Factor Va–Membrane Interaction Is Mediated by Two Regions Located on the Light Chain of the Cofactor†

Michael Kalafatis, Matthew D. Rand, and Kenneth G. Mann*

Department of Biochemistry, Given Building, Health Science Complex, University of Vermont, College of Medicine, Burlington, Vermont 05405-0068

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ABSTRACT: Factor Va was incubated with 1-azidopyrene, a fluorescent lipophilic probe, in the presence of phospholipid vesicles composed of various proportions of phosphatidylcholine (PC) and phosphatidylserine (PS). The majority of the label was associated with the light chain of factor Va. The light chain was found to be labeled in the presence of phospholipid vesicles containing either 100% PC or 100% PS. After cleavage by factor Xa and incubation with PC/PS vesicles composed of 75% PC and 25% PS, label was found both on the M_r = 30 000 fragment, derived from the NH_2-terminal portion of the bovine factor Va light chain (residues 1537–1752), and on the M_r = 46 000/48 000 carboxyl-terminal fragment of the factor Va light chain (residues 1753–2183). The M_r = 46 000/48 000 fragment incorporated 1-azidopyrene independent of the phospholipid composition, while label incorporation into the M_r = 30 000 fragment required phospholipid vesicles containing PC. No labeling of the M_r = 30 000 fragment was observed with phospholipid vesicles composed of 100% PS. The label incorporation into the two portions of the molecule was found to be independent of the ionic strength in the presence of phospholipid vesicles containing 75% PC and 25% PS. In contrast, the labeling of the M_r = 46 000/48 000 fragment with phospholipid vesicles composed of 100% PS was ionic strength dependent. These data suggest that two regions of factor Va light chain interact with the lipid bilayer and have different requirements for interaction: the binding site located on the M_r = 30 000 fragment of the cofactor (A3 domain) interacts with phospholipid vesicles containing neutral phospholipid and is most likely hydrophobic in nature whereas the binding site located on the M_r = 46 000/48 000 carboxyl-terminal fragment (C1–C2 domains) interacts with membranes composed of anionic and neutral phospholipid and displays partly ionic binding characteristics.

Factor Va is an essential cofactor for the activation of prothrombin (Mann et al., 1988; Kane & Davie, 1988). Factor V circulates in plasma as a large protein of M_r = 330 000 (Nesheim & Mann, 1979). The cDNA sequence and derived amino acid sequence for human and bovine factor V have been determined (Kane & Davie, 1986; Jenny et al., 1987; Guinto et al., 1992). Factor V (human and bovine) is composed of three "A" domains, two "C" domains, and a connecting "B" region. Proteolysis of factor V by thrombin or factor Xa (Nesheim & Mann, 1979; Foster et al., 1983; Monkovic & Tracy, 1990) leads to the formation of the active cofactor (factor Va) which is composed of a heavy chain (M_r = 105 000 in the human molecule and 94 000 in the bovine molecule) containing two A domains (A1–A2) and a light chain (M_r = 74 000 for both species) containing an A domain and two C domains (A3–C1–C2). There is 84% identity between the human and bovine heavy chains and 86% identity between the two light chains (Guinto et al., 1992). The two chains of the active cofactor are noncovalently associated in the presence of Ca^{2+} ions.

The assembly of the *prothrombinase* complex involves protein–protein interactions and protein–phospholipid interactions. The functional factor Va–phospholipid interaction is Ca^{2+}-independent, involves the light chain of the cofactor (Bloom et al., 1979; Higgins & Mann, 1983), and requires anionic phospholipid. Conflicting data exist as to whether the binding of factor Va (or factor V) to phospholipid vesicles is ionic strength dependent (Higgins & Mann, 1983; van de Waart et al., 1983; Pusey & Nelsestuen, 1984). Factor Va binds to phospholipid vesicles composed of 75% phosphatidylcholine (PC) and 25% phosphatidylserine (PS) with a K_d = 2.7 nM (Krishnaswamy & Mann, 1988). In our hands, the dissociation constant (K_d) and the stoichiometry of binding of factor V to similar phospholipid vesicles appear minimally affected by the ionic strength of the solvent (Higgins & Mann, 1983). A previous report from our laboratory suggested the presence of a phospholipid binding domain on the NH_2-terminal portion of the A3 domain of factor Va light chain (Krishnaswamy & Mann, 1988). Using peptide fragmentation, this lipid binding site was localized to a peptide containing residues 1654–1752 of the bovine cofactor (Kalafatis et al., 1990). Recently, a study using 100% PS-coated microtiter plates reported that a binding site exists on the C2 domain of the factor Va light chain (Ortel et al., 1992) (corresponding to residues 2024–2183 in the bovine factor V sequence) (Guinto et al., 1992). The present study was undertaken in order to address the location of the phospholipid binding sites within the factor Va light chain and their specificity with respect to the membrane composition.

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 (BSA), 1-palmitoyl-2-oleoylphosphatidylserine (PS), and 1-palmitoyl-2-oleoylphosphatidylcholine (PC) were purchased from Sigma (St. Louis, MO). 1-Azidopyrene (Nieva-

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* To whom correspondence should be addressed.

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Gomez & Gennis, 1977) was purchased from Molecular Probes Inc. (Eugene, OR). 1-Azidopyrene was dissolved in dimethyl sulfoxide (DMSO; Baker Analyzed, Phillipsburg, NJ) at a concentration of 54.7 mM and kept in aliquots in the dark at −20 °C. D-Phenylalanylprolylarginyl chloromethyl ketone (FPR-ck) was purchased from Calbiochem (San Diego, CA). The fluorescent thrombin inhibitor dansylarginine *N,N*-(3-ethyl-1,5-pentanediyl)amide (DAPA) was obtained as previously described (Nesheim et al., 1979a) and was a gift of Dr. Paul Haley (Haematologic Technologies Inc., Essex Junction, VT). Purified annexin V (Funakoshi et al., 1987) was provided by Dr. Kazuo Fujikawa (Department of Biochemistry, University of Washington, Seattle, WA). Bovine prothrombin, thrombin, and factor Xa were purified according to previously described methods (Bajaj & Mann, 1973; Lundblad et al., 1976; Krishnaswamy et al., 1987).

Purification of Factor Va and Factor Va Subunits. Bovine factor Va was obtained as previously described by our laboratory (Nesheim et al., 1980; Katzmann et al., 1981). Purified bovine factor Va displays heterogeneity in the heavy and light chains, both subunits migrating as doublets on sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) (Kalafatis et al., 1993a). All purified factor Va components, M_r = 94 000 heavy chain (H94), M_r = 90 000 product of the heavy chain (H90) which lacks the acidic COOH-terminal end, and M_r = 74 000/72 000 doublet representing the light chain of the cofactor, were obtained using (ethylenedinitrilo)tetraacetic acid (EDTA) and Q-Sepharose and an anti-light chain immunoaffinity column as recently described (Kalafatis et al., 1993b).

Preparation of Phospholipid Vesicles. Phospholipid vesicles composed of various weight percentages in PC and PS were prepared as previously described (Barenholz et al., 1977). Briefly, PC and PS were combined at various weight percentages, and the different mixtures were dried under a gentle stream of nitrogen. Phospholipid mixtures were resuspended in 11 mL of cold buffer composed of 20 mM Hepes/0.15 M NaCl, pH 7.4, and sonicated for 30 min on ice. The mixtures were centrifuged for 30 min at 115000*g*, then the speed was increased to 152000*g*, and centrifugation was allowed to proceed for 210 min at 18 °C. The concentration of the phospholipid vesicles within each mixture was determined by phosphorous assay (Gomori, 1942).

Assay Measuring Thrombin Formation. The formation of thrombin was analyzed using the fluorescent thrombin inhibitor DAPA. In a typical experiment, prothrombin (1.4 μM), DAPA (3 μM), and phospholipid vesicles (20 μM) composed of either 100% PC, or 75% PC/25% PS, or 100% PS were incubated in the dark for 30 min, in order to allow interaction of the components with the phospholipid vesicles and in order to allow DAPA to interact with residual contaminating thrombin. An aliquot of the mixture (1.85 mL) was added to a cuvette containing 10 nM factor Xa, and the base line was monitored for 2 min at room temperature using a Perkin Elmer MPF-44A fluorescence spectrophotometer with λ_{ex} = 280 nm, λ_{em} = 550 nm, and a 500-nm long pass filter in the emission beam. Factor Va was then added at a concentration of 1 nM, and the fluorescence intensity was monitored with time. The initial rate (nanomolar IIa per minute) was calculated as described (Nesheim et al., 1979a).

Labeling with 1-Azidopyrene. 1-Azidopyrene is a specific probe which can bind to the portion of a protein which is in contact with a phospholipid surface. This probe has been shown to bind to the amino acid portion of vimentin (Perides et al., 1986, 1987) and rhodopsin (Klip et al., 1976), which penetrate the lipid bilayer. Annexin V and factor Va were labeled in the presence of phospholipid vesicles containing various amounts of PC and PS using 1-azidopyrene. Annexin V and bovine factor Va (3 μM) in 20 mM Hepes, 0.15 M NaCl, and 5 mM $CaCl_2$, pH 7.4, were incubated with phospholipid vesicles composed of various weight percentages of PC and PS (200 μM) at room temperature for 5 min, in a quartz cuvette. Labeling with 1-azidopyrene was performed essentially as described (Perides et al., 1986). Briefly, 1-azidopyrene was added to the quartz cuvette containing the protein/phospholipid mixture to a final concentration of 36.5 μM (the final DMSO concentration was 0.67%). The mixture was further incubated in the dark for 30 min followed by illumination (5 min) with a long-wave UV light box (UV chromato-vue transilluminator; UVP, Inc., San Gabriel, CA). Upon illumination, the azide is converted to a nitrene which can react with adjacent C=C and C—H bonds in order to form chemically stable derivatives. Once illuminated, the mixture was immediately transferred to an Eppendorf tube containing 1% SDS and 2% β-mercaptoethanol, incubated at 90 °C for 5 min, and stored at −20 °C prior to analysis by SDS–PAGE.

Gel Electrophoresis. SDS–PAGE analysis was performed using 5–15% and 8–18% gradient gels using the method of Laemmli (1970). Proteins were 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. Densitometric analyses of the Coomassie Brilliant Blue-stained SDS–PAGE and of the negative of the fluorescent picture representing incorporation of 1-azidopyrene in different portions of factor Va were performed using a Microscan 1000 scanning densitometer (TRI Inc., Nashville, TN). Results were obtained as integrated volumes for each protein band using arbitrary density units.

RESULTS

Using semiquantitative binding experiments with phospholipid-coated plates, several studies have shown that factor Va binds with high affinity to PS-coated microtiter wells. We have reproduced these binding studies using 100% PS-coated and 75% PC/25% PS-coated microtiter wells according to a recently described method (Ortel et al., 1992). The affinity of ^{125}I-bovine factor Va for PS-coated plates was 1.7 times higher than for PC/PS-coated plates. However, in the presence of phospholipid vesicles composed of 75% PC and 25% PS, the initial rate of thrombin formation in three different experiments was 804 ± 33 nM IIa/min. In the presence of phospholipid vesicles composed of 100% PS, the generation of thrombin was slow with an initial rate of 25 ± 3 nM IIa/min. In the absence of a phospholipid surface, the generation of thrombin was undetectable under our experimental conditions. Thus, even though the affinity of factor Va for PS-coated plates is greater than for PC/PS-coated wells, the efficiency of the *prothrombinase* components with phospholipid vesicles composed of 100% PS represents only 3% of the initial rate of the formation of thrombin when the complex is assembled on phospholipid vesicles composed of 25% PS and 75% PC. Therefore, as previously reported by others (Nesheim et al., 1979b; Jones et al., 1985; Gerads et al., 1990), the preferred surface for *prothrombinase* complex assembly is composed of phospholipid vesicles containing a mixture of PC and PS.

Labeling of Annexin V with 1-Azidopyrene. In order to test the lipid binding protein specificity of 1-azidopyrene, we

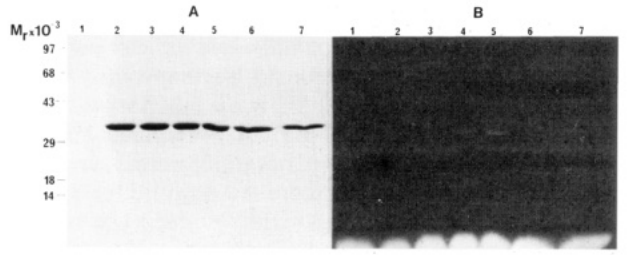

FIGURE 1: Labeling of annexin V in the presence of phospholipid vesicles. Annexin V (3 μM) was incubated with phospholipid vesicles (200 μM) and labeled with 1-azidopyrene as described under Experimental Procedures. The samples were prepared for SDS–PAGE (8–18% linear gradient) under reducing conditions. The gel was stained with Coomassie Blue (A). Prior to staining the gel, the incorporation of 1-azidopyrene in annexin V was visualized by excitation with a long-wave UV light box and photographed through a 418-nm cut-off filter (B). Lane 1, 1-azidopyrene in the presence of phospholipid vesicles composed of 75% PC and 25% PS; lane 2, annexin V in the absence of phospholipid vesicles, but in the presence of 1-azidopyrene; lane 3, annexin V in the presence of 1-azidopyrene and phospholipid vesicles composed of 100% PC; lane 4, annexin V as in lane 3 but in the presence of phospholipid vesicles composed of 75% PC and 25% PS; lane 5, annexin V as in lane 3 but in the presence of phospholipid vesicles composed of 100% PS; lane 6, annexin V as in lane 4 but in the presence of 100 mM EDTA; lane 7, annexin V as in lane 4 but in the presence of 1.5 M NaCl. Molecular weight markers are indicated on the left of panel A.

used annexin V, a well-characterized lipid binding protein. Annexin V is an $M_r = 36\,500$ soluble cellular protein composed of 319 amino acid residues which binds to phospholipid vesicles composed of 80% PC and 20% PS with a K_d = 1.2 nM (Funakoshi et al., 1987; Tait et al., 1989). The binding of annexin V to phospholipid vesicles is PS and ionic strength dependent (Reutelingsperger et al., 1988; Meers et al., 1991). Annexin V binding was not observed in the presence of vesicles containing only PC (Reutelingsperger et al., 1988). Annexin V binding to a lipid surface requires the presence of Ca^{2+} ions, and it has been shown that concentrations as low as 10 μM $CaCl_2$ are sufficient to promote annexin V binding to PC/PS vesicles (Meers et al., 1991). It has been suggested, using electrical measurements, that only a small portion of the molecule interacts with the lipid bilayer (Rojas et al., 1990), but it is not certain whether annexin V penetrates the lipid bilayer.

In the absence of protein and in the presence of PC/PS vesicles, no labeling was observed (Figure 1, lane 1). Annexin V in the absence of phospholipid vesicles or with phospholipid vesicles composed of 100% PC did not incorporate significant label (Figure 1, lanes 2 and 3). Annexin V incorporated label in the presence of phospholipid vesicles composed of either 75% PC and 25% PS (Figure 1, lane 4) or 100% PS (Figure 1, lane 5). When 100 mM EDTA was included in the mixture, no labeling of annexin V was observed (Figure 1, lane 6). A 50% decrease in the labeling of annexin V was observed in the presence of high ionic strength (1.5 M NaCl) (Figure 1, lane 7). Thus, the labeling of annexin V with 1-azidopyrene occurs only in the presence of the conditions which have been established to permit membrane interaction of the protein. It is noteworthy that in the absence of phospholipid or in the presence of 100% PC vesicles the labeling of annexin V represents approximately 12% of the labeling observed in the presence of PC/PS or 100% PS vesicles as assessed by scanning densitometry. Thus, label incorporation into annexin V under conditions which do not allow interaction with a membrane surface or in the absence of a phospholipid surface may be considered as nonspecific.

A comparison of Figure 1B, lane 2, with Figure 1B, lanes 4 and 5, demonstrates that there is no influence of potential vesicle aggregation (i.e., in the presence of 5 mM $CaCl_2$) since the labeling of annexin V is lipid dependent and occurs in the presence of 100% PS. Moreover, the labeling of annexin V observed in the presence of phospholipid vesicles composed of 75% PC and 25% PS (Figure 1B, lane 4) is similar to the labeling of the molecule in the presence of vesicles composed of 100% PS (Figure 1B, lane 5). Altogether these data support the conclusion that 1-azidopyrene is a specific lipophilic probe which can be used to identify the portions of molecules which interact with the lipid bilayer (Nieva-Gomez & Gennis, 1977; Perides et al., 1986, 1987; Klip et al., 1976).

Labeling of Factor Va with 1-Azidopyrene. When purified bovine factor Va was incubated with 1-azidopyrene in the absence of phospholipid vesicles, trace amounts of labeling were incorporated into the heavy and light chains of the cofactor (Figure 2B, lane 2). Factor Va incubated with phospholipid vesicles composed of 100% PC showed a 3-fold increase in the incorporation of 1-azidopyrene in the light chain and the $M_r = 46\,000/48\,000$ carboxyl-terminal portion of the light chain (Figure 2B, lane 3). An increase in the content of PS (to 5%) within the phospholipid vesicles led to a 5-fold increase in the incorporation of label by the light chain and to a more intense labeling of the $M_r = 46\,000/48\,000$ fragment (compare Figure 2B, lane 4, with the sample in lane 2, in the absence of phospholipid vesicles). A slight increase in the labeling of factor Va on the light chain was observed with vesicles which contained increasing concentrations of PS (Figure 2B, lanes 5–12). Figure 2 also shows that labeling of the light chain as well as of the $M_r = 46\,000/48\,000$ fragment is more intense in the presence of phospholipid vesicles composed of 100% PS than in the presence of phospholipid vesicles composed of 100% PC. A relative comparison of the sample shown in Figure 2B, lane 3, with the sample shown in lane 12 as assessed by scanning densitometry demonstrated a 1.5-fold increase in the labeling of the light chain in the presence of PS vesicles when compared to the labeling of the light chain in the presence of PC vesicles. Our data support the conclusion that a lipid binding domain is located on the light chain of the cofactor as well as on the $M_r = 46\,000/48\,000$ fragment.

The heavy chain of the cofactor also incorporates trace amounts of label. In the absence of a membrane surface, factor Va heavy and light chains incorporate approximately equimolar levels of label (Figure 2B, lane 2). In the presence of PC vesicles, there is approximately a 2-fold increase in the labeling of the heavy chain which remains unchanged in the presence of any combination of PC and PS vesicles (Figure 2B, lanes 3–12). The labeling of the heavy chain represents 10–15% of the labeling of the light chain. It is well established that factor Va heavy chain, as well as annexin V, does not interact with PC vesicles. Faint labeling was observed with PC vesicles and annexin V (Figure 1, lane 3) which represented approximately 12% of the total label incorporated in the presence of PC/PS and PS vesicles. Thus, the faint labeling on the heavy chain of the cofactor which is quantitatively similar to the nonspecific labeling of annexin V may be considered as nonspecific. This phenomenon, to a greater extent, was observed in a previous study using radioactive lipophilic photoreagents (Krieg et al., 1987). However, incorporation of label into the heavy chain was not observed by Lecompte et al. (1987) using 1-azido-5-[^{125}I]iodonaphthalene, whereas it was significant when using [^{3}H]adamantanediazirine or 3-(trifluoromethyl)-3-(*m*-[^{125}I]iodophenyl)-

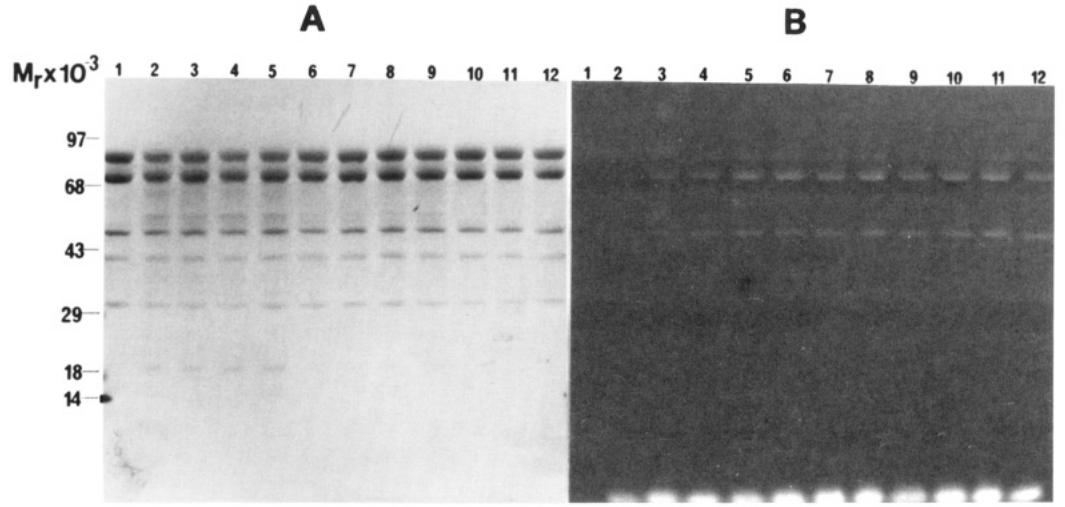

FIGURE 2: Labeling of factor Va in the presence of phospholipid vesicles with varying weight percentages of phosphatidylserine (PS) and phosphatidylcholine (PC). Factor Va was incubated in the presence of phospholipid vesicles and labeled with 1-azidopyrene as described under Experimental Procedures. The samples were prepared for SDS–PAGE (5–15% linear gradient) under reducing conditions. The gel was stained with Coomassie Blue (A). Prior to staining the gel, the portions of factor Va which specifically incorporated 1-azidopyrene were visualized by excitation with a long-wave UV light box and photographed through a KV 418-nm Schott filter (B). Lane 1, factor Va control; lane 2, factor Va in the absence of phospholipid vesicles, but in the presence of 1-azidopyrene; lanes 3–12, factor Va in the presence of 1-azidopyrene and 200 μM phospholipid vesicles composed of 100% PC, 95% PC/5% PS, 90% PC/10% PS, 85% PC/15% PS, 80% PC/20% PS, 75%PC/25% PS, 70% PC/30% PS, 50% PC/50% PS, 30% PC/70% PS, and 100% PS. Molecular weight markers are indicated on the left of panel A.

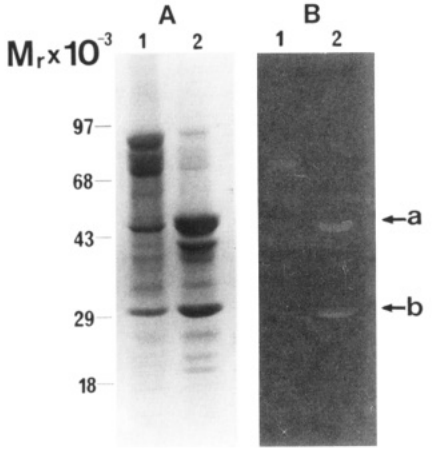

FIGURE 3: Labeling of factor Va before or after cleavage by factor Xa. Factor Va (3 μM) was digested with factor Xa using an enzyme: substrate ratio of 1:10 for 4 h at 37 °C. The mixture was then incubated with phospholipid vesicles composed of 25% PS and 75% PC, and the labeling with 1-azidopyrene was performed as described under Experimental Procedures. (A) SDS–PAGE (8–18% linear gradient) stained with Coomassie Blue; (B) fluorescent photograph of the gel. Lane 1, labeled factor Va control; lane 2, factor Va digested with factor Xa prior to labeling with 1-azidopyrene. The positions of the molecular weight markers are depicted at the left of panel A. The positions of the M_r = 46 000/48 000 fragment (a) and the M_r = 30 000 fragment (b) are also indicated.

diazirine (Krieg et al., 1987). Altogether these data demonstrate that nonspecific label incorporation in the heavy chain is dependent on the lipophilic probe used within each study.

Labeling of Factor Va before and after Cleavage by Factor Xa. In the presence of 1-azidopyrene and phospholipid vesicles composed of 75% PC and 25% PS, the majority of the label was found in the light chain of factor Va (Figure 3B, lane 1). Fluorescence was also detected in the M_r = 30 000 and M_r = 46 000/48 000 fragments of the light chain. Factor Xa cleavage of the heavy chain results in M_r = 56 000 and 45 000 fragments (Odegaard & Mann, 1987). Factor Xa cleavage of the light chain at Arg_{1752} generates an M_r = 30 000 fragment, the NH_2-terminal part of the light chain, and an M_r = 46 000/48 000 fragment containing the COOH-terminal part of the light chain (Figure 3A, lane 2) (Odegaard & Mann, 1987). After interaction with factor Xa and incubation with PC/PS vesicles and 1-azidopyrene, the label appears in both factor Xa-derived fragments of the light chain (Figure 3B, lane 2). These data suggest that at least two distinct regions of the light chain interact with PC/PS vesicles.

Differential Labeling of Factor Va in the Presence of Phospholipid Containing Different Weight Percent Compositions of PC and PS. Factor Va was digested with factor Xa (4 h, at 37 °C) and incubated with 1-azidopyrene and phospholipid vesicles composed of 100% PC (Figure 4, lane 2), 75% PC and 25% PS (Figure 4, lane 3), and 100% PS (Figure 4, lane 4). In order to normalize the results and to correct for the error due to the loading of the SDS–PAGE gel, the Coomassie Blue-stained gel and the negative of the fluorescent picture were subjected to scanning densitometry. The densities of the M_r = 46 000/48 000 fragment as well as of the M_r = 30 000 fragment were recorded. Figure 5 shows the ratio of the density of the fluorescence divided by the density of the two fragments stained with Coomassie Blue.

When 1-azdiopyrene was incubated with factor Xa-treated factor Va in the absence of phospholipid vesicles, no significant amounts of 1-azidopyrene were incorporated in the factor Va light chain fragments (Figure 4B, lane 1). In the presence of phospholipid vesicles composed of either 100% PC or 75% PC and 25% PS, both fragments were labeled (Figure 4B, lanes 2 and 3, and Figure 5). However, when factor Xa-treated factor Va was incubated with phospholipid vesicles composed of 100% PS, only the M_r = 46 000/48 000 fragment was found to be labeled (Figure 4B, lane 4). The amount of label incorporated into this fragment appears to be higher than that incorporated in the presence of other phospholipid compositions (Figure 5, lane 4). These data suggest that the two lipid binding domains located on the light chain of the cofactor have different requirements with respect to the composition of the lipid bilayer. A direct comparison of Figure 5, lane 4, with Figure 2B, lane 12, indicates that the labeling of factor Va in the presence of phospholipid vesicles composed of 100% PS is exclusively due to the portion of the light chain

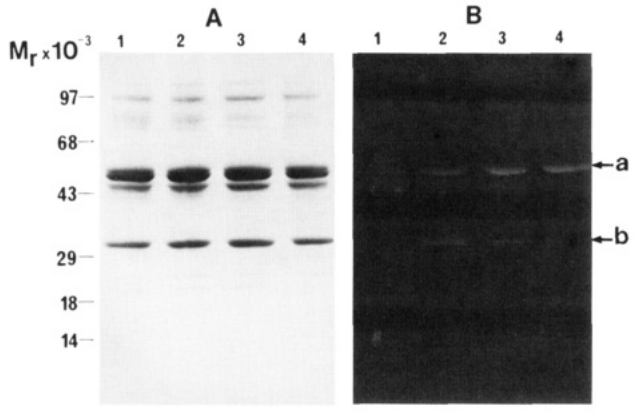

FIGURE 4: Labeling of factor Va lipid binding sites in the presence of phospholipid vesicles composed of 100% PC, 25% PS/75% PC, or 100% PS. Factor Va was digested with factor Xa as described in the legend of figure 3. The mixture was then incubated with phospholipid vesicles (200 μM) composed of either 100% PC, 25% PS/75% PC, or 100% PS. Labeling with 1-azidopyrene was performed as described under Experimental Procedures. (A) SDS–PAGE (8–18% linear gradient) stained with Coomassie Blue; (B) fluorescent photograph of the gel. Lane 1, factor Va digested with factor Xa and incubated with 1-azidopyrene in the absence of phospholipid vesicles; lane 2, factor Va digested with factor Xa and labeled with 1-azidopyrene in the presence of phospholipid vesicles composed of 100% PC; lane 3, sample as in lane 2 but in the presence of phospholipid vesicles composed of 25% PS and 75% PC; lane 4, sample as in lane 2 but in the presence of phospholipid vesicles composed of 100% PS. The positions of the molecular weight markers are indicated at the left of panel A. The positions of the M_r = 46 000/48 000 fragment (a) and the M_r = 30 000 fragment (b) are also indicated.

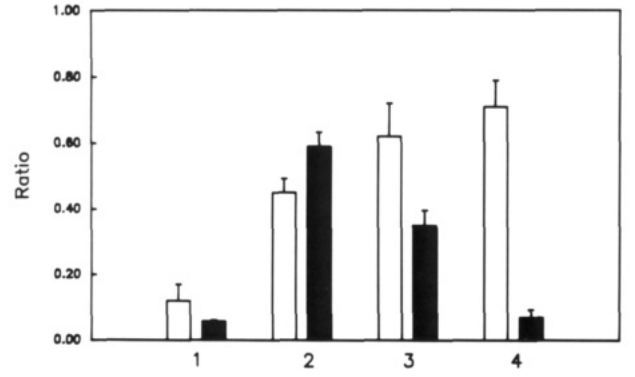

FIGURE 5: Analysis of the interaction of factor Va lipid binding domains with phospholipid vesicles containing varying weight percentages of phosphatidylserine and phosphatidylcholine. The SDS–PAGE gel depicted in Figure 4A was subjected to scanning densitometry. The density of the M_r = 46 000/48 000 fragment as well as the density of the M_r = 30 000 fragment was recorded and compared to the fluorescence incorporated into each fragment in the presence of phospholipid vesicles containing varying weight percentages of phosphatidylserine and phosphatidylcholine (Figure 4B). The figure depicts the ratio of the density of the fluorescence divided by the density of each fragment stained with Coomassie Blue. Open bars depict the analysis of the binding of the M_r = 46 000/48 000 fragment whereas filled bars represent the binding of the M_r = 30 000 fragment. The error bars represent the average of two different experiments and three different SDS–PAGE gel analyses. Lanes 1–4 of the histogram correspond to lanes 1–4 of Figure 4.

located at the carboxyl-terminal portion of the light chain (C1 and C2 domains).

Effect of Solvent Ionic Strength on the Binding of Factor Va to Phospholipid Vesicles. Figure 6 shows that there is no significant change in the intensity of the label incorporated in the M_r = 46 000/48 000 fragment (Figure 6, fragment a) and the M_r = 30 000 fragment (Figure 6, fragment b) in the presence of increasing concentrations of NaCl (Figure 6, lanes 1–3). Label incorporation remains approximately the same

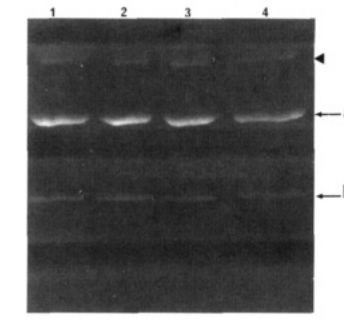

FIGURE 6: Effect of ionic strength on the interaction of factor Va with PC/PS vesicles. Factor Va was digested with factor Xa as described in the legend of Figure 3. The mixture was then incubated with phospholipid vesicles (200 μM) composed of 75% PC/25% PS in the presence of increasing concentrations of NaCl. Labeling with 1-azidopyrene was performed as described under Experimental Procedures. The figure only depicts the fluorescent photograph of the SDS–PAGE (8–18% linear gradient). Lane 1, factor Va digested with factor Xa and incubated with 1-azidopyrene and phospholipid vesicles in the presence of 0.15 M NaCl; lane 2, sample as in lane 1 in the presence of 0.5 M NaCl; lane 3, sample as in lane 1 in the presence of 1 M NaCl; lane 4, sample as in lane 1 in the presence of 1.5 M NaCl. (a) indicates the position of the M_r = 46 000/48 000 fragment and (b) the position of the M_r = 30 000 fragment. The arrowhead indicates the labeling incorporated into the remaining factor Va light chain. Visual inspection of the Coomassie Blue stained gel confirmed that the amount of protein loaded on each lane was approximately similar.

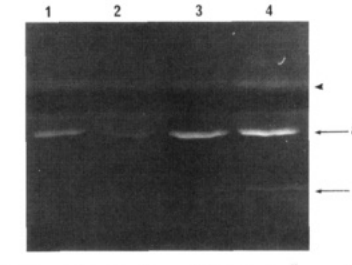

FIGURE 7: Ca^{2+} and ionic strength influence on the interaction of factor Va with PS vesicles. Factor Va was digested with factor Xa as described in the legend of Figure 3. The mixture (2 μM factor Xa-treated factor Va) was incubated with phospholipid vesicles (200 μM) composed of 100% PS and 1-azidopyrene as described under Experimental Procedures. The figure only depicts the fluorescent photograph of the SDS–PAGE (8–18% linear gradient). Lane 1, factor Va digested with factor Xa and incubated with 1-azidopyrene and PS vesicles in the presence of 0.15 M NaCl; lane 2, sample as in lane 1 but in the presence of 1 M NaCl; lane 3, sample as in lane 1 but in the presence of 100 mM EDTA; lane 4, factor Va in the presence of PC/PS vesicles (75/25) and 100 mM EDTA. (a) indicates the positions of the M_r = 46 000/48 000 fragment, and (b) shows the position of the M_r = 30 000 fragment. The arrowhead depicts the labeling incorporated into the remaining factor Va light chain.

(±10% as assessed by scanning densitometry of the negative of the fluorescent picture) even in the presence of 1.5 M NaCl (Figure 6, lane 4). These data demonstrate that there is no effect of increasing NaCl concentrations on the factor Va–PC/PS vesicle interaction.

In order to verify that the binding of factor Va to PS vesicles is ionic strength dependent, factor Xa-treated factor Va was incubated with phospholipid vesicles containing 100% PS in the presence of 0.15 M NaCl (Figure 7, lane 1), 1 M NaCl (Figure 7, lane 2), and 100 mM EDTA (Figure 7, lane 3). A control experiment was performed using phospholipid vesicles composed of 75% PC and 25% PS in the presence of 100 mM EDTA (Figure 7, lane 4). The labeling of the M_r = 46 000/48 000 fragment of the light chain was substantially decreased in the presence of 1 M NaCl (Figure 7, lane 2, fragment a). Scanning densitometric analysis of the sample shown in lane 2 demonstrated a 50% decrease in the labeling

of the light chain of factor Va when incubated with 1 M NaCl and PS vesicles when compared with the sample in lane 1. In contrast, the labeling of the M_r = 46 000/48 000 fragment in the presence of 100 mM EDTA is more intense in the presence of phospholipid vesicles composed either of 100% PS (Figure 7, lane 3) or of 75% PC and 25% PS (Figure 7, lane 4). In both cases, scanning densitometric analyses demonstrated that there is approximately a 2-fold increase in the incorporation of label into the light chain of the cofactor in the presence of 100 mM EDTA as compared to the sample shown in lane 1 (the label incorporation of the samples shown in Figure 7, lanes 3 and 4, represents 187% and 167%, respectively, of the labeling shown in Figure 7, lane 1). These data suggest that the PS-dependent labeling of factor Va on the M_r = 46 000/48 000 fragment is ionic strength dependent. The results depicted in Figures 4–7 suggest that the binding of factor Va to 100% PS vesicles involves Ca^{2+}-independent electrostatic interactions and a binding site located on the carboxyl-terminal part of the light chain.

DISCUSSION

Previous data have demonstrated labeling of factor Va light chain using the lipophilic, photoactivable radioactive probes 1-azido-5-[^{125}I]iodonaphthalene (Lecompte et al., 1987), [^{3}H]-adamantanediazirine (Krieg et al., 1987), or 3-(trifluoromethyl)-3-(*m*-[^{125}I]iodophenyl)diazirine (Krieg et al., 1987) in the presence of phospholipid vesicles containing 75% PC/25% PS. The present data demonstrate that two regions of the light chain of factor Va are able to incorporate the fluorescent lipophilic probe 1-azidopyrene in the presence of phospholipid vesicles composed of either 100% PC or 75% PC and 25% PS. These data suggest that at least two binding sites for the phospholipid vesicles are located within the light chain of factor Va: one on the M_r = 30 000 NH_2-terminal fragment which contains the majority of the A3 domain (residues 1537–1752) and one on the M_r = 46 000/48 000 COOH-terminal portion of the light chain which contains the C1 and C2 domains of the cofactor (Figure 8).

The results obtained with phospholipid vesicles containing various weight percentages of PC and PS demonstrate that the incorporation of the lipophilic probe into the lipid binding domain located on the M_r = 30 000 fragment of the light chain is dependent upon the presence of PC within the composition of the phospholipid vesicles (Figure 4). Moreover, in the presence of phospholipid vesicles composed of 100% PS, only the M_r = 46 000/48 000 fragment showed label incorporation. In the presence of phospholipid vesicles containing only PC, both fragments were labeled. In addition, the labeling of the M_r = 46 000/48 000 fragment in the presence of phospholipid vesicles containing 100% PS is ionic strength dependent. Altogether these data indicate that only the M_r = 46 000/48 000 fragment contributes to the binding of factor Va to phospholipid vesicles composed of 100% PS and that electrostatic interactions are involved in the binding of factor Va to phospholipid vesicles containing only PS.

While the assembly of the *prothrombinase* components with phospholipid vesicles composed of 75% PC and 25% PS increases its activity by 5 orders of magnitude when compared to the activity of the complex in the absence of phospholipid (Nesheim et al., 1979b), the initial rate of the generation of thrombin by the *prothrombinase* complex assembled on phospholipid vesicles composed of 100% PS represents only 3% of that observed when the *prothrombinase* complex is assembled on a phospholipid surface composed of 75% PC and 25% PS (Jones et al., 1985; Gerards et al., 1990). Thus,

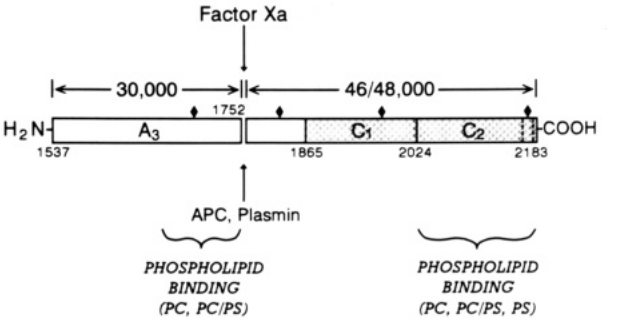

FIGURE 8: Schematic representation of the location of the two phospholipid binding sites contained within factor Va light chain. Factor Va light chain starts at residue 1537 and ends at residue 2183 of the bovine factor V molecule (Guinto et al., 1992). Factor Xa and activated protein C (APC) cleave the light chain between residues 1752 and 1753 to generate an M_r = 30 000 fragment containing the NH_2-terminal part of the molecule and an M_r = 46 000/48 000 fragment containing the COOH-terminal part of the light chain (Guinto et al., 1992 ; Odegaard & Mann, 1987). Plasmin cleavage of the light chain also produces the same fragments, as analyzed by SDS–PAGE (Omar & Mann, 1987); however, the resulting fragments were not yet analyzed for NH_2-terminal sequence. Factor Va light chain contains two phospholipid binding sites possessing different specificities: one located at the central part of the A3 domain (Kalafatis et al., 1990) which interacts with phospholipid vesicles containing neutral phospholipid and one within the C2 domain (Ortel et al., 1992) which interacts with the phospholipid vesicles independent of the composition of the vesicles. The *diamonds* represent the four potential glycosylation sites located on the light chain of factor Va (Guinto et al., 1992). The dashed lines at the carboxyl-terminal portion of the light chain depict two cleavages that must occur since the M_r = 46 000 and M_r = 48 000 fragments which have the same NH_2-terminal sequence (Odegaard & Mann, 1987) differ in relative electrophoretic mobility by 2000 and Cys_{2021} forms a disulfide bridge with Cys_{2180} (Xue et al., 1993). It is also possible that the difference between the two light chain variants is due in differences in glycosylation of one of the four potential glycosylation sites.

phospholipid vesicles composed of 100% PS provide a relatively inactive surface for the assembly and/or expression of the *prothrombinase* complex.

Significant labeling of factor Va light chain occurs in the presence of phospholipid vesicles composed of 100% PC. This observation is most likely reflective of a binding interaction since no label incorporation was observed under similar conditions using annexin V, a well-characterized lipid binding protein which only interacts with membranes containing acidic phospholipid. The K_d of factor Va for a membrane surface exclusively composed of PC is 14 μM as compared to 2.7 nM in the presence of PC/PS vesicles (Cutsforth et al., 1991; Krishnaswamy & Mann, 1988). Collectively these data demonstrate the existence of a binding element within the factor Va light chain which interacts with neutral phospholipid. The observation that the M_r = 30 000 fragment (A3 domain) of the light chain did not show any label incorporation in the presence of phospholipid vesicles composed of 100% PS suggests that the elements responsible for the interaction with phospholipid vesicles containing only PC are located on the M_r = 30 000 fragment (Figure 8). In contrast, the M_r = 46 000/48 000 fragment contains binding elements which interact with phospholipid vesicles of all compositions studied.

The overall data are in agreement with other studies from our laboratory which demonstrate that membranes exclusively composed of PS adsorb the factor Va light chain whereas with PC/PS monolayers penetration of the light chain through the membrane occurs (Lecompte et al., 1991). In the present study, we show that the M_r = 46 000/48 000 fragment (C1–C2 domains) interacts with the lipids independent of their composition whereas the M_r = 30 000 fragment requires PC

for its interaction with the lipid bilayer. Thus, it is likely that hydrophobic regions contained within the M_r = 30 000 fragment are responsible for interaction of the light chain with the phospholipid bilayer whereas the phospholipid binding domain located on the M_r = 46 000/48 000 fragment is responsible for the adsorption of the light chain to the phospholipid bilayer.

Earlier data demonstrated that the light chain of the cofactor alone is involved in the interaction of factor Va with phospholipid vesicles (composed of 75% PC and 25% PS) and/or platelets (Bloom et al., 1979; Higgins & Mann, 1983; Tracy & Mann, 1983). A binding site for PC/PS vesicles has been localized to an M_r = 30 000 portion located on the A3 domain at the NH_2-terminus of the light chain of the cofactor (Krishnaswamy & Mann, 1988). Proteolytic fragmentation and direct binding assays further localized this lipid binding domain to a peptide of 99 amino acids (residues 1654–1752 of the bovine cofactor, Figure 8) (Kalafatis et al., 1990). In contrast, recent studies by Ortel et al. (1992) using recombinant human factor V and PS-coated microtiter wells suggested the presence of a phospholipid binding site located on the C2 domain of the factor Va light chain. These conclusions were based on the observation that recombinant factor V desA3C1, which lacks the A3 and C1 domains (Figure 8), interacted with PS-coated surfaces whereas recombinant human factor V desC1C2, which lacks the C1 and C2 domains of the molecule, did not show interaction with immobilized PS. It was thus concluded that the C2 domain of the factor Va light chain contains a binding domain specific for PS. Recombinant human factor V desA3, which lacks only the A3 domain of the light chain, also did not show any interaction with the PS-coated microtiter wells. The overall data suggested that the binding of factor Va to phospholipid vesicles is a complicated phenomenon, potentially involving more than one binding site.

The recombinant molecule desA3C1 (Ortel et al., 1992), which only possesses the lipid binding site located on the C2 domain, showed a decrease in the binding to immobilized PS with increasing NaCl concentrations, suggesting that electrostatic interactions are involved in the binding of the mutant molecule to PS-coated surfaces. Recombinant factor V desA3C1, which binds to PS-coated microtiter plates in an ionic strength-dependent manner, lacks the hydrophobic lipid binding domain located on the A3 domain, whereas factor V, which binds to PS-coated microtiter wells in an ionic strength independent manner, possesses all phospholipid binding sites (Ortel et al., 1992). The difference in the binding of these two molecules may infer a hydrophilic nature to the lipid binding site located on the C2 domain (Guinto et al., 1992).

Our labeling data are consistent with the studies of Ortel et al. (1992) and with previous reports from our laboratory regarding the identification of factor Va phospholipid binding sites. Our data suggest that the binding of factor Va to membranes is promoted by two regions of the light chain of the cofactor (Figure 8): a binding site located on the A3 domain which involves hydrophobic interactions and requires neutral phospholipid and a binding region located at the COOH-terminal portion of the molecule which involves Ca^{2+}-independent electrostatic interactions and is independent of the phospholipid composition of the vesicles.

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