.6           | R 66.3           | H 8.5            | R 66.3           |
| 9.        | Q 10.7          | H 15.3           | M 14.6           | W 0.5            |
| 10.       | R 36.2          | M 23.2           | K 13.1           | ?                |
| 11.       | R 56.6          | K 21.6           | R 56.6           | Y 3.1            |
| 12.       | H 10.6          | R 43.1           | W 1.5            | F 3.7            |
| 13.       | M 16.5          | W 2.4            | E 1.8            | I 2.9            |
| 14.       | K 12.3          | E 4.7            | Y 9.0            | A 2.7            |
| 15.       | R 22.2          | Y 15.8           | F 9.9            | A 5.4            |
| 16.       | W 1.7           | F 14.1           | I 8.9            | ?                |
| 17.       | E 3.4           | I 12.6           | A 9.6            | ?                |
| 18.       | Y 9.4           | A 20.1           | A 20.1           | Y 3.2            |
| 19.       | F 9.5           | A 17.6           | E 4.9            | I 6.3            |
| 20.       | I 10.3          | E 3.8            | E 3.8            | W 0.3            |
| 21.       | A 11.4          | E 2.2            | V 6.1            | D 1.0            |
| 22.       | A 10.0          | V 9.5            | I 7.3            | Y 1.5            |
| 23.       | E 3.4           | I 10.3           | W 0.1            | ?                |
| 24.       | ?               | W 1.5            | D 1.8            | ?                |
| 25.       | V 5.7           | D 1.8            | Y 4.0            | ?                |
| 26.       | I 6.6           | Y 5.6            | A 3.1            | ?                |
| 27.       | W 0.5           | A 3.1            | P 3.8            | ?                |
| 28.       | D 1.3           | P 3.8            | ?                | ?                |
| 29.       | Y 3.8           | ?                | ?                | ?                |
| 30.       | A 1.0           | ?                | ?                | ?                |
| 31.       | P 3.9           | ?                | ?                | ?                |

*a The number at the right of each amino acid indicates picomoles of amino acid at the given cycle.*
concluded that factor Va structure in the region 306–325 (between the A1 and A2 domains) is altered following binding of the light chain of the cofactor to a membrane surface. Our data demonstrate that this region is actively involved in cofactor function. It is remarkable that this amino acid region, although exposed on the surface of the cofactor in solution as deduced from the modeling of the molecule by using as a template the x-ray structure of ceruloplasmin (26), is not accessible to circulating proteases. This segment, which most likely undergoes conformational changes following binding of factor Va to a membrane surface, could be a "control switch" for factor Va activity prior to its binding to a cell surface and clot initiation. The amino acid segment 306–325 is located within a distorted segment between the A1 and A2 domains of the heavy chain of the cofactor that is most likely a flexible loop structure (26). Prior to clot formation, this region must remain intact to perform its functions (i.e. binding of factor Xa and/or prothrombin). Following clot formation this region becomes vulnerable to antithrombotic proteases (i.e. APC, plasmin), because the clotting process must be terminated.

Our data demonstrate that all plasmin cleavages are considerably accelerated when the cofactor is bound to a membrane surface. Cleavage of the light chain at Arg\textsuperscript{1643} as well as the proteolytic wobble on the light chain at Arg\textsuperscript{1752} and Arg\textsuperscript{1753} and the inactivating cleavages at Lys\textsuperscript{309}, Lys\textsuperscript{310}, and Arg\textsuperscript{313} are observed in the presence of a membrane surface. We have previously demonstrated that cleavage of the human or bovine cofactor at Arg\textsuperscript{306} is anionic-lipid dependent (5, 6). Thus, the binding of the active cofactor to a cell surface during normal hemostasis increases its susceptibility to cleavage and inactivation by antithrombotic proteases (i.e. APC, plasmin), because the clotting process must be terminated.

Inactivation of Membrane-bound Factor Va Plasmin

Our study supports these results. However, the inhibitory peptide was also found to interact with prothrombin. Thus, the peptide region defined by our study may be essential with hemorrhagic events subsequent to the thrombolytic therapy (35). Our data correlates specific cleavages in factor Va with inactivation by plasmin and provides markers that could be used for the detection of factor V following thrombolytic therapy. Recently, we have reported an assay in whole plasma that could differentiate between normal factor V and factor \textit{V}\textsubscript{LEIDEN} (36). Because we have defined the proteolytic fragments derived from factor Va following cleavage by plasmin, a similar assay that would detect factor V fragments in whole plasma might be useful in preventing serious complication following thrombolytic therapy. Furthermore, the presence in plasma of the factor Va-derived peptide containing amino acid residues 314–348 could be an indicator of plasmin activation and ongoing fibrinolysis.

Our data show that the structure of factor Va is equivalent to that of factor VIIIa. Furthermore, the mode of inactivation of the two cofactors, although similar, involves amino acid sequences that are completely opposite in nature. Factor VIIIa cofactor activity involves an acidic peptide located between the A1 and A2 domains (amino acid residues 337–372, with 35 amino acids) (37), whereas our data show that factor Va activity involves an amino acid region containing mostly basic amino acids (307–348, with 42 amino acids). These differences in structure between the two cofactors are most likely related to their participation to the interaction with different proteins that are member of two different complexes with totally different specificity. Factor Va is the cofactor for prothrombinase for the activation of prothrombin, whereas factor VIIIa is the cofactor for the intrinsic tenase for the activation of factor X. Thus, the critical amino acid regions that are responsible for their interaction with the components of prothrombinase and intrinsic tenase must be (and are) unique to each cofactor to ensure their specificity.

A binding domain for factor X has been reported on the COOH-terminal acidic region of factor VIII (amino acid residues 337–372) (37). Similarly, a binding site for prothrombin may be located within the basic amino acid region 314–348 of factor Va. Interestingly, a recent publication by Kojima et al. (38) using synthetic peptides suggested that a binding domain for factor Xa is located within amino acid residues 311–325 of factor Va. Our study supports these results. However, the study by Kojima et al. did not demonstrate direct competition of binding between factor Va and factor Xa by the synthetic peptide(s). Furthermore, in the study of Kojima et al. (38) the inhibitory peptide was also found to interact with prothrombin. Thus, the peptide region defined by our study may be essential...
for the interaction of the cofactor with factor Xa and/or prothrombin, and more data are necessary to establish the exact amino acid residues involved in the interaction of factor Va with the components of prothrombinase.

Recently, the mechanism of cleavage of platelet factor Va by plasmin was also studied on the surface of platelets (39). The addition of a high concentration of plasmin (∼10 nM) to the platelet-bound platelet factor Va resulted in a 4-fold increase in cofactor activity when compared with the activity of the cofactor observed in the absence of plasmin. Under the conditions employed, the increased cofactor activity was sustained as long as 4 h. The increased cofactor activity was not due to a plasmin-mediated release of residual cofactor stores, because the release of additional platelet factor Va was not detected. In marked contrast, only a small increase in cofactor activity (∼1.5-fold) was observed when plasma factor Va was added to activated platelets. Furthermore, in contrast to its platelet counterpart, the plasma factor Va was immediately inactivated by plasmin. When platelets were removed from the mixture and phospholipid vesicles were used to supply a membrane surface, a rapid inactivation of platelet factor Va was observed following a 1-h incubation time period. Based on the epitope specificity of the various monoclonal antibodies used in Western blotting experiments, we hypothesize that plasmin-induced cleavages in the NH₂-terminal portion of the platelet factor Va heavy chain, most likely at amino acid residue 348, are responsible for both the gain and maintenance in the platelet factor Va cofactor activity.

In conclusion, cleavages of factor Va at Arg³⁴⁸ by plasmin (present data) and by factor Xa (5, 24) are not responsible for any substantial loss in cofactor activity. The initial loss in factor Va cofactor activity in the absence of a membrane surface (∼10%, after 1 h; Fig. 2, filled circles) is due to secondary slow cleavages of the cofactor by plasmin at Lys³⁰⁹, Lys³¹⁰, and Arg³¹³. These cleavages are very slow in the absence of a membrane surface as demonstrated in Fig. 3, lane 3, and are accelerated when the substrate is the M, 47,000 fragment (Fig. 3, lanes 4–7). Thus, amino acid region 307–348 is crucial for the expression of factor Va cofactor activity. This region of factor Va is important for factor Va cofactor activity, because it most likely contains a binding site for factor Xa and/or prothrombin. Cleavage of the cofactor by plasmin at Lys³⁰⁹, Lys³¹⁰, and Arg³¹³ will result in the dissociation of the A2 domain of factor Va from the A1 domain/light chain resulting in its inactivation.

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