Roles of Factor V_a Heavy and Light Chains in Protein and Lipid Rearrangements Associated with the Formation of a Bovine Factor V_a-Membrane Complex

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ABSTRACT Factor V_a is an essential protein cofactor of the enzyme factor X_a, which activates prothrombin to thrombin during blood coagulation. Peptides with an apparent M_r of ~94,000 (heavy chain; HC) and ~74,000 or 72,000 (light chain; LC) interact in the presence of Ca^{2+} to form active V_a. The two forms of V_a-LC differ in their carboxyl-terminal C2 domain. Using V_a reconstituted with either LC form, we examined the effects of the two LC species on membrane binding and on the activity of membrane-bound V_a. We found that 1) V_a composed of the 72,000 LC bound only slightly more tightly to membranes composed of a mixture of neutral and acidic lipids, the K_d being reduced by a factor of ~3 at 5 mM and by a factor of 6 at 2 mM Ca^{2+}. 2) The two forms of V_a seemed to undergo different conformational changes when bound to a membrane. 3) The activity of bovine V_a varied somewhat with LC species, the difference being greatest at limiting X_a concentration. We have also addressed the role of the two V_a peptides in membrane lipid rearrangements and binding: 1) V_a binding increased lateral packing density in mixed neutral/acidic lipid membranes. In the solid phase, V_a-HC had no effect, whereas V_a-LC and whole V_a had similar but small effects. In the fluid phase, V_a-HC and whole V_a both altered membrane packing, with V_a-HC having the largest effect. 2) V_a-HC bound reversibly and in a Ca^{2+}-independent fashion to membranes composed of neutral phospholipid ($K_d \approx 0.3$ μM; stoichiometry ≈ 91). High ionic strength had little effect on binding. 3) The substantial effect of V_a on packing within neutral phospholipid membranes was mimicked by V_a-HC. 4) Based on measurements of membrane phase behavior, binding of V_a or its peptide components did not induce thermodynamically discernible lateral membrane domains. These results suggest that the membrane association of factor V_a is a complex process involving both chains of V_a, changes in lipid packing, and changes in protein structure.

INTRODUCTION

Factor V_a, a nonenzymatic protein cofactor, is apparently responsible for mediating several of the protein-protein and protein-membrane interactions required for assembly and functioning of the prothrombinase enzyme complex. This enzyme is required for rapid conversion of prothrombin to thrombin in the blood coagulation cascade. Factor V, which is a single-chain precursor of factor V_a, is hydrolyzed to the active factor V_a by thrombin (Esmon, 1979) or by factor X_a when little or no thrombin is present (Foster et al., 1984). Factor V_a is composed of peptides with an apparent M_r of ~94,000 (heavy chain; HC) and ~74,000 (light chain; LC), which associate noncovalently in solution in the presence of Ca^{2+} ions (Esmon, 1979; Nesheim and Mann, 1979), and may be dissociated, separated, and then reassociated to full activity by the addition of divalent metal ions (Esmon, 1979; Krishnaswamy et al., 1989). In vitro activation of bovine factor V with thrombin results in two forms of light chain (M_r ~74,000 and 72,000), as observed on a polyacrylamide gel. These can be separated by ion-exchange chromatography with a Mono S column (Guinto and Esmon, 1984; Odegaard and Mann, 1987). The difference between these two forms has been attributed to differences in post-translational glycosylation of the second C-type domain of human factor Va light chain (Ortel et al., 1992, 1994). Recently it has been reported (Rosing et al., 1993) that the two forms of human factor V_a have different cofactor activities due to differences in their binding affinities to procoagulant membranes.

By virtue of its high-affinity binding to cell (Tracy et al., 1981; Kane and Majerus, 1982) and phospholipid membranes (Pusey et al., 1982; Krishnaswamy and Mann, 1988; Kop et al., 1989), factor V_a constitutes at least part of the receptor for factor X_a at the membrane surface (Tracy and Mann, 1983). The functional factor V_a-phospholipid interaction is Ca^{2+} independent and requires anionic phospholipid (Bloom et al., 1979). The binding of factor V_a to the platelet membrane (Tracy and Mann, 1983) and to acidic-lipid-containing synthetic membranes (Krishnaswamy and Mann, 1988) is reported to be mediated through the light chain, and seems to involve both hydrophobic and electrostatic interactions with the membrane surface (Kalafatis et al., 1990, 1994; Ortel et al., 1992; Lecompte et al., 1994). It is reported that the heavy chain portion of factor V_a is not directly involved in the interaction of factor V_a with membranes (Krishnaswamy and Mann, 1988; Kalafatis et al., 1994). Binding of factor V_a has generally been acknowledged to require acidic lipids in the target membrane. However, we have reported recently that factor V_a binds to neutral lipid membranes with a K_d of ~3.0 μM (Koppaka and Lentz, 1996) and have suggested further that binding of

Received for publication 5 June 1997 and in final form 1 August 1997.

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0006-3495/97/11/2638/15 $2.00

this protein to membranes is a complex process involving acidic-lipid-independent and acidic-lipid-dependent interactions associated with hitherto undetected conformational changes in factor V_a (Cutsforth et al., 1996).

In this study, we have had two main objectives. Our first objective was to determine differences between the two forms of light chain with respect to formation of an active bovine factor V_a-membrane complex. The 72-kDa form of human factor V_a light chain is reported to form a factor V_a species that binds 45 times tighter to membranes containing anionic phospholipids than does factor V_a containing the 74-kDa form of light chain (Rosing et al., 1993). However, most studies of factor V_a structure and binding have been performed with the bovine protein (Higgens and Mann, 1983; Pusey and Nelsestuen, 1984; Isaacs et al., 1986; Kop et al., 1989; Mosesson et al., 1990; Giesen et al., 1991; Lecompte et al., 1994), and a large difference between bovine factor V_a samples reconstituted with the two light chain species was not found. Our second objective was to examine the interaction of factor V_a and its components with, and their effects on, lipid bilayer membranes. Thereby we hoped to assess the roles of heavy and light chains in the formation of a membrane complex. Our results provide two significant new insights into this process. First, membrane association appears to involve both chains of the cofactor, with the heavy chain especially able to associate with neutral lipid membranes. Second, the association of factor V_a with membranes appears not to cause the lateral rearrangement of lipids into acidic lipid domains, in contrast to previous reports (Mayer and Nelsestuen, 1983), but does invoke changes in membrane packing that, in the case of fluid membranes, are mimicked by the heavy chain alone.

EXPERIMENTAL PROCEDURES

Materials

[^{14}C]DMPC was purchased from Amersham (Arlington Heights, IL). 1,2-Dipentadecanoyl-3-sn-phosphatidylglycerol ($DC_{15}PG$), 1,2-dimyristoyl-3-sn-phosphatidylserine (DMPS), 1,2-dimyristoyl-3-sn-phosphatidylcholine (DMPC), 1,2-dioleoyl-3*sn*-phosphatidylcholine (DOPC), 1,2-dioleoyl-3-*sn*-phosphatidylserine (DOPS), bovine phosphatidylserine (Bov PS), 1-palmitoyl-2-oleoyl-phosphatidylcholine (POPC), and *N*-(lissamine rhodamine B sulfonyl)diacyl phosphatidylethanolamine (Rh-PE) were purchased from Avanti Polar Lipids (Birmingham, AL) and shown to be greater than 98% pure by thin-layer chromatography (Lentz et al., 1982). Solvents were low-residue, HPLC grade. Concentrations of the stock solutions of the phospholipids were established by inorganic phosphate determination (Chen et al., 1956). Phospholipid vesicles were prepared by procedures similar to that described earlier (Tendian and Lentz, 1990; Koppaka and Lentz, 1996). Dansylarginine-*N*-(3-ethyl-1,5-pentanediyl)amide (DAPA) was synthesized and purified by the procedure of Mann et al. (1981) and Nesheim et al. (1979b). 1,6-Diphenyl-1,3,5-hexatriene (DPH) was purchased from Molecular Probes (Junction City, OR). All other chemicals were reagent grade or better.

Bovine prothrombin and factor X were purified from a $BaSO_4$ adsorbate of 12 liters of bovine plasma (Lentz et al., 1994). Factor X was activated with a purified fraction of Russel's viper venom factor X-activating protein (Hematologic Technologies, Essex Junction, VT), which had been covalently linked to agarose beads (Jesty and Nemerson, 1976). Purification of factor V and its heavy and light chain components after activation was performed by the procedure of Esmon (1979). Further purification of the factor V_a components and subsequent labeling of factor V_a heavy chain with fluorescein maleimide was carried out as described earlier (Koppaka and Lentz, 1996). The two forms of factor V_a were also purified directly from activated factor V by separation on a Mono-S column, using a NH_4Cl gradient (Rosing et al., 1993). In this procedure, factor V_a with the light chain of M_r ~74,000 eluted first and the Va containing the light chain of M_r ~72,000 eluted later. The final purity of the proteins was ascertained by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) to be greater than 90%. Factor V_a or its component peptides were precipitated by the addition of solid ammonium sulfate to 80% saturation, collected by centrifugation (Beckman TL-100, 25 min, 30,000 rpm, 4°C), resuspended in 20 mM *N*-[Tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid (TES), 5 mM CaCl, 50% (v/v) glycerol (pH 7.4), and stored at −20°C.

Protein concentrations were determined by absorbance measurements using extinction coefficients at 280 nm, corrected for solution scattering (Donovan, 1969). The molecular weights and extinction coefficients ($E^{1\ mg/ml}_{280\ nm}$) used for the proteins were: factor V, 330,000, 0.96; factor V_a, 150,000, 1.74; factor V_a light chain, 74,000, 2.23; factor V_a heavy chain, 94,000, 1.24 (Nesheim et al., 1981); prothrombin, 72,000, 1.44 (Mann, 1976); factor Xa, 45,000, 0.94 (Jesty and Nemerson, 1976).

Reconstitution of factor V_a

A solution containing a mixture of 2.0 μM light chain and 2.4 μM heavy chain was incubated for 1.5 h at 37°C in 20 mM TES, 150 mM NaCl (pH 7.4) containing 5 mM $CaCl_2$. The assay for activity of reconstituted factor V_a involved direct measurement of prothrombin activation catalyzed by prothrombinase, using the fluorescent α-thrombin inhibitor DAPA (Nesheim et al., 1981) to directly monitor thrombin formation in an SLM 48000 spectrofluorometer (SLM Aminco, Urbana, IL) with an excitation wavelength of 335 nm (slits at 8 nm) and a Schott KV 515 (Schott Glass Technologies, Durea, PA) cutoff filter in the emission path. In these measurements, cuvettes (1.0 ml) contained 1.4 μM prothrombin, 20 μM bov PS/POPC (25/75) vesicles, and 3.0 μM DAPA in 20 mM TES, 0.15 M NaCl, 5 mM $CaCl_2$ (pH 7.4). The reaction mixtures were equilibrated for 10 min at 22°C under constant stirring. Aliquots of reassociated factor V_a (nominal concentration of 1 nM) incubated at the appropriate temperature were added to the reaction mixture. Proteolysis of prothrombin was then initiated by the addition of factor X_a to a final concentration of 10 nM. Initial rates of prothrombin activation were calculated from the initial, steady-state portion of the resulting continuous progress curves and were converted to units describing molar thrombin produced per unit time by assuming that fluorescence intensity at infinite time represented the quantitative conversion of prothrombin to thrombin (Krishnaswamy et al., 1989).

Kinetic measurement with DAPA

Experiments involving prothrombin activation as a function of phospholipid concentration were carried out as described earlier (Pei et al., 1993). These measurements were carried out at 37°C on a Shimadzu RF5000 fluorescence spectrophotometer (Shimadzu Corp., Kyoto, Japan), with excitation at 280 nm (band pass 5 nm) and emission at 540 nm (band pass 5 nm). All buffers were filtered through a 0.45-μm filter (Alltech, Deerfield, IL) to reduce scattered light artifacts. Two sets of experiments were carried out with varying phospholipid concentrations. The reaction mixture (1.0 ml in a continuously stirred cuvette) in set A (limiting factor V_a) contained 20 mM Tris, 0.15 M NaCl (pH 7.4), 5 mM $CaCl_2$, 1.2 nM X_a, 0.2 nM V_a, 500 nM DAPA, and variable concentrations of the phospholipid mixture. In set B (limiting factor X_a), the reaction mixture contained 0.1 nM X_a, 1.2 nM V_a, 500 nM DAPA, and varying concentrations of the phospholipid. The reaction mixtures were equilibrated for 3 min, allowing the prothrombinase to be fully assembled before initiating proteolysis by the addition of prothrombin to a final concentration of 50 nM. We have

used a low concentration of prothrombin to avoid having the substrate compete with the two forms of V_a for the membrane and thus interfere with the sensitivity of the assay for detecting any difference in the activities of the two forms of factor V_a. Rates of change of fluorescence intensity with time were converted into units of active site formation per unit time per unit of hypothetical membrane-bound factor X_a-V_a complex (Pei et al., 1993), using the long-time fluorescence and the known concentration of prothrombin to estimate the change in fluorescence intensity per mole of thrombin formed. The initial slopes for all of the titrations were determined when less then 15% of the substrate had been consumed, thus avoiding the problem of substrate depletion.

Fluorescence anisotropy measurements

In an earlier paper, we used three different probes (two lipid-associated and one protein-associated) to characterize the interaction of factor V_a with phosphatidylcholine membranes (Koppaka and Lentz, 1996). In five different types of binding experiments, all gave consistent results. Here we focus on two of these probes (protein-associated fluorescein and membrane-associated DPH) to characterize further the association of factor V_a with membranes. For measurements with DPH-labeled membranes, a small volume (~1.5 μl) of a stock solution of DPH (0.332 mM) in acetone was added to small unilamellar vesicle (SUV) suspensions to achieve a final dye:lipid ratio of 1:150. The vesicles were then vortexed thoroughly and incubated for 1 h at 40°C before use to achieve maximum partitioning of DPH into the bilayer. DPH-containing vesicles were added to a stirred microcuvette (Helma Cells, Jamaica, NY) containing buffer (20 mM TES, 150 mM NaCl, 5 mM $CaCl_2$, pH 7.4) and protein. The final volume in the cuvette was 1 ml, and the final concentration of phospholipid was 15 μM. The added protein was adjusted so that there was enough protein in a vesicle suspension to saturate the lipid surface-binding sites to better than 95% at every lipid composition. The ratio of protein concentration to lipid concentration used in these experiments was chosen on the basis of previously determined protein binding constants (Krishnaswamy and Mann, 1988; Cutsforth et al., 1996) to achieve this level of binding site saturation.

The mixture in the cuvette was allowed to incubate at 45°C for 10–15 min before the fluorescence measurements were begun during a cooling scan. The cuvette temperature was maintained with a programmable Neslab RTE-8 water bath equipped with an ETP-3 programmer (Neslab Instruments, Portsmouth, NH) and continuously monitored with a model 5810 Digitec digital thermometer and a model 702A Yellow Springs Instrument probe (Yellow Springs, OH) inserted directly into the cuvette. Measurements of DPH fluorescence anisotropy were made with an SLM 48000 spectrofluorometer, using an excitation wavelength of 366 nm. Emission was detected in T-format rough 418-nm cutoff filters. Anisotropy measurements were taken every 0.2°C at scan rates of 30°C/h. Details and interpretations of these measurements are discussed elsewhere (Lentz et al., 1976a,b; Tendian and Lentz, 1990).

Generation of binding isotherms

For the measurements with reconstituted fluorescein-labeled factor V_a and fluorescein-labeled factor V_a-heavy chain, an excitation wavelength of 490 nm was used. Excitation slits were 8 and 4 nm, respectively, and 500-nm cutoff filters were used in the emission path in a T-format. Measurements were performed at 23°C. Aliquots of SUV were added to the cuvette containing the labeled factor V_a in 20 mM TES, 150 mM NaCl (pH 7.5) and an appropriate concentration of $CaCl_2$. The sample was stirred after each addition for 2 min, and readings were taken 45 s after the stirring was stopped. Correction for light scatter of vesicles was made by measuring and subtracting from the observed values the parallel and perpendicular components of intensity due to vesicles alone.

The binding parameters were determined according to the following equation (Krishnaswamy et al., 1986):

$$r_{obs} = r_o + (r_{max} - r_o) \cdot \frac{[P]_b}{[P]_T} \quad (1)$$

where r_{obs}, r_o, and r_{max} are the observed, initial, and maximum anisotropy values corresponding to the protein that is all unbound, partially bound, or all bound to membranes, respectively. $[P]_b$ and $[P]_T$ are the concentrations of the bound and total protein concentrations. The concentration of bound protein, $[P]_b$, is given by

$$[P]_b = \frac{([PL]_T \cdot i + [P]_T + K_d) - \sqrt{([P]_T + [PL]_T \cdot i + K_d)^2 - (4 * [P]_T \cdot [PL]_T \cdot i)}}{2} \quad (2)$$

where K_d is the equilibrium dissociation constant, $1/i$ is the stoichiometry in moles of lipid monomer per mole of protein, and $[PL]_T$ is the total phospholipid concentration.

Fluorescence energy transfer experiments using fluorescein-labeled factor V_a heavy chain were carried out as described previously for labeled factor V_a (Koppaka and Lentz, 1996). An excitation wavelength of 470 nm and slits of 8 and 4 nm were used. Donor (fluorescein) emission was recorded at 520 nm through slits of 4 and 8 nm to monitor energy transfer to Rh-PE (acceptor). Aliquots of SUVs of POPC containing 1% Rh-PE were added to a microcuvette containing fluorescein-labeled factor V_a-heavy chain in 20 mM TES, 150 mM NaCl, 5 mM $CaCl_2$ (pH 7.5). The cuvette contents were constantly stirred, and, after an equilibration time of 2 min, emission was recorded. Corrections for dilution were made by the addition of unlabeled vesicles to the donor.

As described elsewhere (Koppaka and Lentz, 1996), the analysis of the binding data obtained from resonance energy transfer experiments involves a complication in that F_d/F_{da}, the ratio of the fluorescence efficiencies of the donor in the absence and the presence of acceptor, continues to increase in a linear fashion, even after binding is saturated. This linear increase is due simply to the increase in the probability of transfer associated with higher acceptor concentration. The data were thus fit with the following equation:

$$\frac{F_d}{F_{da}} = 1 + \left[\left(\frac{F_d}{F_{da}}\right)_{max} - 1\right] \cdot \frac{[P]_b}{[P]_T} + m \cdot [PL]_T \quad (3)$$

where the first term accounts for binding, with $[P]_b$ given by Eq. 2, and the second term accounts for transfer between acceptor and donor that are not bound to each other. The binding parameter values were obtained by a global analysis as described above. The value of m was estimated in two ways. First, m was taken as the slope of the binding curve at high concentrations of the phospholipid, and then constrained during regression analysis. Second, m was allowed to vary during regression analysis and estimated along with the binding parameters. In both cases, m was found to be similar, and the binding parameters were independent of the way in which the value of m was determined.

Finally, binding was also monitored by the change in the average hydrodynamic SUV radius as protein bound to the vesicle. POPC SUVs suspended (0.2 mM) in 150 mM NaCl, 5 mM $CaCl_2$, and 20 mM TES (pH 7.5) were sized at 22°C by quasielastic light scattering (QELS) in a home-built light-scattering photometer equipped with a Nicomp model 170 computing autocorrelator (Particle Sizing Systems, Santa Barbara, CA), as described in detail elsewhere (Lentz et al., 1992). To this suspension were added small aliquots (~5 μl) of V_a-HC (9 μM). After gentle mixing, samples were allowed to equilibrate for 6–8 min, and then were sized again. A single Gaussian was adequate to describe the distribution of vesicle diameters, except at high (>0.45 μM) V_a-HC concentration, where aggregation became evident. After correcting for the dilution of the original SUV suspension due to added volume, the observed vesicle diameters (D)

were fit to the equation described by Koppaka and Lentz (1996):

$$D_{obs} = D_o + \frac{(D_{max} - D_o)}{2 \cdot [PL]_T} \cdot \left(\frac{[P]_T}{i} + [PL]_T + \frac{K_d}{i} \right) - \frac{(D_{max} - D_o)}{2 \cdot [PL]_T} \cdot \sqrt{\left(\frac{[P]_T}{i} + [PL]_T + \frac{K_d}{i} \right)^2 - \left(\frac{4 * ([P]_T \cdot [PL]_T)}{i} \right)} \quad (4)$$

The binding parameter values, K_d and i, were set as global parameters, whereas the maximum observables (r_{max}, $[F_d/F_{da}]_{max}$, and D_{max}) were set as local parameters during a global analysis of multiple data sets (Koppaka and Lentz, 1996). For studies of factor V_a binding to PS/PC membranes, multiple data sets were fit using the SCoP package (Simulation Control Program; Simulation Resources, Berrien Spring, MI). Single data sets were analyzed with the same program. For studies of V_a-HC binding to POPC SUVs, SCoP was used to fit simultaneously all three types of binding experiment: fluorescence anisotropy, energy transfer, and vesicle diameter.

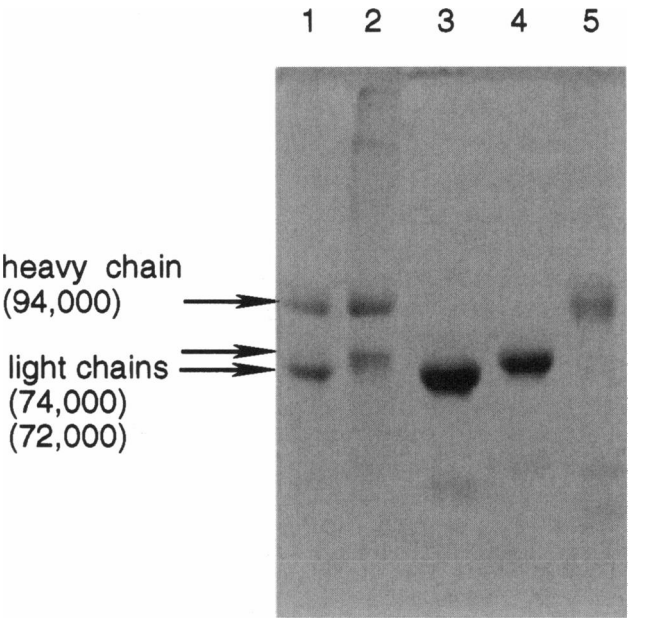

FIGURE 1 SDS-polyacrylamide gel electrophoresis of factor V_a. Purified factor V_a and components were subjected to SDS-PAGE containing 8% acrylamide and stained with Coomassie blue. About 2–2.5 μg of protein was loaded into each well. *Lane 1*, factor V_a (94,000 HC and 72,000 LC) separated by Mono-S; *Lane 2*, factor V_a (94,000 HC and 74,000 LC) separated by Mono-S; *Lane 3*, 72,000 light chain; *Lane 4*, 74,000 light chain; *Lane 5*, factor V_a-heavy chain.

RESULTS

Activity of the two forms of factor V_a

Factor V_a components were purified from activated factor V as described earlier (Koppaka and Lentz, 1996). All experiments were performed using either the purified components of factor V_a or factor V_a reconstituted from the purified components. The two forms of factor V_a were also separated via a Mono-S column (Rosing et al., 1993). The SDS-PAGE gel shown in Fig. 1 demonstrates the purity of the two forms of factor V_a separated via a Mono-S column and of the components of factor V_a isolated from activated factor V (Koppaka and Lentz, 1996). Densitometry measurements indicated that the procedure for separation of the two forms of factor V_a yielded nearly 95% pure protein for factor V_a with the M_r ~72,000 light chain and 85% pure protein for the factor V_a with the M_r ~74,000 light chain. The purified components yielded 72,000 light chain, 74,000 light chain, and heavy chain of 90%, 95%, 85% purity, respectively. Because we wanted to fluorescently label the heavy chain alone instead of whole factor V_a, we have used reconstituted factor V_a for all of our experiments. The activities of the two forms of factor V_a, whether isolated directly or reconstituted from its individual components, were similar under the conditions of our standard factor V_a assay (Experimental Procedures).

Activity of reconstituted factor V_a preparations was normally determined using the DAPA assay at 22°C, under conditions of excess factor V_a and limiting factor V_a, as described in the Experimental Procedures. Identical prothrombinase activities were observed for the two forms of factor V_a reconstituted from the 94,000 molecular weight (MW) heavy chain combined with the 74,000 (1.14 ± 0.08 $\mu MII_a/min/V_a$) and 72,000 (1.23 ± 0.08 $\mu MII_a/min/V_a$) MW light chains. These activities agreed well with the activity of factor V_a reconstituted from immunopurified subunits (1.27 ± 0.05 $\mu MII_a/min/V_a$; Krishnaswamy et al., 1989). However, it has been pointed out (Rosing et al., 1993) that assays carried out at limiting concentrations of factor V_a might not detect differences between the two forms of factor V_a. For this reason, experiments were also carried out to characterize the activity of the two types of factor V_a by varying the concentrations of phospholipid (25/75 PS/POPC) under conditions for which either 1) the cofactor or 2) the enzyme concentrations were limiting. The initial velocities of prothrombin activation for both forms of factor V_a are given in Fig. 2 *A* as a function of phospholipid concentration under conditions for which the concentration of factor V_a was limiting. It was observed, as reported earlier (Pei et al., 1993), that a minimal or critical phospholipid concentration was required to observe significant rates of thrombin formation. At lipid concentrations just above this critical concentration, the activities of both forms of V_a were essentially identical. However, at higher concentrations of phospholipid (~0.5–10 μM), factor V_a reconstituted from the 72,000 MW form of the light chain was found to be slightly more active. Experiments using an excess of factor V_a and a limiting concentration of factor X_a showed a similar behavior (Fig. 2 *B*) in that the 72,000 MW form was found to be increasingly more active at higher phospholipid concentrations. However, the enhanced activity of the 72,000 MW relative to the 74,000 MW light chain forms of factor V_a was noted, even at low phospholipid concentrations, and was dramatic at high phospholipid con-

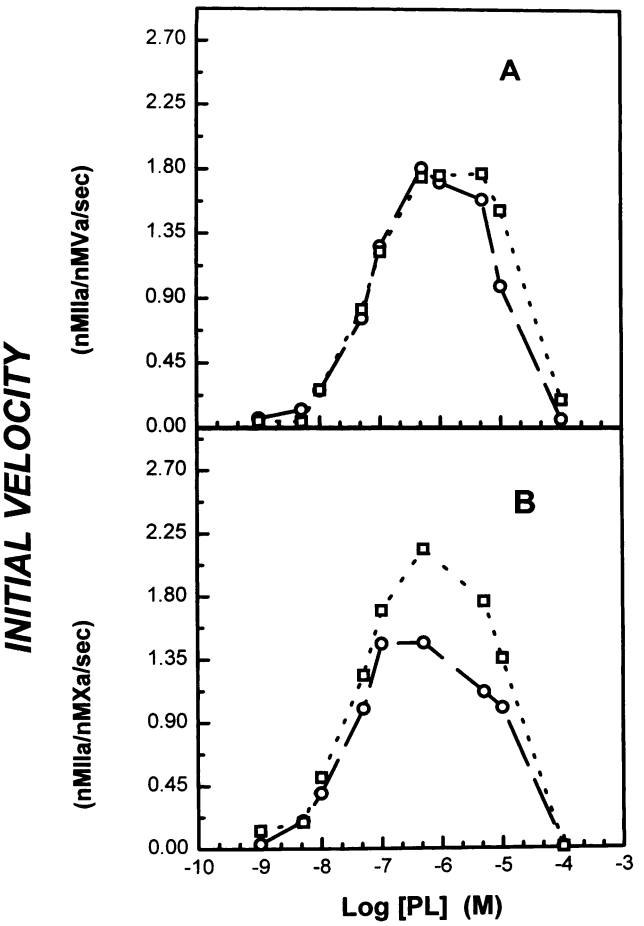

FIGURE 2 Dependence of the initial velocity of prothrombin activation on the concentration of phospholipid. Values of the initial velocity were determined from the slopes of time courses of prothrombin active site development, continuously monitored with the fluorescent thrombin inhibitor DAPA. Data are shown for factor V_a reconstituted using the 74,000 (○) and 72,000 (□) forms of light chain. The reactions were initiated by the addition of prothrombin (50 nM) to a solution of (*A*) 1.2 nM factor X_a, 0.2 nM factor V_a or (*B*) 0.2 nM factor X_a, 1.2 nM factor V_a with 500 nM DAPA and varying concentrations of phospholipid (25:75 Bov PS:POPC) in 20 mM Tris, 150 mM NaCl, 5 mM $CaCl_2$ (pH 7.4) at 37°C.

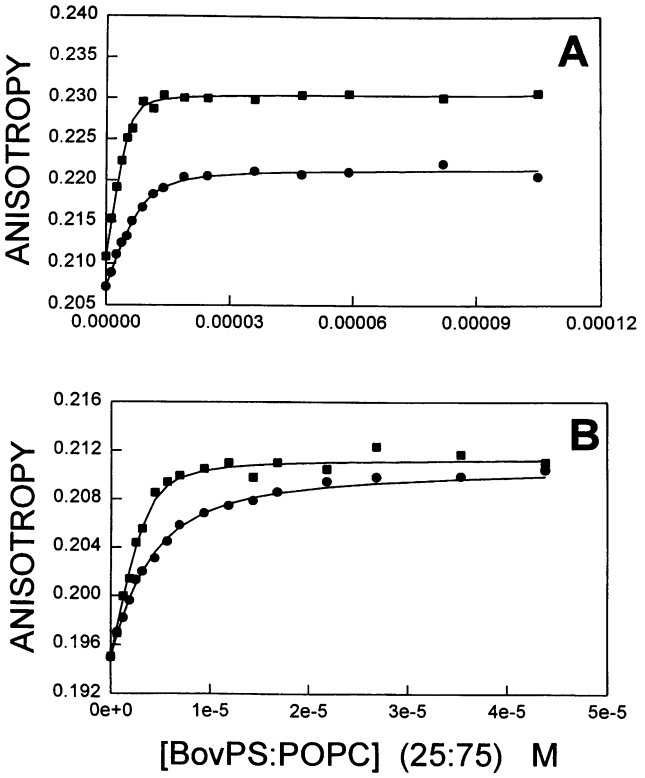

FIGURE 3 Titration of fluorescein-labeled factor V_a with phospholipid vesicles. Effect of increasing concentrations of Bov PS/POPC (25/75) SUVs on the fluorescence anisotropy of fluorescein-labeled factor V_a composed of the 72,000 light chain (■) and the 74,000 light chain (●) at (*A*) 5 mM Ca^{2+} and (*B*) 2 mM Ca^{2+}. The initial concentration of V_a was 0.1 μM in 20 mM TES, 150 mM NaCl (pH 7.5) with appropriate calcium concentrations.

centrations under conditions of limiting factor X_a concentration, as demonstrated in Fig. 2 *B*.

Interaction of the two forms of factor V_a with membranes

The change in anisotropy of fluorescein-labeled reconstituted factor V_a as a function of increasing concentrations of bovine PS/POPC (25:75) SUV at 5 mM and 2 mM calcium are given in Fig. 3, *A* and *B*, respectively. Both curves showed a well-defined saturation behavior of the anisotropy at a high concentration of vesicles. The binding parameters describing these curves (dissociation constant K_d, stoichiometry n, and change in saturating anisotropy, Δr_{sat}) were obtained by nonlinear, least-squares regression analysis (see Experimenatl Procedures); these are given in Table 1. A global analysis for determining the binding parameters was used when there was more than one set of data in an experiment (Table 1). The parameters describing binding of the 72,000 MW species are consistent with those reported previously for the binding of unfractionated pyrene-labeled factor V_a (K_d = 2 nM at 4 mM Ca^{2+}; Cutsforth et al., 1996). It is evident from the data and from these parameters that the binding of the 72,000 MW light chain factor V_a to both bovPS/POPC and DOPS/DOPC SUV was only slightly tighter than that of the 74,000 MW light chain form (K_d related by a factor of less than 3) at 5 mM Ca^{2+}. Binding of both species was somewhat tighter to membranes containing the more unsaturated bov PS species. Of particular note, the values of Δr_{sat}, which reflect the conformational state of the bound protein (Cutsforth et al., 1996), were clearly different at 5 mM Ca^{2+} for the two forms of factor V_a, suggesting that the two forms assume somewhat different conformations on membranes of optimal PS content (Cutsforth et al., 1996). Although the 72,000 and 74,000 MW light chains were of slightly different purity (90 versus 95%), it is not likely that the difference in Δr_{sat} reflects a difference in the amount of uncomplexed heavy chain in the two samples, since reconstitution was always performed with a slight molar excess of labeled heavy chain and

TABLE 1 Binding parameters of the two forms of factor V_a

Factor V_a	Phospholipid	No.*	K_d (nM)#	n#	Δr_{sat}
5 mM Ca^{2+}					
72,000	BovPS/POPC(25/75)	(2)	3.9 ± 1.8	69 ± 24	0.026 ± 0.008
74,000	BovPS/POPC(25/75)	(2)	11.0 ± 1.3	90 ± 18	0.014 ± 0.004
72,000	DOPS/DOPC(20/80)	(1)	8.7 ± 0.5	53 ± 9	0.023 ± 0.009
74,000	DOPS/DOPC(20/80)	(1)	28.5 ± 1.8	48 ± 14	0.014 ± 0.003
2 mM Ca^{2+}					
72,000	BovPS/POPC(25/75)	(2)	6.1 ± 2.0	63 ± 20	0.015 ± 0.003
74,000	BovPS/POPC(25/75)	(2)	32.5 ± 3.8	71 ± 23	0.013 ± 0.004
72,000	DOPS/DOPC(20/80)	(2)	5.58 ± 2.1	61 ± 19	0.025 ± 0.003
74,000	DOPS/DOPC(20/80)	(2)	40.9 ± 9.0	45 ± 8	0.019 ± 0.006

*The number of data sets in experiments done under each condition.
#K_d and n are the dissociation constants and the stoichiometry of the number of phospholipid molecules per protein, obtained by adjusting both parameters as global variables and the values of $\Delta r_{sat}([r_{max} - r_0]$ in Eq. 1) as local variables. Uncertainties in parameter values are given as the standard deviations in the best fit parameters.

because the difference in Δr_{sat} was not observed at 2 mM Ca^{2+}.

At 2 mM Ca^{2+}, the enhanced binding of the 72,000 MW relative to the 74,000 MW light chain form of factor V_a to PS-containing membranes was even clearer (K_d values differed by a ratio of ~6 in Table 1). Unlike at 5 mM Ca^{2+}, there was also no significant difference between data obtained with SUVs composed of bov PS/POPC (25/75) versus DOPS/DOPC (20/80). Also unlike the behavior at 5 mM Ca^{2+}, there was no significant difference between Δr_{sat} values observed for the two factor V_a species. Thus the detailed events leading to formation of a factor V_a-membrane complex are Ca^{2+}-concentration dependent.

Effect of factor V_a and its polypeptide constituents on membranes

Temperature dependence of factor V_a activity

Our second objective was to examine the interaction of factor V_a and its two peptide components with membrane bilayers. First we asked whether factor V_a or its components altered the lipid packing or lateral distribution of acidic and neutral lipids in the plane of the membrane. Our approach was to use a membrane probe to monitor the temperature dependence of lipid packing order and to use this information to construct membrane phase diagrams as a function of acidic lipid content in the presence of factor V_a and its components. To do this, it was necessary to know the effect of temperature on the activity of factor V_a. This is documented in Fig. 4. The data are plotted as a percentage of activity, taking activity at 22°C (see above) as 100%. Factor V_a activity decreased from 22°C to 30°C, remained roughly constant from 30°C to 46°C, and then decreased dramatically from 46°C to 55°C. For this reason, samples for membrane phase behavior measurements were maintained at or below 45°C.

Pure DMPC vesicles

The phase behavior of pure DMPC vesicles in the presence and absence of factor V_a and its constituents was studied by monitoring the temperature dependence of the fluorescence anisotropy of DPH. This is an established method for detecting lipid phase transitions in the absence and presence of proteins (Lentz et al., 1976a,b; Tendian and Lentz, 1990). In addition, changes in the fluorescence anisotropy of this hydrophobic probe serve as an indicator of changes in membrane order due to interaction of a protein with the membrane bilayer (Tendian and Lentz, 1990). Fig. 5 shows the fluorescence anisotropy of DPH incorporated into DMPC small unilamellar vesicles recorded during cooling of vesicles in the presence of factor V_a (*empty triangles*), factor V_a light chain (*filled circles*), heavy chain (*filled triangles*), and in the absence of protein (*empty circles*). All

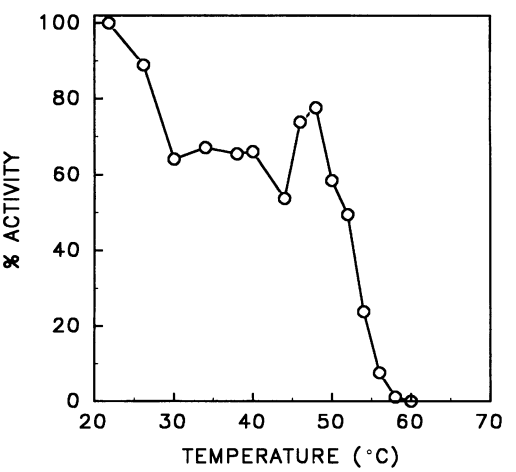

FIGURE 4 Temperature dependence of factor V_a activity. Factor V_a was reconstituted by incubating a mixture containing 0.12 μM light chain and 0.35 μM heavy chain in 20 mM Tris, 150 mM NaCl, 5 mM $CaCl_2$ (pH 7.4) at 37°C for 1.5 h. Aliquots were withdrawn, maintained at the indicated temperatures for 10 min, and assayed for activity at 22°C, using the DAPA assay under conditions of limiting factor V_a (Nesheim et al., 1981).

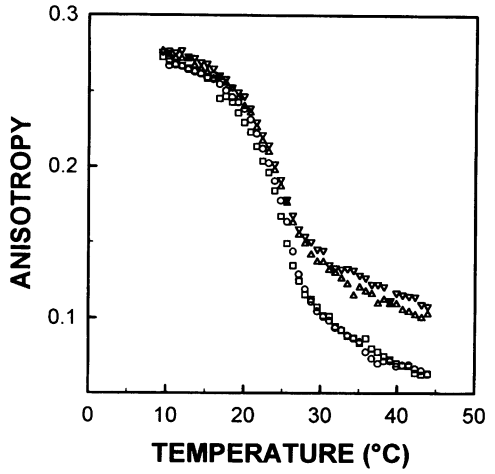

FIGURE 5 Phase transitions of DMPC vesicles as monitored by DPH fluorescence anisotropy. DPH fluorescence anisotropy was measured as described under Experimental Procedures for vesicles alone (○), vesicles in the presence of a native mixture of factor V_a species (△), vesicles in the presence of factor V_a light chain (also a native mixture; □), and vesicles in the presence of factor V_a heavy chain (▽). The probe-to-lipid mol ratio was 1:150. The phospholipid concentration in all experiments was 15 μM. The final concentration of factor V_a and its light chain was 0.5 μM, and the heavy chain concentration was 0.6 μM in 20 mM TES, 150 mM NaCl, 5 mM $CaCl_2$ (pH 7.4).

curves show a dramatic decrease in the anisotropy associated with the main DMPC phase transition occurring at ~24.3°C. Neither factor V_a nor its components altered the phase behavior of DMPC membranes; i.e., neither factor V_a nor its components altered the shape of the phase transition or its temperature range (horizontal position of the curves).

Both factor V_a and its heavy chain caused comparable increases in DPH fluorescence anisotropy over the entire temperature range, but especially in the fluid phase, compared to vesicles without protein (Fig. 5). Table 2 summarizes these observations in terms of the fluorescence anisotropy shifts caused by factor V_a or its heavy chain at temperatures of 5°C above and below the phase boundaries of DMPC SUV. Because these proteins did not shift the phase transition, the observed increase in anisotropy was not due to changes in the physical state of the membrane. This indicates that these proteins interacted with the vesicles and promoted increased lateral packing density in both the fluid and solid phases of the membrane, but especially in the fluid phase. The light chain did not cause any significant change in DPH anisotropy, in either fluid or solid phase vesicles (Fig. 5, *filled circles*; Table 2). Taken together, these observations suggest an interaction of factor V_a with DMPC membranes and that this interaction occurs mainly through the factor V_a heavy chain rather than through the light chain.

Several observations indicate that the observed shifts in DPH fluorescence anisotropy reflect changes in membrane structure due to protein binding rather than partitioning of DPH into the protein. In control experiments performed in the absence of lipid, we observed a sevenfold increase in DPH fluorescence intensity in the presence of factor V_a but no change in fluorescence anisotropy ($0.008 \pm 0.006 \rightarrow 0.002 \pm 0.004$). In contrast, there was a 34-fold increase in intensity accompanied by a significant increase in anisotropy (0.008 → 0.128) in the presence of POPC SUVs. Factor V_a heavy chain did cause an increase in DPH anisotropy (0.172) but an insignificant increase in intensity (1.8-fold). In both cases, the combination of either no anisotropy change and/or insignificant intensity change made it impossible to estimate, by previously described procedures (Lentz et al., 1976b), the partition coefficient of DPH between the membrane and protein. However, these observations also made it clear that the observed fluorescence anisotropy shifts of DPH in the presence of both lipid and factor V_a or V_a heavy chain were due almost exclusively to DPH associated with the lipid rather than with the protein component of a test sample. The fact that DPH accurately detected the lipid order/disorder phase transition supports this conclusion, as does the observation that the DPH anisotropy shifts due to factor V_a or its heavy chain were different for different membranes (see below). Thus the increase in DPH anisotropy observed in the presence of membrane and factor V_a or its heavy chain must be due to the interaction of the

TABLE 2 Summary of effects of factor V_a and its heavy and light chain constituents on membranes of different phospholipid compositions

	Δ_{aniso} (fluid)‖			Δ_{aniso} (gel)‖		
Lipid	V_a	V_a-LC	V_a-HC	V_a	V_a-LC	V_a-HC
DMPC	0.040	0.003	0.040	0.017	0.001	0.023
DMPS/DMPC*	0.018§	0.004§	0.024#	0.017§	0.018§	0.009#
$DC_{15}PG$/DMPC*	0.008¶	0.003¶	0.025¶	0.006¶	0.014¶	0.000¶

*The values given are the average of the delta fluorescence anisotropy values (lipid plus protein − lipid alone) obtained for membranes composed of varying DMPS and $DC_{15}PG$ concentrations at 5°C above (fluid) or below (gel) the phase boundaries. Values have been obtained by taking an average of two or five observations.

#These values were determined from data obtained in two sets of experiments at compositions of 26/74 DMPS/DMPC and 31/69 DMPS/DMPC.

§These values were determined from data obtained in experiments at compositions of 6/94 DMPS/DMPC, 10/90 DMPS/DMPC, 15/85 DMPS/DMPC, 26/74 DMPS/DMPC, and 31/69 DMPS/DMPC.

¶These values were determined from data obtained in experiments at compositions of 30/70 $DC_{15}PG$/DMPC, 40/60 $DC_{15}PG$/DMPC, 50/50 $DC_{15}PG$/DMPC, 58/42 $DC_{15}PG$/DMPC, and 77/33 $DC_{15}PG$/DMPC.

‖The reproducibility of anisotropy measurements was ±0.001.

protein with the membrane and not to binding of DPH to the protein.

DMPS/DMPC SUVs

To examine the influence of factor V_a binding on the packing and lateral organization of lipids within PS-containing membranes, we monitored the effects of protein binding on the phase behavior of membranes with varying acidic lipid contents. The effects of factor V_a and its light and heavy chains on DMPS/DMPC SUVs with three different compositions can be seen from the DPH fluorescence anisotropy data recorded in Fig. 6. These data show only a small effect of factor V_a binding to DMPS-containing membranes on the phase behavior of these membranes (i.e., on the shapes or horizontal positions of these curves). Both factor V_a and its light chain slightly raised the temperature of the gel-phase boundary in these membranes. The presence of these proteins did not measurably affect the fluid-phase boundary of the membranes. The failure of factor V_a or its heavy or light chain components to alter the phase behavior of DMPS/DMPC SUV indicates that binding of these proteins does not significantly affect the intermixing of these lipids in the plane of the membrane bilayer.

The effects of factor V_a and its heavy and light chain constituents on the fluorescence anisotropy of DPH incorporated into DMPS/DMPC membranes are shown in terms of average fluorescence anisotropy shifts (averaged over membranes with different DMPS contents) in Table 2. Factor V_a slightly increased the DPH fluorescence anisotropy just below the gel and above the liquid-crystalline phase boundaries of the lipid vesicles. From these data, we conclude that factor V_a binding stabilized or favored increased lateral packing of lipids in both the fluid and solid phases of the membranes. At the gel-phase boundary, factor V_a light chain had effects similar to those of whole factor V_a (Table 2), whereas the effect of the heavy chain was less pronounced. In the fluid phase, however, factor V_a heavy chain seemed to have contributed most to the anisotropy shift induced by whole factor V_a, in agreement with the observations on pure DMPC vesicles (Fig. 5 and Table 2). In general, however, although factor V_a heavy chain had a pronounced effect on the order within DMPC-containing membranes, its effects on molecular order within DMPS-containing vesicles were smaller (Table 2).

$DC_{15}PG$/DMPC SUVs

Fig. 7 shows the effect of temperature on the fluorescence anisotropy of DPH in vesicles composed of three different $DC_{15}PG$/DMPC ratios in the presence and absence of factor V_a and its components. As for DMPS/DMPC vesicles, there was little change in the shape or horizontal position of the anisotropy curves in the presence of factor V_a or its components. As for DMPS/DMPC vesicles, factor V_a and its light chain slightly raised the temperature of the gel-phase boundary, but had no clear effect on the temperature of the fluid-phase boundary (Fig. 7). Again, these results argue against protein-induced $DC_{15}PG$-rich domains associated with factor V_a or V_a heavy or light chain binding.

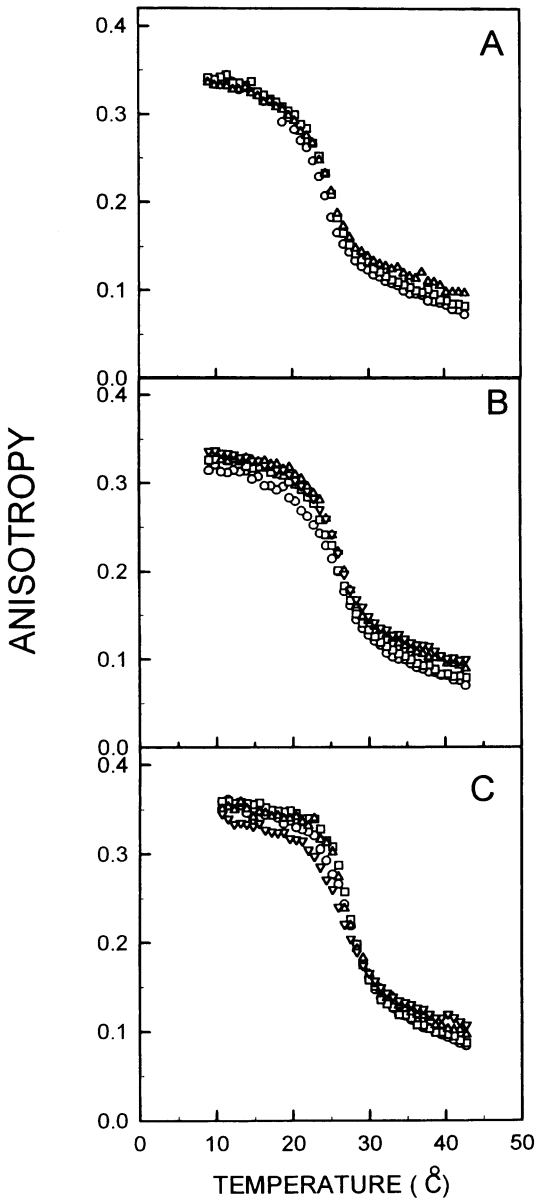

FIGURE 6 Phase transitions of DMPS/DMPC small unilamellar vesicles of varying DMPS content. (*A*) 6/94 DMPS/DMPC; (*B*) 26/74 DMPS/DMPC; (*C*) 31/69 DMPS/DMPC. DPH fluorescence anisotropy is plotted as a function of temperature for vesicles alone (○), vesicles in the presence of a native mixture of factor V_a species (△), vesicles in the presence of factor V_a light chain (also a native mixture; □), and vesicles in the presence of factor V_a heavy chain (▽). The final concentration of V_a and factor V_a light chain was 1.2 μM in *A*, 0.45 μM in *B*, and 0.5 μM in *C*. The concentration of the factor V_a heavy chain was 0.54 μM in *B* and 0.6 μM in *C*.

As for DMPS/DMPC vesicles, the average effects of the proteins on molecular order within various $DC_{15}PG$/DMPC membranes are summarized in Table 2. Factor V_a produced only a small increase in DPH fluorescence anisotropy just

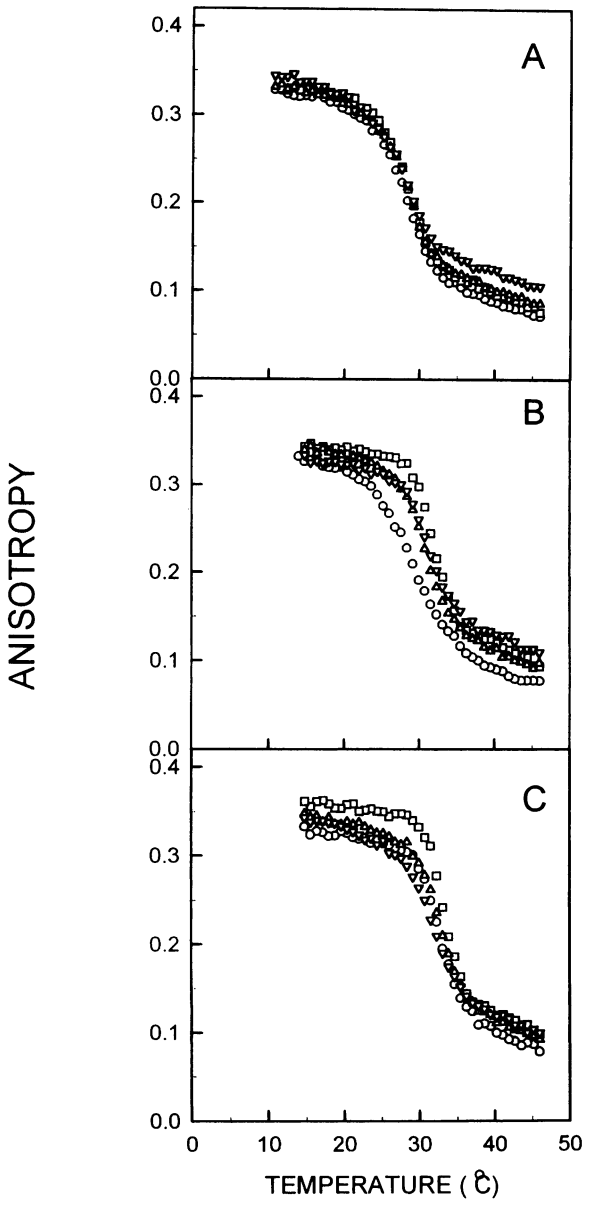

FIGURE 7 Phase transitions of $DC_{15}PG$/DMPC small unilamellar vesicles of varying $DC_{15}PG$ content. (*A*) 30/70 $DC_{15}PG$/DMPC; (*B*) 58/42 $DC_{15}PG$/DMPC; (*C*) 77/23 $DC_{15}PG$/DMPC. DPH fluorescence anisotropy is plotted as a function of temperature for vesicles alone (○), vesicles in the presence of a native mixture of factor V_a species (△), vesicles in the presence of factor V_a light chain (also a native mixture; □), and vesicles in the presence of factor V_a heavy chain (▽). The final concentration of V_a and factor V_a light chain was 0.6 μM in *A*, 0.25 μM in *B*, and 0.2 μM in *C*. The concentration of the factor V_a heavy chain was 0.72 μM in *A*, 0.3 μM in *B*, and 0.24 μM in *C*.

above the fluid phase boundary of the membranes and virtually no effect at the gel phase boundary. Factor V_a heavy chain clearly altered packing in the fluid phase of both $DC_{15}PG$/DMPC and DMPS/DMPC vesicles (Table 2), but had very little effect on packing within the gel phase, which was altered, however, by the light chain. Interestingly, although factor V_a light and heavy chains had similar effects on DMPS- and $DC_{15}PG$-containing membranes, whole factor V_a had very little effect on the packing in $DC_{15}PG$/DMPC vesicles but did slightly alter the molecular packing within DMPS/DMPC vesicles. This suggests that factor V_a has different interactions with membranes of varying acidic lipid compositions, consistent with our previous observations (Cutsforth et al., 1996).

Measurement of factor V_a-heavy chain binding with neutral lipid membranes

Because our DPH anisotropy measurements indicated that the effect of factor V_a on lipid packing within DMPC bilayers might be due to the heavy chain component of this protein, we decided to investigate heavy chain binding to neutral lipid membranes by direct titration methods (Fig. 8). The binding of fluorescein-labeled factor V_a-heavy chain to POPC vesicles was monitored by changes in fluorescein fluorescence anisotropy and by fluorescence energy transfer from fluorescein-labeled heavy chain to rhodamine-labeled phospholipid, and the binding of unlabeled V_a-HC was detected by the increase in vesicle hydrodynamic diameter as protein binds.

A small and saturable increase in the anisotropy of protein-bound fluorescein was observed with increasing lipid concentrations (Fig. 8 *A*). We note that binding of pyrene-labeled factor V_a to neutral membranes produced no change in anisotropy (Cutsforth et al., 1996) and that binding of fluorescein-labeled factor V_a to neutral membranes also produced a much smaller change in anisotropy (Δr_{max} = 0.006) than did binding to acidic-lipid-containing membranes (Δr_{max} = 0.23; Koppaka and Lentz, 1996). Although the anisotropy changes seen in the presence of neutral lipid membranes are small, they are not due to aggregation-induced light-scattering artifacts or to other irreversible changes in the protein brought about by the presence of lipid. This is demonstrated by the inset in Fig. 8 *A*, which shows the effect of adding unlabeled V_a-HC to a sample that had ~63% 0.2 μM labeled peptide bound to POPC SUVs. The fluorescence anisotropy of fluorescein-labeled V_a-HC clearly approaches the value recorded for unbound, labeled V_a-HC (*dashed line*) as it is competed off of the membrane by unlabeled protein. We conclude that the increase in fluorescein fluorescence anisotropy seen upon the addition of POPC vesicles must be due to protein-membrane interactions, which, we have noted previously (Cutsforth et al., 1996), cause changes in the segmental flexibility of the protein-attached probe.

To determine whether ionic composition had a significant impact on the interaction of V_a-HC with neutral lipid membranes, we determined binding isotherms in buffer containing 25 mM Na_2EDTA to complex Ca^{2+} (*filled circles* in Fig. 8 *A*) or 400 mM (instead of 150 mM) NaCl (*filled triangles* in Fig. 8 *A*). Although the figure illustrates that the anisotropy shift associated with the membrane-bound state was sensitive to buffer ionic composition, the binding in-

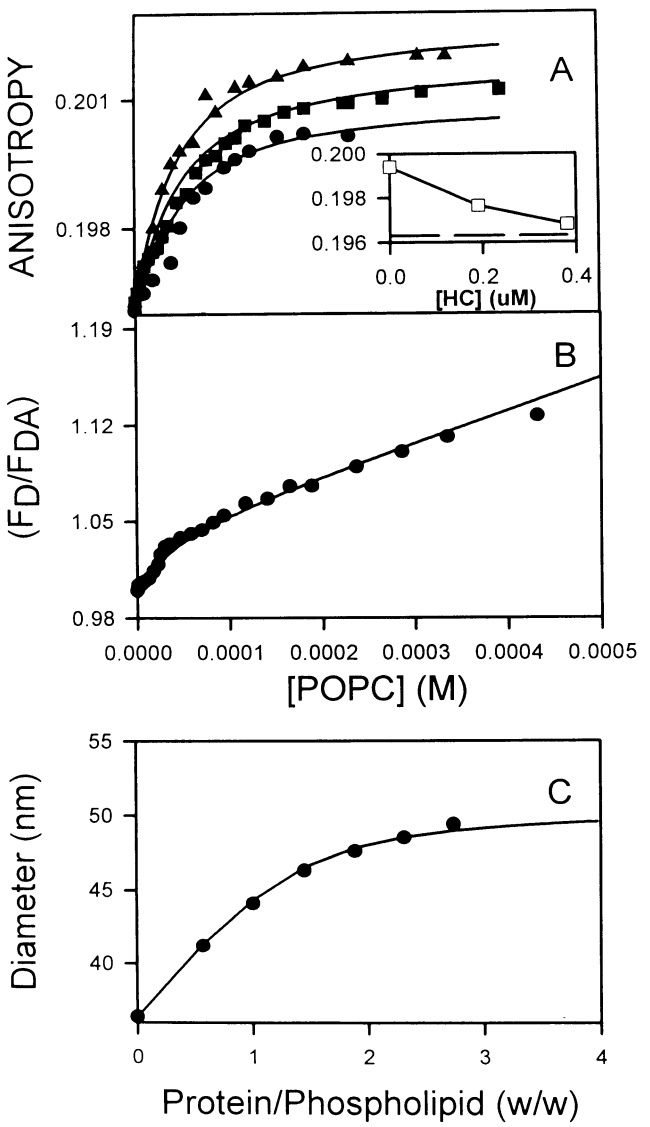

FIGURE 8 Binding of factor V_a-heavy chain to phospholipid vesicles. (*A*) Effect of increasing concentrations of POPC SUVs on the fluorescence anisotropy of 0.2 μM fluorescein-labeled factor V_a heavy chain in 20 mM TES, 150 mM NaCl, 5 mM $CaCl_2$, pH 7.5 (■); in the same buffer also containing 25 mM sodium EDTA (●); and in buffer containing 400 mM NaCl (▲). The inset shows the effect of adding unlabeled V_a-HC to a sample containing 0.2 μM fluorescein-labeled V_a-HC and 70.5 μM POPC SUVs in standard buffer. (*B*) Quenching of fluorescein-labeled factor V_a heavy chain fluorescence at 520 nm (0.3 μM in 20 mM TES, 150 mM NaCl, 5 mM $CaCl_2$, pH 7.5) due to fluorescence resonance energy transfer was monitored as a function of increasing concentrations of Rh-PE/POPC (1/99) SUVs. F_D and F_{DA} are the fluorescence intensities of fluorescein-V_a heavy chain in the absence and presence of Rh-PE acceptor. (*C*) Increase in the mean hydrodynamic diameter of POPC SUVs (0.2 mM lipid) upon the addition of unlabeled V_a-HC, as determined by QELS. The curves drawn through the binding isotherms were obtained by square residual minimization performed globally on all data sets obtained in a common buffer, using the SCoP package, as described in Experimental Methods.

teraction was not altered significantly by the absence of free Ca^{2+} (K_d = 0.6 μM, $1/i$ = 133 lipids/bound protein) or by high salt (K_d = 0.3 μM, $1/i$ = 115 lipids/bound protein). Because Ca^{2+} is needed for the interaction of the light and heavy chains to form active factor V_a, it is not surprising that the segmental flexibility of the bound fluorescein was altered or even that the K_d is slightly altered in the presence of EDTA.

For energy transfer experiments, a clear saturation was not observed at high concentrations of vesicles (Fig. 8 *B*). Rather, the quenching of the donor fluorescence became linear at high acceptor concentrations. We note in the Experimental Procedures (see Eq. 3) that this linear dependence on acceptor concentration is the behavior expected for a noninteracting donor-acceptor pair in solution (Koppaka and Lentz, 1996). The increase seen at low concentrations of membranes, however, must be due to close juxtaposition of donor and acceptor fluorophores and, as such, provides clear evidence of V_a-HC binding to POPC membranes.

As a final measure of V_a-HC binding to POPC SUVs, we monitored by QELS the rate of diffusion of vesicles in the absence and presence of unlabeled V_a-HC added to the external vesicle compartment. The diffusion coefficient of a sphere is related to its hydrodynamic radius. When V_a-HC was added to POPC SUVs, the hydrodynamic vesicle diameter was observed to increase in a saturable fashion from an initial value of 364 Å to a saturation value of 505 Å (Fig. 8 *C*). If V_a-HC were closely packed on the vesicle surface (density = 1.37 g/ml), this change in diameter would correspond to one protein bound per 29 lipid molecules in a vesicle (Pei et al., 1992).

The data obtained from all three experiments were analyzed together by a global nonlinear regression procedure using ScOP (see Experimental Procedures) to obtain the empirical parameters describing binding of factor V_a-heavy chain to neutral lipid membranes ($K_d \approx 0.30 \pm 0.02$ μM; stoichiometry $\approx 101 \pm 33$). We have reported (Koppaka and Lentz, 1996) that whole factor V_a also binds to neutral phospholipid membranes, but with a somewhat larger K_d (~3.0 μM) and stoichiometry (~200 phospholipids/V_a).

DISCUSSION

With respect to our two original objectives, several conclusions are supported by the results presented here. First, with respect to the behavior of the two forms of factor V_a containing the two light chain species,

1. The apparent values of K_d for membrane binding of bovine factor V_a reconstituted with either the 72,000 or the 74,000 MW form of the light chain are only slightly different and are dependent on the concentration of calcium and on lipid chain unsaturation.

2. The two forms of factor V_a seem to undergo different conformational changes when bound to a membrane at 5 mM Ca^{2+} but not at 2 mM Ca^{2+}.

3. The activities of prothrombinase assembled with these two forms of factor V_a differ slightly, probably because of different interactions between the enzyme and the cofactor.

Second, with regard to the influence of factor V_a and its heavy and light chains on lipid packing and lateral rearrangement,

1. Neither factor V_a nor its light and heavy chains induce detectable lipid demixing (lipid domains) within membranes containing mixtures of acidic and neutral lipids.

2. Factor V_a and factor V_a-heavy chain binding favors increased lateral lipid packing density in a fluid membrane, especially one containing only neutral lipids.

3. Both factor V_a and factor V_a-heavy chain interact in a reversible, Ca^{2+}-independent fashion with neutral lipid membranes.

These conclusions will be discussed in order.

Behavior of the two forms of factor V_a

Binding

The light chain of bovine factor V_a has always appeared as a doublet on SDS gels in all of our preparations. This light chain doublet has been reported previously by Nesheim et al. (1984) and Guinto and Esmon (1984), and more recently by Odegaard and Mann (1987). Because the two light chain forms of factor V_a differ in their carboxyl-terminal C_2 segment (Odegaard and Mann, 1987; Ortel et al., 1992), and because the C_2 segment is responsible for the binding of factor V_a to the membrane surface (Ortel et al. (1992), it might be expected that the two forms of light chain would produce bovine factor V_a species differing in their membrane-binding properties. According to our direct binding experiments (Table 1), bovine factor V_a composed of the 72,000 MW light chain binds only about three times tighter than the 74,000 MW form. In contrast, the two forms of human factor V_a are reported to bind very differently (K_d values differ by ~45-fold) to supported planar membranes composed of 20/80 DOPS/DOPC at 5 mM Ca^{2+} (Rosing et al., 1993). There are at least two possible reasons for this difference between our observations and those of Rosing et al. (1993). First, the two forms of factor V_a may well behave differently in the human as opposed to the bovine species. Second, sonicated phospholipid vesicles (used here) probably present a membrane structure very different from that presented by a supported planar bilayer (used by Rosing et al.). Neither possibility can be ruled out, and both may contribute to the differences seen between our results and those of Rosing et al. (1993).

Conformations on a membrane

The binding of factor V_a to membranes is a kinetically complex, multistep process in which an initial rapid association is followed by a slower phase yielding higher affinity binding (Bardelle et al., 1993). We have reported (Cutsforth et al., 1996) that, if the binding of pyrene-labeled factor V_a with membranes were modeled thermodynamically in terms of a two-process binding mechanism, one process would involve a moderately strong acidic-lipid-independent adsorption to a membrane surface, whereas the second process would involve the specific association of acidic phospholipids with a limited number of sites on the protein. Of particular interest was the observation that the segmental flexibility of the pyrene-labeled portion of the protein, as reported by fluorescence anisotropy, varied with the type and amount of acidic lipid incorporated into the bound membranes. This suggested that formation of a factor V_a-membrane complex might involve acidic-lipid-dependent protein conformational changes. The results presented here indicate that the segmental flexibilities and thus the conformations of factor V_a containing the 72,000 MW and the 74,000 MW light chain are different at 5 mM Ca^{2+} (Δr_{sat} values in Table 1). This might help explain the slight differences in apparent binding affinities and activities of these two forms of the prothrombinase cofactor.

Activity

Under both experimental conditions considered (limiting factor X_a or limiting factor V_a), maximum activity of the prothrombinase complex was observed at micromolar concentrations of phospholipid (Fig. 2). In addition, there was very little difference in the activities of the two forms of factor V_a within the rising portion of our lipid titration curves (Fig. 2, *A* and *B*). Because the rising portion of such a phospholipid response curve reflects mainly the membrane binding of factor V_a and the membrane assembly of the prothrombinase (Powers and Lentz, 1993), the similarity of the lipid response curves for the two forms of factor V_a implies very similar membrane binding properties for these two species. This is consistent with the similar K_d values measured by fluorescence techniques at the 5 mM Ca^{2+} concentration used in kinetic measurements (Table 1). Thus the conclusion reached by Rosing et al. (1993) that "the large [membrane] affinity difference likely explains the different cofactor activities" appears not to apply to bovine factor V_a. The main difference between the lipid response curves for the two bovine factor V_a forms was the higher activity observed for the 72,000 MW form at high lipid concentration, especially in an assay system in which factor X_a was limiting (Fig. 2 *B*). This portion of the lipid-response curve reflects competition between free lipid and membrane-bound factor V_a for factor X_a (Powers and Lentz, 1993). Because the membrane affinities of the two forms of bovine factor V_a were quite similar, our data suggest that the 72,000 MW form of light chain may interact more favorably with factor X_a than does the 74,000 MW form, thereby explaining the difference in the cofactor activities of the two forms of bovine factor V_a.

Membrane changes associated with factor V_a binding

Lipid packing and lipid domains

It has often been suggested that the binding site for the components of the prothrombinase complex, including fac-

tor V_a, consists of a membrane patch or "lateral domain" rich in acidic lipids (Lim et al., 1977; Dombrose et al., 1979; Mayer and Nelsestuen, 1981, 1983). We have suggested elsewhere that this is not the case for prothrombin and factor X_a (Cutsforth et al., 1989; Tendian and Lentz, 1990), and the data presented here indicate that this is also not the case for factor V_a. Phase diagrams (not shown) for DMPS/DMPC or $DC_{15}PG$/DMPC vesicles in the absence and presence of either factor V_a or its components were constructed from data such as those illustrated in Figs. 6 and 7, as described by Tendian and Lentz (1990). The shapes of these phase diagrams in the presence or absence of protein indicated that the lateral mixing of neutral and acidic lipids was probably close to ideal under both circumstances but, at any rate, was not altered by protein binding. Formation of protein-induced acidic-lipid-rich domains is not consistent with these observations (Tendian and Lentz, 1990). Consistent with these results, our recent analysis of bovine factor V_a binding data indicates that a very few, rather than a large pool, of acidic lipid molecules are involved in membrane binding (Cutsforth et al., 1996).

Although binding of factor V_a to DMPS- or $DC_{15}PG$-containing vesicles seems not to induce any lateral rearrangement of acidic lipids into pools or domains, it is clear that it does induce a change in membrane order, as revealed by a change in the DPH fluorescence anisotropy (Table 2). A protein-induced increase in membrane order can result from penetration of the bilayer by protein, leading to a reduction of free volume within the bilayer. A number of studies have suggested hydrophobic penetration as one mechanism of factor V_a binding (Krieg et al., 1987; Kalafatis et al., 1990, 1994; Ortel et al., 1992; Lecompte et al., 1994). Alternatively, the observed decrease in fluorescence anisotropy could reflect any other event leading to an increase in the lateral packing of lipids in the bilayer (Tendian and Lentz, 1990). For this reason, our observation of a factor V_a-induced increase in fluorescence anisotropy is consistent with but cannot be taken as proof of hydrophobic penetration of the bilayer.

The effects of factor V_a or its heavy chain on lipid order was greatest for neutral lipid membranes free of acidic lipids (Table 2). We have shown recently that factor V_a interacts with neutral lipid membranes (Koppaka and Lentz, 1996). Our present results extend our earlier report of a factor V_a interaction with neutral lipid membranes, suggesting that this interaction probably involves some contribution from the heavy chain component of factor V_a, as discussed below.

Role of heavy chain in membrane association

The binding experiments illustrated in Fig. 8 directly confirm that factor V_a heavy chain binds to neutral lipid membranes with an affinity comparable to, indeed, somewhat greater than, that of intact factor V_a. This implies that the heavy chain accounts for a significant portion of the free energy driving association of factor V_a with a neutral lipid membrane. This view is supported by the observation that the membrane order within neutral DMPC membranes was altered to a comparable extent in the presence of either whole factor V_a or its heavy chain, but not in the presence of light chain (compare anisotropy changes for V_a, V_a-LC, and V_a-HC in Table 2).

It can be argued that the apparent association of factor V_a heavy chain with neutral lipid membranes is due to impurities in our heavy chain preparations. Such a hypothesis can never be fully refuted, because completely pure preparations of bovine factor V_a and factor V_a components have never been reported. However, we believe for three reasons that this hypothesis is unlikely. First, the major impurity in factor V_a and V_a-HC is usually a ~55-kDa component seen with SDS-PAGE. No fluorescence at ~55 kDa was detectable with SDS-PAGE of labeled V_a heavy chain. Thus the experiments that quantitate factor V_a heavy chain binding using labeled factor V_a heavy chain are not likely to be severely affected by the major impurity in factor V_a heavy chain preparations. Because binding experiments with labeled and unlabeled V_a-HC were consistent with the same set of binding parameters, it is also unlikely that unlabeled V_a-HC binding is much affected by impurities. Second, light chain impurities in heavy chain preparations cannot account for the effects of heavy chain on membrane packing (Table 2), because purified light chain had no effect on DPH anisotropy. Finally, it might be argued that factor V_a could reassemble in the presence of 5 mM Ca^{2+}, using the very small contamination (<5% by SDS-PAGE) of light chain in our heavy chain preparations, and that reassembled factor V_a might be responsible for the effects that we see. If this were the case, we would expect the apparent K_d for heavy chain binding to neutral lipid membranes to be equal to that for factor V_a binding (3 μM; Koppaka and Lentz, 1996) divided by the mol fraction contamination of light chain in our heavy chain preparation. In fact, the opposite is true; the estimated K_d for heavy chain binding to neutral lipid membranes (0.37 μM) is less than that for factor V_a binding. In addition, the apparent binding parameters for V_a-HC to POPC SUVs in the presence and absence of Ca^{2+} were not significantly different (Fig. 8 *A*). Because Ca^{2+} is necessary for the reconstitution of factor V_a from its heavy and light chains, this rules out reassembled V_a impurity as a cause of our results. These comments argue forcefully against the impurity hypothesis.

Our observation of factor V_a heavy chain binding is certainly surprising, because it has been fairly well accepted that factor V_a light chain largely accounts for membrane binding (Higgins and Mann, 1983; Tracy and Mann, 1983; Pusey and Nelsestuen, 1984; Krishnaswamy and Mann, 1988). However, all of these papers report the importance of light chain to factor V_a-membrane association, but none provide direct evidence against a role for the heavy chain. Kalafatis et al. (1994) have reported that both factor V_a heavy and light chains incorporate approximately equimolar levels of a photoactive label, 1-azidopyrene, in the absence of a membrane surface. However, in the presence of 100%

POPC membranes, factor V_a showed an approximately threefold increase in labeling of the light chain and a twofold increase on the heavy chain. Earlier results of Krieg et al. (1987), using another photoactivatable reagent, adamantane diazirine, also showed similar label incorporation into both heavy and light chain in the presence of neutral lipid membranes. In both instances, labeling of heavy chain was roughly half that of light chain for factor V_a binding to membranes containing acidic lipids. Although Kalafatis et al. (1994) and Krieg et al. (1987) interpreted their observations to mean that heavy chain is not involved in the interaction with membranes, their data are, in fact, not inconsistent with our observation that V_a-HC does bind to neutral lipid membranes.

We show that both whole factor V_a and the factor V_a heavy chain bind to and perturb the packing of neutral lipid membranes, whereas the light chain alone did not alter lipid packing. Furthermore, high salt concentration had little effect on the interaction of V_a-HC with POPC SUVs, suggesting that the interaction is not ionic in nature. This and the V_a-HC-induced increase in packing order suggest that the interaction of V_a-HC with a neutral lipid bilayer is at least partially hydrophobic in nature. The perturbation of membrane packing order by factor V_a or its heavy chain was less dramatic for membranes containing acidic lipids (Table 2). We suggest that heavy chain contributes to the acidic-lipid-independent contribution to factor V_a membrane binding, at least in part through a partial penetration into the membrane hydrophobic core, but that the relative magnitudes of this contribution and of the acidic-lipid-dependent contribution depend on membrane acidic lipid content, as our previous analysis of factor V_a binding data has indicated (Cutsforth et al., 1996).

It should be noted that our observations do not imply that an acidic-lipid-independent association site does not exist in the light chain of factor V_a. Indeed, it is known that the A_3 domain of bovine factor V, which comprises the M_r 30,000 fragment of the light chain, has two hydrophobic segments of amino acids that could potentially and probably do interact with a phospholipid vesicle (Kalafatis et al., 1990, 1994). However, there are known to be highly homologous hydrophobic regions in the A_1 and A_2 domains of the heavy chain (Jenny et al., 1987), which could account for the interaction of factor V_a heavy chain with neutral phospholipid membranes. It may be that interaction of these regions of the heavy chain with a membrane can occur in the absence of acidic lipid, whereas membrane interaction with the light chain hydrophobic regions may require occupancy of specific acidic lipid sites or assembly of whole factor V_a. Indeed, our previous results have suggested that the interactions of the various sites on factor V_a with a membrane are highly interdependent and that occupancy of one site probably causes conformational changes that alter the membrane affinity of other sites (Cutsforth et al., 1996).

Finally, it might be argued that the interaction of factor V_a or its heavy chain with neutral membranes is not physiologically significant, because it is much weaker than that observed for intact factor V_a with acidic-lipid-containing membranes ($K_d \approx 2$ nM; Cutsforth et al., 1996). Whereas the major free energy of factor V_a binding to a membrane may well be contributed by the light chain, we argue that less energetic interactions of the heavy chain with the membrane may contribute significantly to the configuration and functioning of the whole molecule on a membrane. Because a clear mechanistic role for factor V_a in prothrombin activation has not been established, it is difficult to judge the relevance of any component of the total factor V_a-membrane interaction to the function of this critical blood coagulation cofactor. However, Gerads et al. (1990) and Govers-Riemslag et al. (1994) observed prothrombin activation on dioleoylphosphatidylcholine membranes, but only in the presence of factor V_a, thus providing indirect support for a functionally significant interaction of factor V_a with neutral lipid membranes.

A model for factor V_a-membrane complex formation

Fig. 9 presents a hypothetical model that summarizes our present understanding of the complexities of factor V_a association with a procoagulant membrane. The model envisions factor V_a binding as involving acidic-lipid-independent adsorption to a membrane surface. The results presented here suggest that the factor V_a heavy chain contributes to this. Based on the results of Cutsforth et al. (1996) from pyrene-labeled factor V_a and on the results presented in this study from fluorescein-labeled factor V_a, this model proposes a conformational change of factor V_a

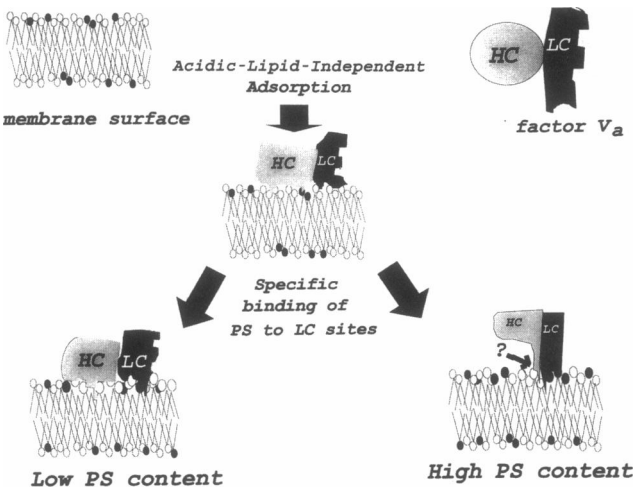

FIGURE 9 Model for molecular events associated with membrane binding of factor V_a. Binding appears to involve both acidic lipid sites and acidic-lipid-independent modes of association (represented by protein "fingers" penetrating the bilayer). The acidic-lipid-independent association of factor V_a with neutral membranes suggested by our observations appears to involve to some extent the heavy chain portion (HC) of factor V_a. Specific binding of acidic lipids (●) to sites on factor V_a (Cutsforth et al., 1996) probably involves the light chain portion (LC) of the molecule. It appears that the binding affinities at different sites are altered by the occupancy of other sites by means of ligand-induced protein conformational changes.

on binding to a surface. The model shows a change in both the heavy and light chains, but there is no way to locate the change in any particular region of factor V_a from our data. We know at this point only that a pyrene probe or a fluorescein probe located on the heavy chain is altered in its segmental motion by binding. This result could be due to direct interaction between the heavy chain in factor V_a and the membrane, although changes in heavy chain conformation could also result from altered interactions between heavy and light chains within factor V_a bound to a membrane. More data will be needed to locate and confirm a membrane-induced conformational change. What is clear from our membrane phase behavior data and from the thermodynamic binding analysis of Cutsforth et al. (1996) is that factor V_a binds only a limited number of acidic lipid molecules rather than gathering a large domain of these molecules. In addition, the conformational states of factor V_a on acidic-lipid-rich versus acidic-lipid-poor membranes appear to be different (Cutsforth et al., 1996), as illustrated in Fig. 9. The conformation suggested in the figure for acidic-lipid-rich membranes is drawn to be consistent with the structural data of Stoylova et al. (1994) and with the small limiting binding stoichiometry reported by Cutsforth et al. (1996). The hydrophobic penetration of the heavy chain segment is suggested but not clearly established by the ionic-strength-independence of our V_a-HC binding data, as well as by the common effect of V_a-HC and V_a on the fluorescence anisotropy of DPH in DMPC membranes.

The purposes of the model shown in Fig. 9 are to help focus the issues involved in factor V_a membrane binding and to stimulate further experiments. Many issues remain to be resolved, such as locating and defining the conformational change(s) associated with membrane binding, quantitating the interaction of heavy chain with membranes containing acidic lipids, establishing the role of Ca^{2+} in complex formation, and determining whether the suspected conformational changes have consequences for the functioning of factor V_a.

We thank Drs. Charles T. Esmon and Sriram Krishnaswamy for their helpful suggestions during the purification of factor V_a.

This work was supported by the U.S. Public Health Service (HL45916).

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