Identification and Characterization of a Phospholipid-binding Site of Bovine Factor Va*

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Michael Kalafatis, Richard J. Jenny, and Kenneth G. Mann‡
From the Department of Biochemistry, Health Science Complex, University of Vermont, College of Medicine, Burlington, Vermont 05405

Coagulation factor Va is a cofactor which combines with the serine protease factor Xa on a phospholipid surface to form the prothrombinase complex. The phospholipid-binding domain of bovine factor Va has been reported to be located on the light chain of the molecule and more precisely on a fragment of Mr = 30,000 which is obtained after digestion of factor Va light chain by factor Xa. This proteolytic fragment is located in the NH2-terminal part of factor Va light chain (residues 1564-1765). In order to further characterize the lipid-binding domain of bovine factor Va, isolated bovine light chain was preincubated with synthetic phospholipid vesicles (75% phosphatidylcholine, 25% phosphatidylserine) and digested with trypsin, chymotrypsin, and elastase. Two peptide regions protected from proteolytic cleavage were identified and characterized from each proteolytic digestion. A comparison of the NH2-terminal sequence and amino acid composition of the two tryptic peptides with the deduced sequence of human factor V indicates a match with residues 1667-1797 of the light chain this lipid-binding region of the bovine factor Va which is the active cofactor of the prothrombinase complex (7, 8, 10). The activation of factor V to factor Va by thrombin involves three enzymatic cleavages and gives rise to two heavily glycosylated activation peptides (Mr = 71,000 and 120,000) and the active cofactor (Mr = 168,000). Factor Va is a heterodimer composed of a heavy chain derived from the NH2-terminal part of factor V (Mr = 94,000) and a light chain (Mr = 74,000) derived from the COOH-terminal end of the factor Va molecule. The two chains are non-covalently associated in the presence of divalent ions (8, 11-13). Although thrombin is the principal activator of factor V, factor Xa likely serves as the initial activator of factor V during early stages of coagulation when little or no thrombin is present. This activation catalyzed by factor Xa requires significantly higher enzyme to substrate ratios than that required for the thrombin-catalyzed reaction (14). Recent data have shown that factor Xa activates human factor V in a time-, phospholipid-, and calcium ion-dependent manner (15). Furthermore, the factor Xa cleavage pattern differed markedly from the one obtained in the bovine system whereas the activation profile obtained using thrombin instead of factor Xa for the activation was slightly different. Moreover, these data indicated that activation by factor Xa gives rise to a cofactor which is active as the thrombin-activated factor V (15). Once formed factor Va may be inactivated by plasmin or activated protein C (APC)† (16, 17). The specific peptide bonds within bovine factor Va which are hydrolyzed by factor Xa and APC have been previously reported, and the derived fragments have been isolated and characterized (18).

A complete understanding of the assembly of the prothrombinase complex and the expression of its activity requires the exact definition of the parameters of the interactions within the complex. A great deal of information now available concerning the factor V/Va membrane interaction has indicated that the factor V- or the factor Va-phospholipid interaction

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The coagulation cascade is regulated by a series of reactions which culminate in the formation of the fibrin clot (1, 2). A central event in the coagulation cascade is the conversion of prothrombin to thrombin. This latter event is catalyzed by the prothrombinase complex which is composed of a non-enzymatic cofactor, factor Va, which combines with the serine protease factor Xa on a cellular or phospholipid surface in the presence of calcium ions. This enzyme complex catalyzes the activation of prothrombin to thrombin at a rate >300,000-fold greater than that of factor Xa alone (3-6). The procofactor, factor V, circulates in plasma as a large monomeric protein with a molecular weight of 330,000 (7, 8). The cDNA sequence for human factor V includes a 6672-base pair coding region and the deduced amino acid sequence consists of 2224 amino acids inclusive of a 28-amino acid leader peptide (9).

Proteolytic cleavage of factor V by thrombin produces factor Va which is the active cofactor of the prothrombinase complex (7, 8, 10). The activation of factor V to factor Va by thrombin involves three enzymatic cleavages and gives rise to two heavily glycosylated activation peptides (Mr = 71,000 and 120,000) and the active cofactor (Mr = 168,000). Factor Va is a heterodimer composed of a heavy chain derived from the NH2-terminal part of factor V (Mr = 94,000) and a light chain (Mr = 74,000) derived from the COOH-terminal end of the factor V molecule. The two chains are non-covalently associated in the presence of divalent ions (8, 11-13). Although thrombin is the principal activator of factor V, factor Xa likely serves as the initial activator of factor V during early stages of coagulation when little or no thrombin is present. This activation catalyzed by factor Xa requires significantly higher enzyme to substrate ratios than that required for the thrombin-catalyzed reaction (14). Recent data have shown that factor Xa activates human factor V in a time-, phospholipid-, and calcium ion-dependent manner (15). Furthermore, the factor Xa cleavage pattern differed markedly from the one obtained in the bovine system whereas the activation profile obtained using thrombin instead of factor Xa for the activation was slightly different. Moreover, these data indicated that activation by factor Xa gives rise to a cofactor which is active as the thrombin-activated factor V (15). Once formed factor Va may be inactivated by plasmin or activated protein C (APC)† (16, 17). The specific peptide bonds within bovine factor Va which are hydrolyzed by factor Xa and APC have been previously reported, and the derived fragments have been isolated and characterized (18).

A complete understanding of the assembly of the prothrombinase complex and the expression of its activity requires the exact definition of the parameters of the interactions within the complex. A great deal of information now available concerning the factor V/Va membrane interaction has indicated that the factor V- or the factor Va-phospholipid interaction

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‡ To whom correspondence should be addressed.

† The abbreviations used are: APC, activated protein C; PCPS, phospholipid vesicles; Pyr-Va, factor Va modified with pyrene maleimide; PS, phosphatidylserine; PC, phosphatidylcholine; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; HPLC, high performance liquid chromatography; PPACK, D-phenylalaninopropylargininyl chloromethyl ketone; TPK, t-1-oxi-2-amino-2-phenylethyl chloromethyl ketone; Hepes, 4-(2-hydroxyethyl)1-piperazineethanesulfonic acid.
requires the presence of negatively charged phosphopholipid (19, 20) and is not dependent on the presence of exogenously added calcium ions (19). Fluorescence polarization studies using factor Va modified with pyrene maleimide (Pyr-Va) have indicated a dissociation constant between Pyr-Va and the phospholipid vesicles of 2.7 x 10^{-9} M with 42 mol of PCPS required/mol of factor Va bound to a membrane (21). The stoichiometry between factor Va and the PCPS vesicles was proportionally altered with increasing PS concentration, but the K_d was essentially independent of the PS content of the vesicles (21). In the case of bovine factor Va the lipid-binding event is mediated by the light chain of the cofactor (21-23) and more precisely by a fragment of M, = 30,000 (21). This fragment, which contains the NH_2-terminal part of factor Va light chain, is produced when the light chain is cleaved by factor Xa, or APC (18). The fragment of M, = 30,000 inhibits Pyr-Va binding to phospholipid vesicles (21), and essentially all the lipid binding capacity of the light chain can be accounted for by this NH_2-terminal fragment. Conflicting reports are available in the literature regarding the contribution of hydrophobic and electrostatic interactions toward stabilization of the factor Va-phospholipid complex (20, 22, 23). Also some results have led to a hypothesis that factor Va binding to platelets is intrinsically different from the binding of factor Va to phospholipid vesicles and may involve specific receptors on the platelet surface (24). In addition the results obtained using Pyr-Va are surprising because the fragment of M, = 30,000 contains a major part of the A3 domain of factor Va which is highly homologous to the A1 and A2 domains of the heavy chain of the cofactor (9) which do not bind to phospholipid vesicles.

In the present paper we study the membrane-binding site of factor Va. We used the assumption that at least part of the factor Va light chain is protected by the phospholipid bilayer. This study identifies an element of the light chain which accounts for the interaction of factor Va with the phospholipid vesicles.

**Experimental Procedures**

**Materials**—All proteolytic enzymes used in this study were purchased from Worthington. Heps, L-α-phosphatidylethanolamine (hen egg) and L-α-phosphatidylserine (bovine brain) were from Sigma. a-Phenylmercuric chloroform (PMAK) was from Calbiochem. Phospholipid vesicles (PCPS), composed of 75% phosphatidylcholine and 25% phosphatidylserine were prepared as previously described (22, 25). and stored in 20

**Protein Purification**—Bovine factor Va was partially purified using the procedure of Nesheim et al. (6) with the omission of the Cibacron Blue chromatography step. This material was resuspended in HBS and activated with thrombin (9 units/ml) at 37 °C for 20 min. The activation was stopped by the addition of 0.5% (v/v) of the activator. CaCl_2 was added to a final concentration of 2 mM, and the mixture was applied to an anti-light chain immunosaffinity column (100-ml packed bed, 300 mg of IgG), and factor Va was purified using the procedure described previously (26). Isolated factor Va heavy chain and factor Va light chain were prepared using Q-Sepharose (Pharmacia LKB Biotechnology Inc.). Briefly, 50 mg of bovine factor Va was dissolved in a solution of 2 mM EDTA, 50 mM NaCl, pH 7.4, and applied to a column of Q-Sepharose (50 mg) equilibrated in the same buffer. The column was washed with five volumes of starter buffer, and the components were eluted with a linear gradient of NaCl from 0 to 0.6 M NaCl. Total yields, as evaluated using clotting assays, were about 70% of the observed for at least 4 months as judged by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

**Peptide Isolation**—Isolated light chain was dialyzed against Hepes-saline buffer, and incubated with PCPS vesicles. Final concentrations were 3 μM light chain and 750 μM PCPS. The sample was incubated for 5 min at 37 °C to ensure that equilibrium had been attained. TPCK-treated trypsin, chymotrypsin, or elastase were added to separate reaction mixtures at a final concentration of 6.4, 10, and 10 μM, respectively. The mixture was incubated at 37 °C for 45 min to allow digestion of the exposed light chain. Preliminary experiments using a mixture of 125I light chain and unlabeled light chain had indicated that these conditions were optimum and gave rise to large amounts of peptides. The sample was then applied to a 7.5 x 50-cm TSK-3000 high performance liquid chromatography (HPLC) gel filtration column (Beckman) equilibrated in Hepes-saline buffer. The column was monitored with a Waters model 484 absorbance detector set at 280 nm. The flow rate was 1 ml/min, and the data were collected on a strip chart recorder operated at 5 mm/min. The protein fraction which comigrated with the PCPS vesicles as determined by running PCPS alone was collected and lyophilized. This procedure was repeated three times for each enzyme. The lyophilized sample was resuspended in 3 ml of H_2O, 0.05% trifluoroacetic acid, centrifuged at 10,000 rpm in an HBI microcentrifuge for 5 min, and filtered through 0.45 μm filters (Millipore) in preparation for reverse-phase HPLC analysis.

**HPLC Analysis** was performed by injecting 1 ml of sample onto a 4.6-mm x 7.5-cm Beckman Ultrasphere RPSC-C3 column operated at 1 ml/min with gradient elution of 0% buffer B to 60% buffer B over 15 min (buffer A, 0.05% trifluoroacetic acid in H_2O and buffer B, 0.05% trifluoroacetic acid in CH_3CN). Column effluent was monitored at 214 nm, and the data were collected on a strip chart recorder operated at 5 mm/min. Control experiments included a complete analysis of PCPS alone and PCPS plus enzyme and light chain alone. The purity of all protein or peptide preparations was evaluated using SDS-PAGE. It must be noted that after 45 to 60 runs with the same column, a shift of the peaks toward the left side of the chromatogram was observed. Although the samples were centrifuged and filtered and no increase in the back pressure of the column was observed some of the specific hydrophobic sites of the column were apparently saturated by the PCPS vesicles. This phenomenon may explain the earlier elution of the proteins. However, the purification step was consistent, always with the elution of three major peaks and this event in no way influences the purity of the peptides.

**Peptide Bound to the Phospholipid Vesicles**—The peptides isolated by HPLC were pooled separately, dried completely in a Savant Speed-Vac concentrator, then redissolved in 60 μl of Hepes-saline buffer. The peptide solution (60 μl) was mixed with 60 μl of a known concentration of freshly prepared PCPS, incubated for 5 min, and applied to the same TSK column which was washed with HBS with appropriate purification. The PCPS peaks were collected in all the cases, even when no significant increase in the optical density at 280 nm was observed. The collected fractions were lyophilized and then redissolved in 1 ml of H_2O, trifluoroacetic acid (0.05%) and applied to the reverse-phase RPSC-C3 column. A control which included HPLC analysis of the PCPS peak after lyophilization was run separately. The only peak which was different from the baseline or the chromatogram obtained after running PCPS alone was collected, dried, redissolved in 30 μl of 0.5 M Tris, 480 mM glycine, 0.25% SDS, and analyzed on 15% silver-stained SDS-PAGE.

**Activity Measurements**—The cofactor activity of factor Va was evaluated using clotting assays.

**Sequence Analysis**—The NH_2-terminal sequence of the purified peptides was determined using automatic Edman degradation on a Applied Biosystem 475A protein sequencing system. The phenylthiohydantoins were identified using high performance liquid chromatography. In a typical experiment a known volume of purified peptide, directly from the HPLC purification step, was concentrated using a filter precoated with Polybrene (Applied Biosystems) and dried in an acid-washed conical 1.5-ml Savant Speed-Vat concentrator, then redissolved in 60 μl of Hepes-saline buffer. The peptide solution (60 μl) was mixed with 60 μl of a known concentration of freshly prepared PCPS, incubated for 5 min, and applied to the same TSK column which was washed with HBS with appropriate purification. The PCPS peaks were collected in all the cases, even when no significant increase in the optical density at 280 nm was observed. The collected fractions were lyophilized and then redissolved in 1 ml of H_2O, trifluoroacetic acid (0.05%) and applied to the reverse-phase RPSC-C3 column. A control which included HPLC analysis of the PCPS peak after lyophilization was run separately. The only peak which was different from the baseline or the chromatogram obtained after running PCPS alone was collected, dried, redissolved in 30 μl of 0.5 M Tris, 480 mM glycine, 0.25% SDS, and analyzed on 15% silver-stained SDS-PAGE.

**Activity Measurements**—The cofactor activity of factor Va was evaluated using clotting assays.
were identical to those obtained using the Applied Biosystems computer program.

Amino Acid Composition Analysis—In a typical experiment, a known volume of purified peptide (usually a third or half of the amount used for the amino acid sequence) was transferred to an acid-washed borosilicate glass culture tube (6 x 50 mm). Noreleucine, 1 nmol, was added and the mixture was completely dried in a Savant Speed-Vac concentrator. The tube was then transferred into an acid washed 19 x 150-mm glass culture tube containing 600 μl of 6 N hydrochloric acid (Pierce Chemical Co.) and 2% thioglycolic acid to avoid oxidation of the methionine and tyrosine. Cysteine was determined as cysteic acid by performic acid oxidation prior to hydrolysis with 6 N HCl (27). The 19 x 150-mm hydrolysis tube was then purged with nitrogen and sealed under vacuum prior to heating for 24 h at 110 °C. The samples were dried completely, resuspended in 200 μl of 0.2 N sodium carbonate buffer, pH 2.2, and filtered through 0.45-μm filters. Amino acid composition was determined using cation-exchange chromatography (Interaction Chemicals AA 911) and employing a pH gradient from 3.17 to 9.94, with post column detection using o-phthalaldehyde. The data were quantified using a computer program, Maxima 820 (Waters).

Protein Labeling—Proteins were labeled with 125I-Na (Amersham Corp.) using Iodo-Gen (Pierce Chemical Co.) as previously reported by Fraker and Speck (28). Free sodium iodide was removed by gel filtration using cellulose F5 desalting columns (Pierce Chemical Co.) or Sephadex G-10 columns (Pharmacia).

In some experiments a trace amount of iodinated light chain was added to a known concentration of unlabeled light chain, and the mixture was incubated with either PCPS or PC vesicles or HBS for 6 h at 37 °C. The final concentration was 6 μM light chain and 750 μM PCPS or PC. Then TPCK-treated trypsin or chymotrypsin were added to a final concentration of 6.4 and 8 μM, respectively, and the blue in 50% glycerol followed by heating at 90 °C for 5 min. In several experiments the time-dependent proteolysis of the light chain-PCPS complex was studied. For these experiments a mixture of 35I light chain and unlabeled light chain and PCPS vesicles was combined with trypsin, chymotrypsin, or elastase using the same conditions described above. After appropriate intervals of time an aliquot of the solution was withdrawn and SDS (1% final concentration) and bromphenol blue, and heating the sample at 90 °C for 5 min. The samples were subjected to SDS-PAGE analysis on 8-18% gradient gels following the method of Laemmli (29). The gels were stained with Coomassie Brilliant Blue dye, then purged with nitrogen and sealed under vacuum prior to heating for 24 h at 110 °C. The samples were dried completely, resuspended using o-phthalaldehyde. The data were quantified using a computer program.

Gel Electrophoresis—SDS-PAGE analysis was performed using homogeneous or 8-18% and 8-20% gradient gels following the method of Laemmli (29). The gels were stained with Coomassie Brilliant Blue R or silver-stained (30).

Analysis of the Lipid-binding Peptide Amino Acid Sequence—Sequence analysis was studied using the University of Wisconsin Genetics Computer Group sequence analysis package. Computer programs contained within this package which were used in the analysis of the factor V sequence included "PEPTIDESTRUCTURE." This program makes secondary structure predictions for a protein or a peptide sequence. Predictions are based upon the rules developed by Chou and Fasman (31) for predicting helices, sheets, and turns. Options also allow for the determination of surface probability according to a formula developed by Emini et al. (32). The predictions are experiment in a graphical form. It was observed clearly and unambiguously that such predictions may be far different from the actual protein structure.

RESULTS

The Light Chain—Vesicle "Footprint"—A mixture of unlabeled and iodinated factor Va light chain was incubated with PCPS, PC, or Hepses-saline buffer. The reaction mixtures were digested with trypsin or chymotrypsin as described under "Experimental Procedures." The resulting mixture was subjected to SDS-PAGE analysis and the proteins were displayed by autoradiography (Fig. 1). The intact native light chain appears as a large band with M, = 76,000, accompanied by fragments of M, = 58,000, 42,000, and 30,000 (Fig. 1, lane 1). When the light chain was incubated with PCPS prior to the trypsin digestion the large band representing the light chain disappeared and two new fragments of M, = 58,000 and 34,000 were present accompanied by two peptides of M, = 18,200 and 14,000. Low molecular weight material is observed at the bottom of the gel which likely represents small peptides not resolved by the gel (Fig. 1, lane 2). When chymotrypsin replaced trypsin for the hydrolysis of the light chain in the presence of PCPS, trace amounts of a species of M, = 58,000 were observed (Fig. 1, lane 5). In addition, a fragment of M, = 36,000 was detected and was accompanied by two peptides of M, = 17,500 and 14,500 (Fig. 1, lane 5). The same low molecular weight material as observed when using trypsin was present at the bottom of the gel. When the light chain was incubated with trypsin in the presence or absence of PC, only low molecular weight material at about 10,000 was present at the end of the digestion (Fig. 1, lanes 3 and 4). In the same manner when the light chain was incubated with chymotrypsin in the presence or absence of PC vesicles (Fig. 1, lanes 6 and 7) only low molecular weight material containing several bands at about 8,000 was present at the bottom of the gel. These data suggest that portions of the light chain are protected from proteolysis by the phospholipid vesicles composed of phosphatidylinerine and phosphatidylcholine. Further, there are two smaller peptides in the factor Va light chain which are protected from enzymatic digestion and which may contain all or part of the phospholipid-binding domain of factor Va.

Time Course Digestion of the Light Chain—Phospholipid Mixture by Various Enzymes—A mixture of iodinated and unlabeled light chain was incubated with PCPS vesicles. Then trypsin, chymotrypsin, or elastase were added to separate reactions, and samples were removed at various times and prepared for SDS-PAGE as described under "Experimental Procedures." The native light chain had the same composition as described in Fig. 1, lane 1. When trypsin was added to the PCPS/light chain mixture, the doublet of M, = 48,000/52,000 as well as a species of M, = 39,000 and two peptides of M, = 18,200 and 14,000 remained even after 24 h (data not shown). In contrast when chymotrypsin or elastase were used instead

**FIG. 1. Protection of factor Va light chain hydrolysis by the PCPS vesicles. A mixture of unlabeled and iodinated light chain was preincubated with PCPS vesicles for 5 min at 37 °C. Then trypsin or chymotrypsin were added as described under "Experimental Procedures." The reaction was stopped by adding 1% SDS (v/v), bromphenol blue, and heating the sample at 90 °C for 5 min. The samples were subjected to an 8 to 18% gradient SDS-PAGE. The position of the fragments was revealed by autoradiography. Lane 1, starting material, factor Va light chain; lane 2, light chain incubated with PCPS vesicles prior to the addition of trypsin; lane 3, light chain incubated with PC vesicles prior to the addition of trypsin; lane 4, light chain incubated with HBS prior to the addition of trypsin; lane 5, sample as in lane 2 but incubated with chymotrypsin; lane 6, sample as in lane 3 after chymotrypsin digestion; lane 7, sample as in lane 4 after incubation with chymotrypsin. Position of the molecular weight markers is indicated at left.**
of trypsin only the two peptides of $M_r = 17,500$ and 14,500 remained after 24 h. It should be noted that regardless of the digestive enzyme used there is ultimately complete loss of the larger ($M_r > 18,200$) fragments. This observation is consistent with the reversible nature of the factor Va light chain interaction with phospholipid. Finally, a comparison of the time course digestion of the factor Va light chain using trypsin, chymotrypsin, and elastase shows that the concentration of all the fragments decreased with time. Therefore, the reaction was stopped at 45 min where the concentration of the set of peptides with $M_r = 18,200/14,000$ and 17,500/14,500 was maximum.

Interaction of the Factor Va Light Chain or Heavy Chain with the PCPS Vesicles—High performance liquid chromatography gel filtration data obtained from five different mixtures of light chain or heavy chain and PCPS are illustrated in Fig. 2. Fig. 2A indicates the position at which PCPS vesicles were eluted from the gel filtration column, which is presumably at the void volume of the column. When the PCPS vesicles (750 $\mu$M) were incubated with the light chain (3 $\mu$M final concentration) prior to the TSK gel filtration, a net increase in the 280 nm absorbance of the void volume peak was observed (Fig. 2B). This increase was the result of the interaction between the light chain and the PCPS vesicles. It must be noted, however, that measurement of the absorbance at 280 nm in Fig. 2B is the result of the intrinsic absorbance of the light chain and the contribution of high scatter due to the phospholipid vesicles. Fig. 2C indicates the position at which native light chain alone was eluted from the column and shows, in comparison with Fig. 2B, that the light chain binds specifically to phospholipid vesicles. The amount of the light chain recovered after HPLC gel filtration of the light chain-phospholipid complex (Fig. 2B, first peak) was verified by silver stained SDS-PAGE and compared with the peak of light chain alone (Fig. 2C) and with the material which does not bind to the PCPS vesicles (Fig. 2B, second peak). More than 95% of the starting material was found to coelute with the PCPS vesicles (Fig. 2B, first peak). Fig. 2D illustrates the chromatogram obtained when the heavy chain of factor Va was incubated with the phospholipid vesicles. A comparison of this chromatogram with that obtained with the heavy chain alone (Fig. 2E) or with PCPS alone (Fig. 2A) indicates no interaction between the factor Va light chain and PCPS vesicles. This was also confirmed by SDS-PAGE analysis of the fractionated material (data not shown). These data indicate that this technique can be used to isolate lipid-bound protein or peptide in a quantitative manner. This technique can also give a qualitative answer as to whether or not a protein binds to the phospholipid vesicles and shows that the heavy chain of factor Va does not bind to the PCPS. The slight increase in the absorption of the PCPS peak was the result of the coelution of the high molecular weight contaminant of the factor Va heavy chain preparation with the PCPS peak.

Proteolytic Processing of Factor Va Light Chain by Trypsin and Chymotrypsin in the Presence of PCPS Vesicles—When trypsin alone was incubated with PCPS vesicles and subjected to HPLC gel filtration analysis two separate peaks were observed corresponding to free PCPS and to free trypsin (Fig. 3A); no interaction occurs between the PCPS vesicles and trypsin. Fig. 3B illustrates the profile obtained when the light chain-phospholipid complex was incubated with trypsin (45 min) prior to chromatographic analysis. The net increase in the 280-nm absorbance of the PCPS peak is attributed to the portion of the factor Va light chain which remains associated with the phospholipid vesicles. Similarly, chymotrypsin shows no interaction with the PCPS vesicles (Fig. 3C). The greater absorbance of the second peak which represented free chymotrypsin compared with the trypsin peak in Fig. 3C is the result of the higher chymotrypsin concentration and the higher extinction coefficient of chymotrypsin ($E_{280}^\text{av} = 20.4$) compared with $E_{280}^\text{av} = 14.3$ for trypsin. When the light chain was incubated with the PCPS vesicles prior to the chymotrypsin digestion and analyzed by gel filtration, an increase of the 280-nm absorbance of the phospholipid peak (Fig. 3D) was observed which was presumably the result of the interaction of the lipid binding portion of the light chain with the PCPS vesicles. Therefore, it appeared that this technique could be used to isolate the lipid-bound peptide of the factor Va molecule.

Enzyme Digests Isolated by Gel Filtration—The fragments which were coeluted with the PCPS vesicles were lyophilized, filtered, and subjected to reverse-phase high performance liquid chromatography. Fig. 4 illustrates a chromatogram obtained when the undigested light chain-phospholipid complex was analyzed. The peak at the beginning of the chro-
A Phospholipid-binding Domain of Factor Va

46,000 which bound to the fragment of $M_r = 30,000$ copurified with the light chain.

Fig. 5 shows the results obtained from reverse-phase HPLC when the lipid-peptide complex resulting from trypsin digestion and resolved by gel filtration was analyzed. The chromatogram illustrates a peak at 27.6 min which represents a peptide of $M_r = 18,200$ (ts) as shown in Fig. 5, inset, lane 1. A second peak at 28.3 min contained intermediates resulting from the incomplete digestion of the light chain with trypsin (Fig. 5, inset, lane 2). A third peak occurs at 30.0 min and contained a peptide of $M_r = 14,000$ (ts, Fig. 5, inset, lane 3). The fourth peak at 33.5 min with the small shoulder at 32.5 min contained the residual light chain and a fragment of $M_r = 46,000$ (Fig. 5, inset, lanes 4 and 5).

The chromatogram resulting from reverse-phase HPLC analysis of the PCPS-light chain chymotrypsin mixture gave products which eluted at 28.1 min (Fig. 6) and contained a peptide of $M_r = 17,500$ (ct1, Fig. 6, inset, lane 2). A second peak at 29.7 min represented a peptide of $M_r = 14,500$ (ct2, lane 3).

The conditions for the enzymatic digestion of the light chain-PCPS complex are described under "Experimental Procedures." HPLC gel filtration was performed as described in the legend for Fig. 2. Elution profiles of samples and controls are illustrated as follows: A, PCPS and trypsin; B, PCPS-light chain after trypsin treatment; C, PCPS and chymotrypsin; D, PCPS-light chain after chymotrypsin treatment.

The sample was prepared as described under "Experimental Procedures," and the HPLC analysis was performed as described in the legend for Fig. 4. The inset shows a silver-stained 8 to 20% gradient SDS-PAGE. Lanes 1–5 correspond to peaks 1 to 5 of the chromatogram. The position of the molecular weight markers is indicated on the left.

The inset shows a silver-stained gel which flowed directly through the column. The peak at 28 min contained a fragment of $M_r = 30,000$ as shown by a silver-stained gel (Fig. 4, inset, lane 1). The large peak at 32.0 min represented the light chain and the doublet of $M_r = 48,000/46,000$ (Fig. 4, inset, lane 2). These results show that the two components previously reported to bind specifically to the PCPS vesicles (the fragment of $M_r = 30,000$ and the light chain) can be separated. The doublet of $M_r = 48,000/$
Fig. 6, inset, lane 2). Fig. 6 (inset) lanes 3 and 4, illustrates the proteins included in the third peak at 32.0 min and in the shoulder at 33.5 min. These proteins were the remaining light chain and a species of _M_ = 46,000.

An identical digestion/isolation procedure was used with elastase and gave rise to results similar to those obtained using chymotrypsin. A peptide of _M_ = 17,500 (t3) was eluted at approximately the same anodictin concentration as ct3, a second peak containing a peptide of M = 14,500 (e2) was recovered at about 30.0 min, and a third peak containing fragments of _M_ = 42,000, 39,000, and 35,000 was eluted close to the end of the chromatogram (data not shown).

**NH2-terminal Sequence of t1, t2, ct1, ct2, e1, and e2—Edman degradation followed by phenylthiohydantoin analysis was employed to determine the NH2-terminal sequence of the isolated peptides.** The resulting sequences are presented in Table I. The peptide t1 which is generated when the light chain/lipid complex is incubated with trypsin yielded a sequence which matches with the portion of human factor V sequence starting at amino acid residue 1657 (Table I). The second tryptic peptide (t2) has an NH2-terminal sequence identical to the sequence previously reported by our laboratory for the NH2-terminal end of the bovine or human factor Va light chain and for the fragment of _M_ = 30,000 produced after factor Xa and/or APC digestion of bovine factor Va light chain (Table I).

The NH2-terminal sequence of peptides ct1 and e1 which are the peptides isolated after chymotrypsin and elastase digestion of the lipid/light chain mixture was identified with a sequence which matches with a portion of human factor V starting at amino acid residue 1667 (Table I). The second peptide isolated after chymotrypsin and/or elastase digestion had the same NH2-terminal sequence as t3, which is the NH2-terminal end of the bovine factor Va light chain (Table I). An important observation is that each time that the potential amino acid expected was asparagine at position 1675 of the light chain (indicated by an arrow on the first column in Table I), no residue was detected, and no quantification was possible for the next cycle. In addition the recovery of the three or four following amino acids was 80% less than expected. This residue corresponds to a potential glycosylation site in the human factor V molecule. It is most likely that the peptide binds to the glass fiber filter of the sequencer by this carbohydrate moiety, and most of the peptide is washed out following Edman degradation of this residue.

**Amino Acid Composition Analysis of the Tryptic and Chymotryptic Peptides**—The results obtained from amino acid composition analysis of the tryptic and chymotryptic peptides are presented in Table II, and are compared with the composition found in the corresponding regions of human factor V. The difference between the calculated amino acid composition and the number of residues expected is attributed to 1) a comparison of the sequence of the same peptide region but from two different species (human and bovine), and 2) the accuracy of the method which is 5 to 10%. The only significant difference was the number of glycine (and alanine residues in the case of t1 and ct1). Most other quantifications are in good agreement with respect to the two major reasons reported above. It must be noted that no methionine or cysteine were found either in t3 and ct3 as well as in the corresponding regions of human factor V (Table II).

**Direct Binding of the Peptides to PCPS Vesicles**—The four isolated peptides from the trypsin and chymotryptic digests were assayed for their ability to bind directly to PCPS

<table>
<thead>
<tr>
<th>Human light chain 1657-1681*</th>
<th>Trypsin</th>
<th>Chymotrypsin</th>
<th>Elastase</th>
<th>Intact bovine light chain†</th>
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* From Ref. 9.
† From Ref. 18.
‡ From Ref. 33.
§ Not identified.
¶ Not quantitated.
vesicles as described under “Experimental Procedures.” ct1 was able to interact directly with the phospholipid vesicles as shown by the significant increase in the optical density at 280 nm compared with the absorbance of the complex PCPS-ct, with PCPS alone. When t1 was assayed in a similar fashion for the direct binding to PCPS vesicles it showed an interaction as judged by the increase of the optical density at 280 nm compared with the free PCPS peak. The species contained in the phospholipid peak obtained after incubation of ct1 with the PCPS vesicles were collected, lyophilized, and submitted to reverse-phase HPLC analysis. Fig. 7 shows the result of three different chromatograms. The base line (short dashed line) indicated a small negative shift at 11 min which is probably the result of a change in the trifluoroacetic acid concentration of the solvent. This shift was consistent and observed in all three chromatograms. The chromatograms obtained with lyophilized phospholipid (long dashed line) showed a large peak at the beginning of the chromatogram which is a consequence of the buffer components flowing through the column. The other small peaks represented contaminants in the buffer and/or the initial PCPS preparation. When the lyophilized PCPS/t1 mixture was analyzed (Fig. 7, continuous line), the only peak significantly different from the base line or from running lyophilized PCPS alone was detected at about 29.0 min. The components contained within this peak were analyzed by SDS-PAGE (Fig. 7, inset). The gel showed only one band of Mr = 18,200 which corresponded to peptide t1. In contrast, when t1 and ct1 were tested for interaction with the PCPS vesicles no significant increase in the absorbance at 280 nm of the phospholipid peak was observed. Further, when the species contained within the lyophilized PCPS peak obtained after incubation of t1 and phospholipid were submitted to reverse-phase HPLC no peak significantly different from the base line was detected.

### DISCUSSION

The protocol described in this paper has permitted us to show that proteolysis of the bovine factor Va light chain bound to phospholipid vesicles lead to protection of two peptide segments which are located in the A3 domain of factor Va. One of the peptide regions is protected from enzymatic digestion in the presence of an acidic lipid bilayer, is capable of binding to membranes which contains acidic phospholipid, and most likely contains a phospholipid-binding site of factor Va.

The proteolysis of the light chain of bovine factor Va bound to PCPS vesicles by trypsin, chymotrypsin, and elastase was studied. In each case, two peptide regions were protected. The amino-terminal sequences of the two peptides derived from the A3 domain of the light chain were obtained and compared with the deduced amino acid sequence of human factor V (9). Among 20 NH2-terminal amino acids determined for each peptide, an identity of 80% was observed between the human and bovine sequences, indicating that these two portions of the molecule are highly conserved between the two species. These two peptide regions contain a large number of potential cleavage sites for trypsin and chymotrypsin, as shown by the amino acid composition of each peptide. Although some of these sites are inaccessible in the native factor Va light chain structure most of them are exposed and are effectively cleavage by these two enzymes when PCPS is not present. This

### Table II

Amino acid composition of the tryptic and chymotryptic peptides and comparison with the number of residues found in the corresponding region of human factor V

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latter phenomenon occurs whether or not the light chain was in buffer or preincubated with phospholipid vesicles composed of 100% phosphatidylycholine, prior to the addition of the enzymes. In contrast, proteolysis of the light chain bound to PCPS vesicles by TPCK-treated trypsin occurs between amino acids 1566 and 1657, and gives rise to a peptide of $M_r =$ 14,000 containing the NH$_2$-terminal part of the light chain and of the A3 domain (9, 18, 33). The other peptide of $M_r =$ 18,200 contains the central part of the A3 domain of factor Va and shows an interaction with the phospholipid vesicles (Fig. 8).

The results of the enzymatic digestion obtained using trypsin or chymotrypsin and factor Va light chain to vesicles composed of 25% PS and 75% PC or 100% PC showed a net protection of the factor Va light chain only by phospholipid vesicles containing PS. These results are consistent with most other studies (21, 23, 24), and they clearly demonstrate that phosphatidylserine is necessary to support the factor Va-PCPS interaction.

The results obtained following proteolysis of the factor Va light chain with other enzymes are quite consistent. Two peptides derived from the NH$_2$-terminal part of the light chain were obtained as a consequence of proteolysis between residues 1666 and 1667 by both chymotrypsin and elastase. Based upon the deduced human factor V amino acid sequence (9), the relative molecular weight of the two peptides and their amino acid compositions, it is most probable that the enzymatic cleavage which releases these two peptides must occur within the region 1790-1797 of the factor Va light chain. The only potential cleavage site for trypsin within this region as compared with the deduced amino acid sequence of human factor V is located between amino acids 1791-1792 (9) where chymotrypsin and elastase may cleave between residues 1783-1794 or 1797-1798 (Fig. 8). The chymotrypsin-derived peptide containing the central part of the A3 domain of the factor Va light chain binds to the phosphatidylserine-containing phospholipid vesicles in a similar manner to the corresponding trypsinic peptide. It is interesting to note that since trypsin cleaves between amino acids 1566 and 1657 and chymotrypsin at position 1666 and both peptides contain the central part of the A3 domain of the factor Va light chain bound to PCPS vesicles, the 10-residue region between 1657 and 1666 must be exposed to the solvent and probably does not interact with the PCPS vesicles (Fig. 8). The other set of peptides $t_3$ (1546-1656) and $t_5$ (1546-1666) did not show evidence of direct interaction with the PCPS vesicles. Since $t_3$ and $t_5$ did not show any affinity for the PCPS vesicles but are protected from enzymatic digestion, it is most likely that these peptide regions are intimately associated by non-covalent interaction with the peptide(s) which bind to the phospholipid vesicles.

The processing of bovine factor Va light chain by factor Xa and/or APC gives rise to a fragment of $M_r =$ 30,000 (18) containing the amino-terminal portion of the native light chain (residues 1546-1765) and to an insoluble doublet ($M_r =$ 46,000/48,000) which is derived from the carboxyl-terminal portion of the molecule (Fig. 8). Krishnaswamy and Mann (21) showed that the fragment of $M_r =$ 30,000 is involved in the factor Va-PCPS interaction and could quantitatively account for the light chain membrane interaction. These data could not discount participation of the COOH-terminal-derived doublet of $M_r =$ 46,000/48,000 of the light chain in the interaction of factor Va with the phospholipid vesicles because after HPLC purification of the 46,000/48,000 doublet all attempts to remove acetonitrile from the protein solution resulted in irreversible insolubility; hence, measurement was not possible. The factor Xa-APC cleavage site of the light chain occurs at residue 1765 and the resulting A3 domain is a lipid-binding segment of the molecule (Fig. 8). The exact positions of the carboxyl termini of $t_1$ and $c_1$ are estimated on the basis of their amino acid composition and apparent molecular weights on SDS-PAGE. These peptides include a glycosylation site at position 1675, and the carbohydrate at this site provides for some uncertainty with respect to the molecular weight of the peptides. Therefore, with respect to the considerations reported above (comparison of the NH$_2$-terminal sequence of the peptides with the corresponding regions within the human factor V sequence, $M_r$ excluding carbohydrate and amino acid composition of each peptide) the chymotrypsin and trypsin cleavage probably occurs at position Trp$^{1764}$ and Arg$^{1765}$ of the light chain respectively (Fig. 8). These cleavage sites may coincide with the cleavage at position 1765 of the factor Va light chain by factor Xa and/or APC (18). Our present data, combined with the competitive binding experiments using the isolated fragment of $M_r =$ 30,000 from the NH$_2$-terminal part of the factor Va light chain (21), leads to the conclusion that the lipid-binding region can be narrowed to a 99-amino acid segment corresponding to residues 1667-1765 (Fig. 8). A hydropathy plot in which the lipid-binding segment of 99 amino acids is identified is illustrated in Fig. 9. Within this region are two hydrophobic segments, i.e. residues 1716-1727 and 1741-1748, segments of 12 and 8 amino acids, respectively. It is conceivable that one or both of these two segments are involved in the interaction of the light chain with the phospholipid vesicles.
structure (or receptor) of the platelet membrane which is different from that occupied by factor V. All these results, including ours, imply that the two cofactors may interact with separate components of the activated platelet membrane. However, the possibility that other domains of factors V and VIII are implicated in the phospholipid binding must not be excluded.

The regulation and the formation of the prothrombinase complex includes the interaction of factor Va with prothrombin, factor Xa, and APC on a phospholipid surface in the presence of CaCl2. The prothrombin-binding site was previously localized to the heavy chain of the factor Va (37). A portion of the factor Xa and the entire APC binding sites are located on the light chain of the cofactor (38, 39). Recent data from Walker et al. (40) using recombinant DNA fragments of factor VIII and synthetic peptides of both factor V and factor VIII suggested that the binding domain for APC is localized within the COOH-terminal end of the A3 domain of factor V (between residues 1865–1874). The bulk of information regarding the ability of factor V or factor Va to bind to phospholipid vesicles has been obtained from various studies of the factor Va-PCPS interaction using modified factor Va or modified phospholipid vesicles (20–22, 34). Although these previous studies are not consistent with respect to the dissociation constant for the factor Va-phospholipid interaction, and/or the number of specific sites for factor Va on the phospholipid surface, they all agree with the fact that only the light chain of the active cofactor can account for the interaction of factor Va with the PCPS vesicles.

Our data clearly show that phosphatidylserine is necessary for the binding of the factor Va light chain to phospholipid vesicles. This latter interaction is essential for the protection of part of the light chain from enzymatic degradation. We therefore found that this protected region contains a binding domain of factor Va to phospholipid vesicles. This interactions with the phospholipid vesicles involves a peptide of 99 amino acids (residues 1667–1765) located on the central part of the A3 domain of factor Va (9, 18). It should be interesting to find whether all of the peptide sequence is necessary for the binding of the light chain to PCPS vesicles, and, consequently, if the tertiary structure of this peptide or of the light chain plays a role in the binding of factor Va to the phospholipid bilayer.

Acknowledgments—We wish to thank Dr. Srinam Krishnaswamy for reviewing this manuscript and for his general support to this project. We wish to acknowledge Matthew Rand for excellent technical assistance, Xavier C. Villarreal and Robert J. Kelm for the protein sequencing and amino acid composition, and Mary E. Mahoney and Lisa McNaney for their patient assistance in the preparation of this manuscript.

REFERENCES


Fig. 9. Hydropathy plot of the factor Va-derived phospholipid-binding domain. The hydrophobic and hydrophilic regions within the 99-amino acid region of factor Va (residues 1667–1765) were determined as described under “Experimental Procedures.” The center line in the plot is the boundary between the hydrophobic and hydrophilic properties of each amino acid. The area below the center line indicates hydrophilic regions, and the area above indicates hydrophobic regions. An increase in distance from the centerline indicates that a region is either more hydrophobic or hydrophilic. The hydrophobic and hydrophilic areas within this peptide coincide with the probability of the same regions to be in contact with the solvent (second line). The two hydrophobic regions located between the chymotrypsin and factor Xa and/or APC cleavage sites (residues 1716–1727 and 1741–1748) are indicated by the vertical arrows.
A Phospholipid-binding Domain of Factor Va

Identification and characterization of a phospholipid-binding site of bovine factor Va.

M Kalafatis, R J Jenny and K G Mann


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