Phosphatidylserine Binding Alters the Conformation and Specifically Enhances the Cofactor Activity of Bovine Factor V_a†

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ABSTRACT: Factor V_a is a cofactor for the serine protease factor X_a that activates prothrombin to thrombin in the presence of Ca^{2+} and a platelet membrane surface. A platelet membrane lipid, phosphatidylserine (PS), regulates the proteolytic activity of factor X_a as well as the structure of prothrombin. Here we ask whether PS also regulates the structure and cofactor activity of factor V_a, which is a heterodimer composed of one heavy chain (A1-A2 domains) and one light chain (A3-C1-C2 domains). We use fluorescence, circular dichroism, equilibrium dialysis, and activity measurements to demonstrate the following: (1) Factor V_a has four sites for dicaproyl-*sn*-glycero-3-phospho-L-serine (C_6PS, a soluble form of PS); the heavy and light chains each bind two C_6PS molecules. (2) In the absence of Ca^{2+}, only two sites remain, one in the heavy chain and another in the light chain. (3) Binding to these sites causes conformational changes evidenced by changes in intrinsic fluorescence and in CD spectra and changes in cofactor activity. (4) At least some of the four lipid binding sites are nonspecific with respect to soluble lipid species, but the site(s) that regulate(s) cofactor activity is (are) specific for C_6PS, phosphatidic acid, or phosphatidyl-(homo)serine and produce a response comparable to that seen with a PS-containing membrane. (5) Like Ca^{2+}, C_6PS also mediates the interaction between factor V_a heavy (V_a-HC) and light (V_a-LC) chains. We conclude that PS regulates both the cofactor and the enzyme of the prothrombin-activating complex.

During blood coagulation, the zymogen prothrombin is activated to thrombin, the central enzyme of the homeostatic process (*13*). This proteolytic activation is catalyzed by the factor X_a−factor V_a complex on membranous vesicles released from activated platelets (*6, 42, 43*). Activated factor V (V_a) is the protein cofactor to the serine protease (factor X_a). The resting platelet plasma membrane has nearly all its phosphatidylserine (PS)[1] located on its cytoplasmic face, but release of platelet vesicles is associated with trans-bilayer migration of nearly half of this to the extracytoplasmic leaflet (*4, 5*). It appears that the appearance of PS on the surface of these platelet-derived vesicles is essential for the assembly of an active prothrombinase (*10, 18, 43*).

The specific effects of PS-containing membranes on bound prothrombin structure (*9, 27*) and on the apparent kinetic constants for the assembled prothrombinase (*11, 37*) suggest that PS may play a specific regulatory role in the activation of prothrombin. Any such regulatory role could originate from PS-specific effects on prothrombin, factor X_a or factor V_a, or any combination of these. Previously, we have shown substantial effects of soluble short-chain PS (C_6PS) both on the proteolytic activity of factor X_a toward prothrombin (*3, 24*) as well as on factor X_a structure (*2, 46, 49*). Is factor V_a also regulated by soluble, short-chain PS?

Little is known about the effect of PS on factor V_a structure and cofactor activity. Unlike other components of the prothrombinase, factor V_a interacts with neutral membranes free of PS (*23*). It has been reported that factor V_a binds by a different mechanism to PS-rich versus PS-poor or PS-free membranes and has a different conformation on a PS-poor versus a PS-rich membrane surface (*12, 24*). This raises the possibility of PS-regulation of factor V_a.

Because binding to a membrane involves interactions with the membrane surface as well as with individual lipid molecules within the membrane, it is difficult to discern from membrane binding studies whether a protein might be regulated by specific interactions with individual lipid molecules. In the present work, we take a different approach by examining the effects of various soluble PS analogues

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[1] Abbreviations: C_6PS, 1,2-dicaproyl-*sn*-glycero-3-phospho-L-serine; C_6PG, 1,2-dicaproyl-*sn*-glycero-3-phospho-*rac*-1-glycerol; C_6PC, 1,2-dicaproyl-*sn*-glycero-3-phosphocholine; C_6PA, 1,2-dicaproyl-*sn*-glycero-3-phosphate; LysoC6PC, 1-caproyl-2-hydroxy-*sn*-glycero-3-phosphocholine; LysoC6PA, 1-caproyl-2-hydroxy-*sn*-glycero-3-phosphate; C_6P(D)S, 1,2-dicaproyl-*sn*-glycero-3-phospho-D-serine; C_6P(h)S, 1,2-dicaproyl-*sn*-glycero-3-phospho-D-homoserine; C_6P(N-CH_3)S, 1,2-dicaproyl-*sn*-glycero-3-phospho-L−*N*-methylserine; GPS, L-α-glycerophosphorylserine; bovPS, bovine brain phosphatidylserine; DOPC, 1,2-dioleoyl-3-*sn*-phosphatidylcholine; PS, phosphatidylserine; PL, phospholipid; DAPA, dansylarginine-*N*-(3-ethyl-1,5-pentanediyl)amine; ANS, 8-anilino-1-naphthalenesulfonic acid; V_a-LC, factor V_a light chain; V_a-HC, factor V_a heavy chain; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; CD, circular dichroism; QELS, quasi-elastic light scattering; CMC, critical micelle concentration; Tris, tris(hydroxymethyl)aminomethane.

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on the structure and cofactor activity of factor V_a. We demonstrate that both heavy and light chains of factor V_a bind soluble lipids, with each chain binding two molecules of C_6PS in the presence and one in the absence of Ca^{2+}. Our results have also shown that, like factor X_a, factor V_a has preference for L-PS. All the PS analogues used in this study bound to factor V_a and induced a conformational change. However, only C_6PS, dicaproylphosphatidic acid (C_6PA), and dicaproylphosphatidyl(home)serine [$C_6P(h)S$] enhanced the cofactor activity of factor V_a, with the full functional response of factor V_a seen only when the binding sites were occupied with C_6PS molecules. Finally, soluble C_6PS induced an interaction between the heavy and light chains of factor V_a in the absence of Ca^{2+}.

MATERIALS AND METHODS

Materials. Lipid stock suspensions in buffer were prepared fresh for titration experiments by measuring aliquots of appropriate lipid stocks in chloroform, evaporating the chloroform under a stream of nitrogen, dissolving the lipid in cyclohexane, and then lyophilizing these frozen solutions overnight. The resulting dry powder was dispersed in the appropriate buffer and vortexed thoroughly. Lipid stock suspensions were observed to be stable for several weeks when frozen at −70 °C but were generally used without freezing and within 15 h of preparation. 1,2-Dicaproyl-*sn*-glycero-3-phospho-L-serine (C_6PS),[1] 1,2-dicaproyl-*sn*-glycero-3-phospho-*rac*-1-glycerol (C_6PG),[1] 1,2-dicaproyl-*sn*-glycero-3-phosphocholine (C_6PC),[1] 1,2-dicaproyl-*sn*-glycero-3-phosphate (C_6PA),[1] 1-caproyl-2-hydroxy-*sn*-glycero-3-phosphocholine ($LysoC_6PC$),[1] and 1-caproyl-2-hydroxy-*sn*-glycero-3-phosphate ($LysoC_6PA$)[1] were purchased from Avanti Polar Lipids (Alabaster, AL). L-α-Glycerophosphorylserine (GPS)[1] was purchased from Sigma Chemical Co. (St. Louis, MO). 1,2-Dicaproyl-*sn*-glycero-3-phospho-D-serine [$C_6P(D)S$],[1] 1,2-dicaproyl-*sn*-glycero-3-phospho-D-homoserine [$C_6P(h)S$],[1] and 1,2-dicaproyl-*sn*-glycero-3-phospho-L-*N*-methylserine [$C_6P(N\text{-}CH_3)S$][1] were synthesized by a phospholipase D catalyzed transphosphatidylation reaction, using a one-phase system as described previously (*2*). The chemical structures of these lipid analogues are shown in Figure 1. Bovine brain phosphatidylserine (bovPS)[1] and 1,2-dioleoyl-3-*sn*-phosphatidylcholine (DOPC)[1] were purchased from Avanti Polar Lipids Inc. (Alabaster, AL). DAPA,[1] bovine factor X_a, bovine thrombin, and bovine prothrombin were obtained from Haematologic Technologies Inc. (Essex Junction, VT). 8-Anilino-1-naphthalenesulfonic acid (ANS)[1] was from Sigma Chemical Co. All other chemicals were ACS reagent grade or the best available grade.

Isolation of Factor V_a, V_a-LC,[1] and V_a-HC.[1] Factor V_a and its light and heavy chains were prepared as previously described (*23*). The two forms of factor V_a (factor V_{a1} and V_{a2}) were purified directly from activated factor V by separation on a Mono-S column, using a NH_4Cl gradient (*23*, *39*). Human factor V_{a1} differs from human factor V_{a2} in that it contains a polysaccharide group at Asp^{2181} in the C2 domain of the light chain (*20*) and it binds somewhat less tightly to PS-containing membranes and perhaps to factor X_a (*20*, *39*). The two forms of the bovine protein are less well characterized but seem to behave similarly (*23*). The final purity of the proteins was ascertained by sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE)[1] to be greater than 90%. Protein concentrations of factor V_{a1} and V_{a2} were determined by absorbance (*32*), using an extinction coefficient of 1.74 $(mg/mL)^{-1}$ at 280 nm.

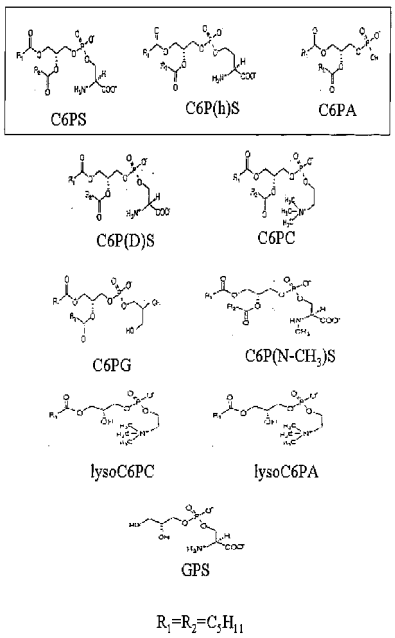

FIGURE 1: Structures of the various C_6PS analogues used for binding studies are shown. R_1═R_2 represents C_5H_{11}.

Fluorescence Measurements. The intrinsic fluorescence intensity of factor V_a or of its isolated subunits was recorded with an SLM 48000-MHF spectrofluorometer (SLM Aminco, Urbana, IL) at 24 °C. The excitation wavelength was 285 nm (band-pass 4 nm), and the emission intensity was recorded at 340 nm (band-pass 4 nm). Slits were kept closed except during the measurements to avoid photodegradation of the sample. All buffer solutions were filtered through a 0.2 μm polycarbonate filter (white AAWP, 47 mm, Millipore Corp., Bedford, MA) to decrease light scattering. Titrations were performed at 23 °C. Upon each addition of lipid, the cuvette solution was stirred for at least 4 min before reading the fluorescence intensity to ensure that equilibrium had been established. The total volume added to a sample during the titration was always <5% of the total sample volume, and corrections were made for sample dilution. For each addition, several intensity measurements were performed and averaged. As a control, buffer in the absence of protein was titrated. The fluorescence from a standard containing 0.17 μM factor V_a in buffer containing 1 mM Ca^{2+} was used as a reference (F_0) for all experiments. Experiments on samples from a given protein preparation were performed in duplicate, with results being essentially identical (∼0.2% variation in absolute intensities between experiments).

Assay for Prothrombin Activation in the Presence of Short-Chain Phospholipids. The rate of prothrombin activation was estimated from the time-dependent fluorescence change of DAPA bound to the activation products. Stopped-flow measurements were performed at 37 °C using an SLM-Aminco Milliflow stopped-flow reactor (Spectronic Instruments, Inc., Rochester, NY) attached to the SLM 48000 spectrofluorometer (Spectronic Instruments) with 280 nm excitation (4 nm slit) and a 515 nm cutoff filter. Reactions were initiated by rapidly mixing equal volumes (200 μL) of

the contents of the two driving syringes. Syringe A contained 2 μM prothrombin and 10 μM DAPA in 50 mM Tris, 150 mM NaCl, 5 mM $CaCl_2$, pH 7.5. Syringe B contained the same buffer plus 2 nM factor X_a, and 0.8 mM phospholipid either in the absence or in the presence of 10 nM factor V_{a2}. We used factor V_{a2} instead of the natural mixture of V_{a1} and V_{a2} for the activity measurements because factor V_{a2} binds nearly 3 orders of magnitude tighter to factor X_a in the presence of C_6PS than does factor V_{a1}, allowing assembly of a fully functional prothrombinase complex in solution (*29*). Control experiments were performed in exactly the same way except syringe B did not contain soluble phospholipids. An additional control was performed using 200 μM bovPS/DOPC (25:75) large, unilamellar vesicles (*31*) in place of soluble lipids. Prior to mixing, both syringes were allowed to incubate at 37 °C for 4−5 min. Fluorescence intensity at the completion of the reaction (infinite time) was considered to represent quantitative conversion of prothrombin to thrombin. The initial rate of thrombin generation was then determined from the initial rate of fluorescence intensity change normalized to the intensity at complete thrombin formation (*33*, *37*).

Circular Dichroism Measurements. Circular dichroism (CD) spectra were recorded from 250 to 200 nm on an Aviv model 620S spectrometer (Aviv Associates, Inc., Lake Wood, NJ) in a 1 cm path-length cell at 24 °C with a bandwidth of 1.0 nm. Data were collected at every 0.5 nm with an average time of 5 s. The digital data were processed, smoothed, baseline-corrected, and converted to molar ellipticity units. For baseline correction, CD spectra of buffer (0.8 mM Tris, 120 mM NaCl, 1.0 mM $CaCl_2$, pH 7.5) containing various concentrations of soluble C_6PS were collected and were subtracted from sample spectra. We used the ellipticity ratio $\Theta_{222}/\Theta_{208}$ (*15*) as a convenient parameter to follow changes in the secondary structure of factor V_a upon addition of C_6PS. In addition, we estimated α-helix content using the average of results from the two published software packages CDSSTR and CONTIN, which are available in the program CDPRO (*44*), in order to give context to the $\Theta_{222}/\Theta_{208}$ ratio.

Determination of the Stoichiometry of C_6PS Binding to Factor V_a. The stoichiometry of soluble C_6PS binding to factor V_a in the presence of 1 mM Ca^{2+} or 1.2 mM EDTA was determined by equilibrium dialysis experiments in which the difference in total phosphate concentration between the two chambers of the dialysis cell was monitored (*2*, *45*, *46*). Due to the limited sensitivity of the phosphate assay, this experiment had to be carried out at fairly high lipid concentrations, although these needed to be below the critical micelle concentration (CMC) of C_6PS. It was also necessary to saturate factor V_a binding sites, and, to preserve protein, we had to work with reasonably low protein concentrations. Therefore, we performed dialysis experiments at a fixed C_6PS concentration and varied factor V_a concentrations such that greater than 97% of protein sites were occupied by lipid for each experimental point determined. Experiments were performed using 2.0 mL Teflon dialysis cells (Spectrum Medical, Los Angeles, CA) with the two cells separated by a 6000−8000 dalton molecular mass cut-off membrane. Both chambers contained 0.29 mM C_6PS in 50 mM Tris, 150 mM NaCl, pH 7.5, and either 1 mM Ca^{2+} or 1.2 mM Na_2EDTA. Factor V_a was added to one set of cells to final concentrations of 1.7, 2.7, 4.2, or 6.3 μM and allowed to equilibrate with the corresponding protein-free cells at room temperature for 24 h while being rotated horizontally at 20 rpm. The difference in total phosphate concentration between the two chambers (ΔP) was then measured by taking eight aliquots (100 μL) out of each chamber and assaying these for total phosphate content according to the method of Chen et al. (*8*). Total phosphate content was also measured for buffer plus protein alone as a control. Based on simple equilibrium binding expressions for binding to n equivalent and independent sites, ΔP should vary linearly with factor V_a concentration with a slope equal to $n[L]/[k_d(1 + [L]/k_d)]$, where [L] is the free lipid concentration, k_d is the effective site dissociation constant for lipid binding (taken as 9 μM based on titrations of intrinsic fluorescence or CD changes; see Results), and n is the stoichiometry of binding. We calculated [L] under the conditions of each point from assumed integer values of n (integer values from 1 to 8) and then calculated expected values of ΔP using the stated equation. To estimate n, we compared the plot of expected ΔP versus factor V_a concentration with the observed plots, with the calculated curve that best matched the observed data defining the stoichiometry.

Determination of C_6PS Critical Micelle Concentration (CMC).[1] To verify that we were determining the effect of soluble C_6PS and its analogues on factor V_a, we needed to determine the effect of factor V_a on the CMC of short-chain phospholipids. We did not have sufficient quantities of all analogues to determine their CMCs in the presence of factor V_a, but we have found previously that none of these formed micelles below the CMC of C_6PS in the presence of Ca^{2+} and factor X_a (*2*, *24*). Thus, we focused on the CMC of C_6PS under the conditions of the relevant lipid titrations, since Ca^{2+}, ionic strength, and proteins all cause variation in the CMC (*24*). The diameter of C_6PS aggregates was measured by quasi-elastic light scattering (QELS)[1] in the presence of factor V_a, as described earlier (*30*). Increasing concentrations of lipid were added to the sample cell, a 6 × 50 mm borosilicate glass disposable culture tube (Baxter Healthcare Corp., McGraw Park, IL), containing 0.4 mL of 0.17 μM bovine factor V_a in 50 mM Tris, 3 mM $CaCl_2$, 150 mM NaCl at pH 7.4. All buffer solutions were filtered through an 0.8 μm polycarbonate filter (white AAWP, 47 mm, Millipore Corp., Bedford, MA) to minimize light scattering from dust particles. The sizes of the various aggregates were measured as intensity-weighted diameters, which were calculated from the Z-averaged diffusion coefficient. The fluorescence of the hydrophobic dye ANS was also recorded as described previously (*24*) to provide a second measure of the CMC.

DATA ANALYSIS

The response of soluble lipids to factor V_a and their isolated chains (V_a-LC and V_a-HC) was fitted to a single binding site model:

$$R = R_0 + \Delta R_{sat}\frac{[L]}{k_d + [L]};\ \Delta R_{sat} = R_{sat} - R_0 \qquad (1)$$

where R and R_0 are the observed response and the response at zero lipid concentration, respectively. k_d is the effective site dissociation constant, and [L] is the free soluble lipid concentration, which, under our conditions, is approximately

the total lipid concentration added to a cuvette. Note that k_d is not an actual site dissociation constant, but an effective site dissociation constant that reflects the shape of the binding curve and not the number of sites present on the macromolecule. If n equivalent and independent sites exist on a macromolecule, the number of bound ligands is $n[L]/(k_d + [L])$.

In cases where a single-binding-site model did not adequately describe the binding curve, the data were fitted to a model assuming two independent binding sites:

$$R = R_0 + \Delta R_{sat1}\frac{[L]}{k_{d1} + [L]} + \Delta R_{sat2}\frac{[L]}{k_{d2} + [L]} \quad (2)$$

where ΔR_{sat1} and ΔR_{sat2} are the changes in the observable response at saturation for sites 1 and 2, respectively. k_{d1} and k_{d2} are the effective site dissociation constants for sites 1 and 2, respectively.

Data were fit to these equations using a Marquardt−Levenberg weighted, nonlinear least-squares algorithm supplied with Sigmaplot (v 4.2; Jandel Scientific, Corte Madera, CA).

Effect of Soluble Lipids on the Cofactor Activity of Factor V_a. Enhancement of cofactor activity (E) by soluble lipids was measured using the following equation:

$$E = \frac{r_{Xa\cdot Va\cdot PL}/r_{Xa\cdot PL}}{r_{Xa\cdot Va}/r_{Xa}} = \frac{r_{Xa\cdot Va\cdot PL}/r_{Xa\cdot Va}}{r_{Xa\cdot PL}/r_{Xa}} \quad (3)$$

Here, $r_{Xa\cdot Va\cdot PL}$ is the rate of prothrombin activation measured as the initial slope of the rate of change of normalized DAPA fluorescence with time by enzyme in the presence of factor V_a and lipid, and other terms are defined analogously. The ratio $r_{Xa\cdot Va}/r_{Xa}$ was determined as the average from five independent experiments, while $r_{Xa\cdot Va\cdot PL}/r_{Xa\cdot PL}$ was the average of three independent measurements for each soluble lipid. The physical meaning of this expression can be seen best in the rearranged right-most form. The ratio $r_{Xa\cdot Va\cdot PL}/r_{Xa\cdot Va}$ gives the effect of phospholipid binding to both X_a and V_a, while the ratio $r_{Xa\cdot PL}/r_{Xa}$ gives the effect of phospholipid binding to factor X_a. The ratio of these two quantities gives the rate enhancement that is specific to the presence of factor V_a.

RESULTS

Effect of Ca^{2+} on the Intrinsic Fluorescence of Factor V_a. To determine the optimal Ca^{2+} concentration for studying lipid binding to factor V_a, the intrinsic fluorescence of factor V_a at 1 mM Ca^{2+} was titrated with additional Ca^{2+} and Na_2-EDTA (Figure 2). Upon addition of Ca^{2+} beyond 1 mM, the total fluorescence intensity of factor V_a decreased by as much as 6% at saturation (closed circles). Upon addition of EDTA to factor V_a in the presence of 1 mM Ca^{2+}, the fluorescence intensity decreased by 4% at saturation (Figure 2, inset). These data are also plotted versus calculated (see Materials and Methods) free Ca^{2+} concentration in Figure 2 (open circles).

The variation of the intrinsic fluorescence of factor V_a with free Ca^{2+} concentration in solution suggests that factor V_a light and heavy chains continue to bind Ca^{2+} beyond concentrations needed for reconstitution. Our experiments were performed with 1 mM Ca^{2+}, sufficient to reconstitute factor V_a and support prothrombinase activity.

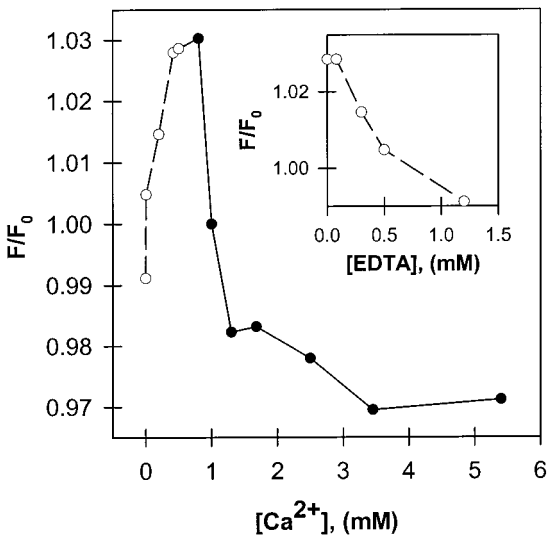

FIGURE 2: Factor V_a (0.17 μM) in 50 mM Tris, 150 mM NaCl, 1.0 mM $CaCl_2$, pH 7.5, was titrated with $CaCl_2$ (closed circles) and Na_2EDTA (open circles, shown in inset). The free Ca^{2+} concentration in solution in the presence of Na_2EDTA was calculated using Chelator (1992, Th. Schoenmakers, The Netherlands), and fluorescence intensity is plotted as open circles versus the calculated free Ca^{2+} concentration in the main figure. The fluorescence intensity in the presence of 1 mM Ca^{2+} was arbitrarily taken as 1.

Effect of Soluble Short-Chain Lipids on the Intrinsic Fluorescence of Factor V_a. The various PS analogues shown in Figure 1 were used to titrate factor V_a. The effects of representative lipids on the intrinsic fluorescence of factor V_a are shown in Figure 3A. The intrinsic fluorescence of factor V_a decreased with the addition of soluble C_6PC (circles), C_6P(N-CH_3)S (squares), and C_6P(h)S (triangles). Saturation occurred at about 0.1−0.15 mM in all cases. The binding curves in all cases were well fitted by a single-binding-site model, and the binding parameters (k_d = effective site dissociation constant; ΔF_{sat} = fluorescence change at saturation) derived from the titrations are given in Table 1. As can be seen in Table 1, soluble C_6PS and all other PS analogues bound to factor V_a with similar affinities and responses at saturation.

Effect of Ionic Strength on Soluble C_6PS Binding to Factor V_a. To obtain insight into the mechanism of soluble C_6PS binding to factor V_a, 0.17 μM factor V_a was titrated with soluble C_6PS at 0.15 M (closed circles) and 0.8 M (open circles) NaCl, with the results shown in Figure 3B. The binding parameters obtained from fitting these curves to a single-binding-site model are reported in Table 2. Ionic strength did not affect the binding affinity of soluble C_6PS for factor V_a, but it had significant effect on the intrinsic fluorescence change at saturation (ΔF_{sat}).

Effect of Factor V_a on the CMC of C_6PS. Since factor V_a and its individual chains are known to bind to PS-containing membranes (*12*, *23*, *25*), it is essential to know that the interaction recorded here is with C_6PS in a single-molecule state rather than in an aggregated or micellar state. We have previously characterized carefully the formation of micelles by C_6PS and have reported the CMC of C_6PS at 1 mM Ca^{2+} as 5.3 mM in a buffer containing 50 mM Tris, 175 mM NaCl, 0.6 wt % PEG, pH 7.4 (*24*). However, we have also shown that factor X_a (but not prothrombin) had substantial effects on short-chain lipid CMC's (*24*). Thus, controls were

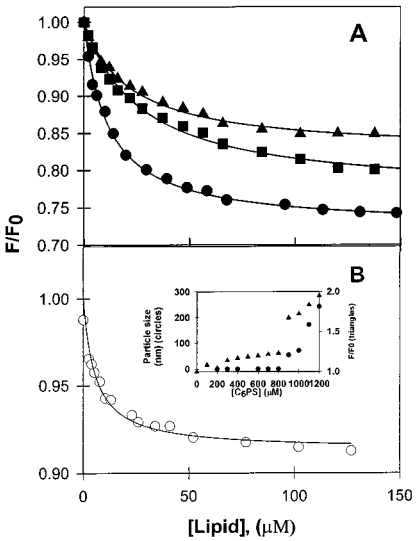

FIGURE 3: Intrinsic fluorescence of bovine factor V_a (0.17 μM) in 50 mM Tris, 150 mM NaCl, 1.0 mM Ca^{2+}, pH 7.5, is plotted versus concentration of soluble C_6PC (closed circles), $C_6P(N\text{-}CH_3)S$ (squares), and $C_6P(h)S$ (triangles) at 24 °C in frame A. The intrinsic fluorescence of factor V_a in 800 mM NaCl and 1.0 mM Ca^{2+} is recorded as open circles in frame B. The solid lines passing through the curves are the fits of the data to a single-binding-site model. The binding parameters obtained from these fits are given in Table 1. The size of C_6PS aggregates in the presence of factor V_a in this buffer was measured with increasing C_6PS concentration by QELS. The variation of particle size (circles) and ANS fluorescence (triangles) with soluble C_6PS concentration is shown in the inset, establishing that the C_6PS CMC under these conditions was 800−900 μM.

Table 1: Parameters[a] Associated with Binding of Various PS Analogues to Bovine Factor V_a and with the Effect of Binding on Cofactor Activity

lipid	K_d (μM)	ΔF_{sat} (fraction)	E^b
C_6PS	9.0 ± 0.6	−0.26 ± 0.03	15.1
C_6PA	44.4 ± 1.2	−0.22 ± 0.02	8.3
$C_6P(h)S$	19.9 ± 1.3	−0.17 ± 0.03	7.7
$C_6P(D)S$	39.7 ± 4.6	−0.17 ± 0.04	1.1
C_6PC	11.0 ± 1.1	−0.22 ± 0.02	1.0
C_6PG	5.0 ± 0.5	−0.22 ± 0.03	0.98
$C_6P(N\text{-}CH_3)S$	29.4 ± 1.2	−0.24 ± 0.04	0.92
LysoC_6PC	9.3 ± 1.1	−0.18 ± 0.03	1.0
LysoC_6PA	10.7 ± 1.3	−0.22 ± 0.04	0.92
GPS	45.8 ± 3.3	−0.10 ± 0.01	0.93

[a] Binding parameters (K_d and ΔF_{sat}) were obtained from fitting the data to a simple hyperbolic model, with errors being taken as the standard errors of the hyperbolic parameters. ΔF_{sat} is the fluorescence change at saturation relative to the fluorescence of factor V_a in 1 mM Ca^{2+} (see Materials and Methods). Note that K_d values are simply phenomenological and have no relationship to the affinities of individual sites on factor V_a for soluble lipids. [b] E: Enhancement of the cofactor activity of factor V_a by the addition of soluble lipids as calculated from eq 3 under Materials and Methods.

performed under exactly the conditions of the 150 mM NaCl titration using QELS and ANS fluorescence (inset to Figure 3B). Both methods established the CMC of C_6PS under these conditions as 800−900 μM. Thus, factor V_a dramatically lowered the C_6PS CMC, as was seen for factor X_a (*24*). However, this value is much larger than the highest lipid concentration used for any of our lipid titrations.

Since the limit of resolution of our QELS instrument is about 5−10 nm, one could argue that very small aggregates might account for our data. This possibility can be discounted both by the observation that C_6PS forms aggregates containing roughly 220 molecules (*24*) and by our equilibrium dialysis stoichiometry measurements showing just 4 or 2 molecules bound to factor V_a in the presence or absence of Ca^{2+}, respectively (Figure 8). We have further shown that a single C_6PS molecule binds to and alters the conformation of the *C2* domain of the factor V_a light chain (*45*). These observations and our controls make a compelling argument that the effects we report are of individual C_6PS molecules binding to sites on factor V_a. Since C_6PS has the lowest and most Ca^{2+}-sensitive CMC of the various lipid analogues we have considered (*2*, *24*), it is safe to assume that the titrations performed with other lipids were also performed well below their CMC's.

Effect of Soluble Lipids on the Cofactor Activity of Factor Va_2. The time courses of activation of prothrombin by the species X_a·C_6PS (open circles), Xa·V_a·C_6PS (closed circles), Xa·V_a·C_6PA (closed squares), and Xa·V_a·$C_6P(h)S$ (closed triangles) are shown in Figure 4. Soluble C_6PS, C_6PA, and $C_6P(h)S$ all enhanced the catalytic ability of factor X_a in the presence of factor V_{a2}. We show also the activity of factor X_a in the presence of C_6PS without factor V_{a2} (open squares). Because C_6PS and some other phospholipids enhance the rate of thrombin generation by factor X_a (*3*), the ratio of the rates of thrombin generation by X_a·V_a·PL to X_a·PL ($r_{Xa\cdot Va\cdot PL}/r_{Xa\cdot PL}$) was measured to determine the effect of factor V_a in the presence of phospholipid. The cofactor effect of factor V_{a2} in the absence of lipid was also measured as the ratio of the rate of thrombin generation by X_a·V_a to the rate by factor X_a alone ($r_{Xa\cdot Va}/r_{Xa}$, data not shown). Factor V_{a2} alone, in the absence of lipid, increased the rate of prothrombin activation by only about 2-fold (data not shown), a result that was expected, since the K_d of factor X_a binding to factor V_a in the absence of PS is roughly 1−3 μM (*7*), meaning that very little X_a is bound to V_a under the conditions of our assay. We have found, however, that the presence of C_6PS causes the K_d for the interaction between factor X_a and factor V_{a2} to drop to 3 nM (*29*). Thus, the ratio of $r_{Xa\cdot Va\cdot PL}/r_{Xa\cdot PL}$ to $r_{Xa\cdot Va}/r_{Xa}$ (see eq 3; values reported in Table 1) essentially describes the abilities of various lipids to influence the ability of factor V_a to bind to factor X_a and to alter its enzymatic properties. Among all the PS analogues studied, C_6PS, C_6PA, and $C_6P(h)D$ enhanced the cofactor activity of factor V_a by 15, 8, and 8 times, respectively. By comparison, bovPS/DOPC (25:75) membrane vesicles increased this ratio by only 1.8-fold relative to the value obtained with C_6PS. This demonstrates that the greatest effect of a PS-containing membrane on factor V_a cofactor activity is due to molecular PS rather than to a membrane surface. None of the other analogues enhanced cofactor activity. Clearly, C_6PS demonstrated a specific ability to enhance the cofactor activity of factor V_a toward factor X_a.

Effect of C_6PS on the Secondary Structure of Factor V_a. The effect of C_6PS on the secondary structure of factor V_a was investigated using circular dichroism spectroscopy. The CD spectra of factor V_a (1.3 μM) at various concentrations of soluble C_6PS (Figure 5, inset) were analyzed in terms of the ratio of ellipticity at 222 nm to 208 nm. Although we could obtain spectra only to 200 nm under our conditions, estimates of α-helix content can also be made with good accuracy from such spectra (*17*) by published procedures (*44*). The variations of Q_{222}/Q_{208} and α-helicity with the addition of C_6PS are shown in Figure 5. The ellipticity ratio

Table 2: Parameters[a] Associated with the Binding of Soluble C_6PS to Factor V_a, V_a-LC and V_a-HC at 0.15 and 0.8 M NaCl

protein/chain	K_{d1} (μM)	K_{d2} (μM)	$\Delta F_{sat,1}$ (fraction)	$\Delta F_{sat,2}$ (fraction)
		C_6PS at 0.15 M NaCl		
FV_a at 1.0 mM Ca^{2+}	9.0 ± 0.6	NA	-0.26 ± 0.03	NA
FV_a-LC at 1.0 mM Ca^{2+}	$1^b \pm 2$	$47^b \pm 28$	-0.003 ± 0.01	-0.017 ± 0.001
FV_a-HC at 1.0 mM Ca^{2+}	2.2 ± 0.7	$260^b \pm 1000$	-0.021 ± 0.001	-0.032 ± 0.002
		C_6PC at 0.15 M NaCl		
FV_a-LC at 1.0 mM Ca^{2+}	31 ± 3	NA	-0.043 ± 0.002	NA
FV_a-HC at 1.0 mM Ca^2	$4^b \pm 2$	$200^b \pm 800$	-0.026 ± 0.008	$-0.043^b \pm 0.10$
		C_6PS at 0.15 M NaCl and 1.2 mM EDTA		
FV_a	15.4 ± 1.7	NA	-0.094 ± 0.003	NA
FV_a-LC	20 ± 1.2	NA	-0.0144 ± 0.0003	NA
FV_a-HC	3.3 ± 0.2	NA	-0.0259 ± 0.0003	NA
		C_6PS at 0.8 M NaCl		
FV_a at 1.0 mM Ca^{2+}	7.7 ± 0.4	NA	-0.09 ± 0.010	NA
FV_a-LC at 1.0 mM Ca^{2+}	NA	30.0 ± 4	NA	-0.012 ± 0.002
FV_a-HC at 1.0 mM Ca^{2+}	NA	NA	NA	NA

[a] Binding isotherms were fit to the simplest model that would give an adequate description of the data, either a single- or two-independent-site model (see Materials and Methods). ΔF_{sat} is the fluorescence change at saturation relative to the fluorescence of factor V_a in 1 mM Ca^{2+} (see Materials and Methods). K_d values are in general phenomenological and do not represent site binding constants. [b] These parameters were not well-defined by regression analysis, with a *t*-test giving $P > 0.05$.

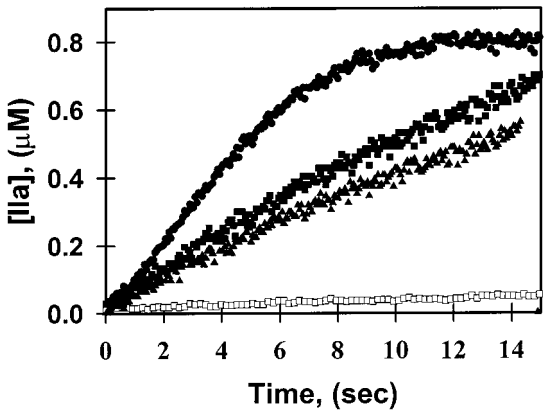

FIGURE 4: Effect of soluble lipids on the rate of prothrombin activation. Activation was initiated by stopped-flow mixing of equal volumes of prothrombin and DAPA in one syringe with enzyme (X_a, V_a, lipid) in another syringe at 37 °C. Final concentrations were: 0.82 μM prothrombin, 5 μM DAPA, 1 nM X_a, 5 nM V_a, and 400 μM C_6PS (closed circles), C_6PA (closed squares), or $C_6P(h)S$ (closed triangles). Controls were also done in the absence of factor V_a but in the presence of 400 μM lipid. In agreement with our previous results (*2*), the rate of prothrombin activation in the absence of factor V_a was nearly the same for all three of these lipids, so only the results for C_6PS are shown by open circles. The absolute rates of thrombin generation by enzymes $X_a{\cdot}C_6PS$, $X_a{\cdot}V_a{\cdot}C_6PS$, $X_a{\cdot}V_a{\cdot}C_6PA$, and $X_a{\cdot}V_a{\cdot}C_6P(h)S$ were 0.0020, 0.10, 0.061, and 0.049 μM/s, respectively. The CMC for C_6PS under these conditions was determined by both ANS fluorescence and QELS to be 650−700 μM with substrate and >800 μM without substrate (*29*).

and α-helicity both decreased hyperbolically with the addition of soluble C_6PS, yielding apparent single-site dissociation constants of 9 and 10 μM, respectively. The fact that these two parameters decreased in a parallel fashion indicates that the major change in factor V_a secondary structure associated with C_6PS binding was a decrease in helical content.

C_6PS Binding to Factor V_a Light and Heavy Chains. Factor V_a is a large protein that has two noncovalently associated chains (V_a-LC and V_a-HC) with five domains (A1, A2 in V_a-HC; and A3, C1, and C2 in V_a-LC). The capability to separate the two subunits provides an approach for determining the distribution of lipid sites between the two chains of

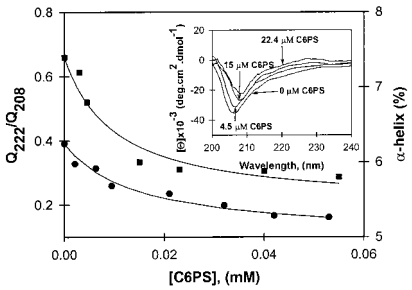

FIGURE 5: Variations of the molar ellipticity ratio (Q_{222}/Q_{208}) (left-hand scale) and α-helicity (right-hand scale) of factor V_a with addition of soluble C_6PS are shown with circles and squares, respectively. The solid lines passing through the curves represent the fit of the variation of Q_{222}/Q_{208} and α-helicity with C_6PS to a single-binding-site model, to yield apparent binding constants of 9.3 and 10.2 μM, respectively. The CD spectra of factor V_a (1.3 μM) in the presence of 0, 4.5, 15.0, and 22.4 μM C_6PS and 1 mM Ca^{2+} are shown in the inset for reference.

factor V_a. Factors V_a-LC and V_a-HC in the standard 1 mM Ca^{2+} buffer were titrated with soluble C_6PS in the presence of 0.15 M (closed circles) and 0.8 mM NaCl (open circles), and the results are shown in Figure 6A,B, respectively. Small but reproducible decreases in the intrinsic fluorescence demonstrate that factors V_a-LC and V_a-HC both bound to soluble C_6PS at 0.15 M NaCl (closed circles, Figure 6A,B). While both the V_a-LC and the V_a-HC data were approximately described by a single-site-binding model (dashed lines in Figure 6), both were statistically better described by a two-independent-binding-site model (solid lines; see Materials and Methods), with parameters obtained from these fits reported in Table 2. Repeat experiments on both LC and HC were nearly identical to those recorded here and also showed that the two-binding-site model gave statistically improved descriptions of the titration data. Both chains bound C_6PS under these normal ionic strength conditions, although factor V_a-HC apparently bound C_6PS substantially more tightly than did V_a-LC. At 0.8 M NaCl, V_a-HC did not bind at all to C_6PS (open circles in Figure 6B), while V_a-LC binding was not much affected by high ionic strength. However, in contrast to the data at 0.15 M NaCl, a single-site model was fully adequate to fit the 0.8 M NaCl data (open circles in Figure 6A), with the parameters recorded in

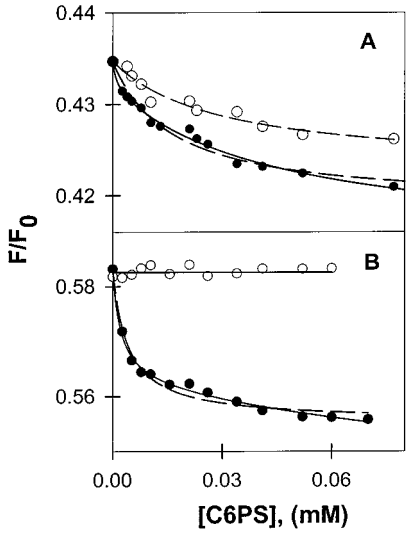

FIGURE 6: Changes in factor V_a light-chain and heavy-chain fluorescence in response to C_6PS titration. Factor V_a light chain (0.69 μM) (frame A) and heavy chain (0.69 μM) (frame B) were titrated with soluble C_6PS in 50 mM Tris, 1.0 mM $CaCl_2$, pH 7.5, in the presence of either 150 mM NaCl (filled circles) or 800 mM NaCl (open circles). In the presence of 0.15 M NaCl, the binding isotherms were best described by a two-independent-binding-site model (solid lines through closed circles). Single-site fits are shown for reference by dashed lines. In the presence of 0.8 M NaCl, the titration of V_a-LC was well described by a single-binding-site model (dashed line through open circles in frame A). The binding parameters associated with the fits are tabulated in Table 2.

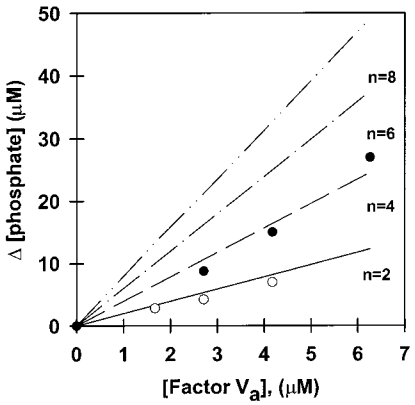

FIGURE 7: Determination of the stoichiometry of C_6PS binding to factor V_a. Predicted values of the difference of phosphate concentrations (Δ[phosphate]) between the two chambers of equilibrium dialysis cells (as described under Materials and Methods) are plotted as a function of increasing concentrations of factor V_a for four assumed values of stoichiometry [$n = 2$ (solid line), 4 (dash−dash line), 6 (dash−dot line), and 8 (dash−dot−dot)]. The experimentally determined Δ[phosphate] values in the absence (closed circles) and in the presence (open circles) of Ca^{2+} show clearly that the stoichiometry of C_6PS binding to factor V_a under these two conditions is 4 and 2, respectively.

Table 2. This suggests that at least one of the sites in V_a-LC may be sensitive to charge−charge interactions, as are both sites in V_a-HC.

Stoichiometry of C_6PS Binding to Factor V_a. The titrations so far presented (CD and intrinsic fluorescence) demonstrate binding of soluble lipids indirectly, through the structural changes they induce. To confirm binding by a direct method and to determine the stoichiometry of binding, we measured soluble C_6PS binding to factor V_a by equilibrium dialysis in the presence 1 mM Ca^{2+} or 1 mM Ca^{2+} and 1.2 mM Na_2-EDTA (Figure 7). The experimentally observed differences in phosphate concentration between the two dialysis chambers in the presence (closed circles) and absence (open circles) of Ca^{2+} were compared to values calculated for various assumed values of binding stoichiometry (see Materials and Methods). The results show clearly that factor V_a binds four molecules of C_6PS in the presence of Ca^{2+} and two in its absence.

Effect of C_6PS on the Interaction of V_a-LC and V_a-HC in the Absence of Ca^{2+}. The aims of this experiment were, first, to ask whether there are C_6PS binding sites in both the light and heavy chains of factor V_a in the absence of Ca^{2+}, and, second, to determine whether occupancy of these sites causes factors V_a-LC and V_a-HC to interact with each other in the absence of Ca^{2+}. Factor V_a-LC, factor V_a-HC, and factor V_a all bound to soluble C_6PS in the absence of Ca^{2+} (Figure 8). Factor V_a, after incubation for an hour with 1.2 mM Na_2-EDTA, has been shown to dissociate into a 1:1 mixture of light chain and heavy chains (*48*). If we assume that V_a-LC and V_a-HC chains do not interact with each other in the absence of Ca^{2+}, then the C_6PS binding isotherm for factor V_a in the presence of EDTA (Figure 8C, filled circles) should be a simple sum (Figure 8C, open circles) of the curves obtained with V_a-LC (Figure 8A) and V_a-HC (Figure 8B) chains also in the presence of EDTA. This expectation clearly was not met. All three curves in Figure 8 were well fitted to a single-binding-site model with the binding parameters reported in Table 2. Since only two molecules of C_6PS bind to factor V_a in the absence of Ca^{2+}, this must mean that that one C_6PS bound to each of the chains in the absence of Ca^{2+}, and that this binding somehow mediates an interaction between factor V_a light and heavy chains.

In the presence of Na_2EDTA to chelate Ca^{2+} and dissociate the heavy and light chains of factor V_a, the C_6PS CMC was found to be 0.9−1 mM (inset to Figure 8A), compared to 8 mM in the absence of Ca^{2+} in a similar buffer (*24*). Despite the dramatic effect of the light and heavy chains of factor V_a on C_6PS aggregation, the CMCs we have measured in the presence of these proteins under our experimental conditions are well below the concentrations needed to see structural and functional responses from factor V_a and its subunits to binding of C_6PS and its analogues. It is clear, then, that the effects we report are due to binding of molecular, not aggregated, C_6PS to individual sites.

Soluble C_6PC Binding to Factor V_a Light and Heavy Chains in the Presence of Ca^{2+}. The purpose of this experiment was to test whether binding sites located in the light or heavy chains of factor V_a are specific for C_6PS by testing for binding to C_6PC. The observed variation of intrinsic fluorescence intensity of factor V_a light and heavy chain upon titration with soluble C_6PC is shown in Figure 9A,B, respectively. The light and heavy chains both bound to C_6PC, but the binding data require description in terms of two, independent binding sites only for V_a-HC (Figure 9B, solid line), with the V_a-LC data well described by a single-binding-site model (Figure 9A, dashed curve). The parameters for these fits are reported in Table 2. This is similar to what we saw with C_6PS binding, except that binding of this lipid to both V_a-HC and V_a-LC was better described by the two-independent-site model (Figure 6). Overall, the binding data are consistent with there being two

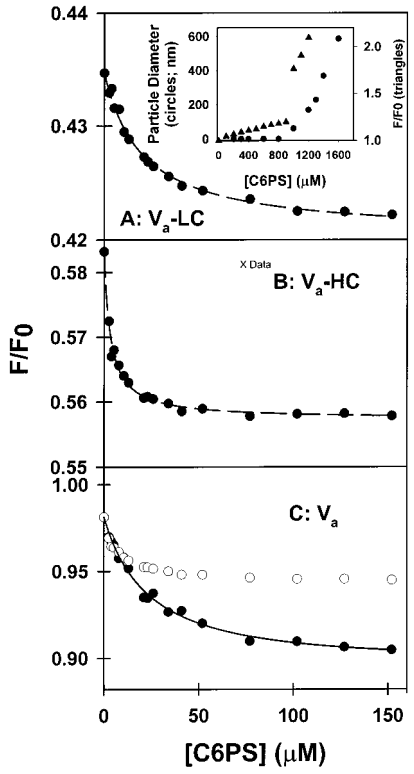

FIGURE 8: Intrinsic fluorescence changes of factor V_a, V_a-LC, and V_a-HC in response to C_6PS in the absence of Ca^{2+}. Factor V_a light chain (0.69 μM) (frame A), factor V_a heavy chain (0.69 μM) (frame B), and factor Va (0.69 μM) (frame C) were incubated with 1.2 mM Na_2EDTA in a buffer of 50 mM Tris, 150 mM NaCl, 1 mM$CaCl_2$, pH 7.5, for an hour at 23 °C and then titrated with soluble C_6PS in the same buffer. Since factor V_a subunits dissociate slowly in the absence of Ca^{2+} (*48*), this assured that frame C represents titration of an equal mixture of dissociated factor V_a heavy and light chains. The fluorescence intensities of V_a-LC, V_a-HC, and factor V_a were normalized to the fluorescence intensity of factor V_a in the presence of 1.0 mM Ca^{2+}. Lines passing through the filled circles are single-site (dashed lines) or two-site (solid line) fits to the data. The sum of the results in frames A and B was calculated, assuming no interaction between light and heavy chains, and normalized to the observed fluorescence intensity for factor V_a in the presence of Na_2EDTA and no C_6PS. This sum is plotted as the open circles in frame C. The binding parameters associated with fits are reported in Table 2. The variation of particle size (circles) and ANS fluorescence (triangles) with soluble C_6PS concentration is shown in the inset, establishing that the C_6PS CMC under these conditions (separated light and heavy chains in the absence of Ca^{2+}) was 900−1000 μM.

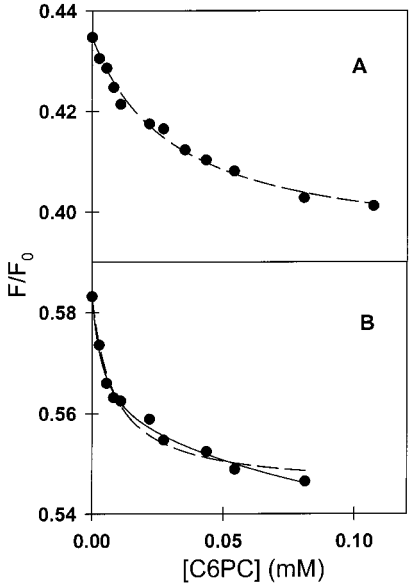

FIGURE 9: Intrinsic fluorescence changes of V_a-LC and V_a-HC in response to C_6PC in the presence of Ca^{2+}. Factor V_a light chain (0.69 μM) (frame A) and heavy chain (0.69 μM) (frame B) were titrated with C_6PC in 50 mM Tris, 1.0 mM $CaCl_2$, 150 mM NaCl, pH 7.5. The solid line passing through the frame B curve was obtained by fitting the data to a two-binding-site-model, while the dashed lines represent a single-site model. The binding parameters associated with the fits are recorded in Table 2.

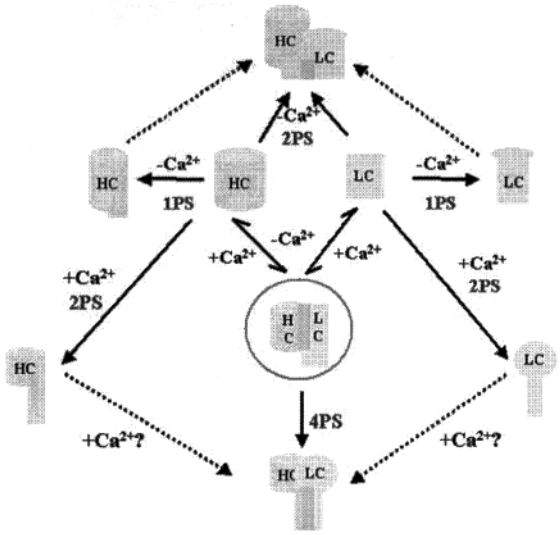

FIGURE 10: Cartoon summarizing the main conclusions.

sites in both the heavy and light chains that bind short-chain phospholipids in a nonspecific manner.

DISCUSSION

The purpose of this work was to ask whether short-chain, soluble phospholipids interact with and regulate factor V_a as they do with factor X_a and, if so, to locate the lipid binding sites in bovine factor V_a heavy or light chain and then to define their specificity for different lipid species. The main conclusions of this paper are summarized below and in the cartoon given in Figure 10: (1) Factor V_a binds four molecules of soluble C_6PS in the presence of Ca^{2+}, two in the light and two in the heavy chain. Each chain binds only one C_6PS molecule in the absence of Ca^{2+}. (2) Binding of soluble C_6PS as well as of all the other short-chain lipids to factor V_a induces a conformational change in factor V_a and in its light and heavy chains. The conformational changes induced by C_6PS in factor V_a light and heavy chains influence the interaction between the chains. (3) Binding of C_6PS, C_6-PA, or C_6P(h)S all specifically enhance the cofactor activity of factor V_a for factor X_a activation of prothrombin. Other lipids, including the D-stereoisomer of C_6PS, have no effect on cofactor activity. Each of these conclusions is discussed below.

Factor V_a Has Four C_6PS Sites, Two in Factor V_a-LC and Two in Factor V_a-HC. Equilibrium dialysis measurements unequivocally establish that four soluble C_6PS molecules bind to factor V_a in the presence of 1 mM Ca^{2+} (Figure 7). V_a-LC and V_a-HC also bound soluble C_6PS in the presence of 1 mM Ca^{2+} (Figure 6). Out of four C_6PS binding sites on factor V_a, two are probably located in the light chain and

two in the heavy chain. This interpretation is based on the fact that the two-binding-site model gave statistically better descriptions of our fluorescence titration data (Figure 6) than did a single-site model. Because the difference between the single-site and two-site descriptions was not large, our data do not rule a 3/1 or 1/3 distribution of C_6PS sites between the LC and HC, but it seems most likely at this point that two C_6PS sites exist in each of the factor V_a chains.

It is worth asking whether these C_6PS sites are involved in factor V_a binding to PS-containing membranes. Consistent with this possibility, we showed that binding of two to four molecules of PS to sites on factor V_a, along with a substantial PS-independent interaction (*22*), can account for PS-dependent binding of factor V_a to a membrane (*12*). Binding of factor V_a to PS/PC membranes is generally thought to occur through the light chain, with many studies pointing to the C2 domain (*19, 21, 25, 26, 35, 36, 38, 41*). Consistent with this, recent direct measurements have demonstrated C_6PS binding to a single site on the C2 domain of V_a-LC with a k_d of 2 μM (*45*). Our results could not clearly define the affinities of the two sites that appear to exist in the LC (Table 2), but do indicate that one is fairly tight ($k_d \approx 1 \pm 2$ μM) and the other one roughly 2 orders of magnitude less tight ($k_d \approx 47 \pm 28$ μM). The similarity in apparent k_d values suggests that the tight site might correspond to the C_6PS site located in the C2 domain (*45*). Binding of the C2 domain to PS-containing membranes occurs with a K_d of 0.17 μM (*45*), meaning that the C2 site must contribute significantly to membrane binding but that at least one other site must exist to account for the much smaller K_d of whole factor V_a binding to a similar membrane (2 nM) (*12*).

Our data also clearly show that at least one and likely two molecules of C_6PS bind to the factor V_a heavy chain (Figure 6B). Remarkably, this binding appeared to be considerably tighter than was the binding to light chain (Figure 6). However, our two-site binding analysis suggests this is caused by the tight site contributing roughly one-fifth of the overall intrinsic fluorescence change of the V_a-LC but about half of the intrinsic fluorescence change associated with C_6PS binding to the V_a-HC (see ΔF_{sat} values in Table 2). The site binding constants derived from this analysis (Table 2) showed that the C_6PS binding affinity to V_a-HC was actually comparable to the V_a-LC affinity for the tight site, but was considerably weaker for the weak site. Is it possible that C_6PS sites in the factor V_a-HC are involved in providing binding free energy needed for association of whole factor V_a with a PS-containing membrane? Current models of the domain organization of factor V_a on a membrane would dispute this possibility (*47*), but these models are far from proven. A stronger argument against this possibility is that V_a-LC, but not V_a-HC, competes factor V_a off of a PS-containing membrane with an apparent affinity close to that seen with whole factor V_a (*25*). However, given the results presented here and the fact that V_a-HC has been shown to interact weakly with membranes (*23*), we are open to the possibility that the domain structure of factor V_a on a membrane may be other than is currently envisioned and that the heavy chain may at some point contact the membrane and that this contact could contribute to membrane binding or to PS regulation of cofactor activity. Alternatively, the binding of C_6PS- and PS-containing membranes to V_a-HC may be anomalous. In this regard, we have recently shown that one of two C_6PS sites on factor X_a is not involved in membrane binding or in regulation of that enzyme but rather is part of the substrate recognition site (*46*). Similarly, one or both of the C_6PS binding sites in V_a-HC could be associated with protein recognition sites, since the V_a-HC contains recognition sites for factor X_a (*1, 34*) and for prothrombin (*16, 28*). We will need more information about the location and specificities of the C_6PS sites in the V_a-HC and about the structure of factor V_a on a membrane before we can resolve this issue.

While one or more of the four C_6PS sites we have defined may be involved in other than membrane binding, at least one of these sites enhances factor V_a's cofactor activity during prothrombin activation in a C_6PS-specific fashion (Table 1), just as PS-containing membranes have been reported to do (*14, 18, 37*). Since the *C2* domain site is not specific for C_6PS (*45*), the activity-regulating site(s) may be elsewhere. At this point, we have no information on where the regulatory site(s) may be, but we remain open to the possibility that it could be in either the light or the heavy chain.

Soluble C_6PS Binding to Factor V_a Induced a Conformational Change in Whole Factor V_a and Probably in Its Isolated Subunits. Upon addition of soluble C_6PS, the fluorescence intensities of factor V_a, V_a-LC, and V_a-HC decreased in the presence (Figures 3A and 6) and in the absence (Figure 8) of Ca^{2+}. This suggests that factor V_a, V_a-LC, and V_a-HC all undergo conformational changes upon binding to soluble C_6PS. While the intrinsic fluorescence changes we have observed were small, they were completely reproducible. Of course, a small decrease in intrinsic fluorescence can mean that one or two Trp residues become exposed to water upon C_6PS binding, not an event that would be described as a substantial conformational change. However, our conclusion is supported by the fact that C_6PS also induced secondary structural changes in factor V_a (Figure 5). Upon addition of a saturating concentration of C_6PS, the α-helical content of factor V_a decreased from 7.4% to 5.6%, confirming that a significant change in global factor V_a conformation did occur upon binding of factor V_a.

It appears that the conformational changes induced by C_6PS binding can induce intersubunit interactions within factor V_a. This conclusion is based on the observation that binding of C_6PS to the light- and heavy-chain subunits is not additive under conditions that these subunits are presumably dissociated by removal of Ca^{2+} (*32*). C_6PS apparently induces some interaction between subunits even in the absence of Ca^{2+} (Figure 8). This means that lipid binding to the light chain may alter the conformation of the heavy chain and thus the interaction of factor V_a with factor X_a or prothrombin.

At Least One Site on Factor V_a Alters Cofactor Activity in a Stereospecific Manner. All PS analogues shown in Figure 1 bound to factor V_a with similar affinities and induced similar changes in intrinsic fluorescence (Table 1). This differs from the behavior seen for the binding of C_6PS analogues to factor X_a, where one set of lipids gave a full structural response, another set gave partial responses, and many did not bind at all (*2*). This difference in the response of factor X_a and its cofactor to soluble C_6PS lipid analogues could be because there are four C_6PS sites on factor V_a and only two on factor X_a. Thus, there are many more opportunities for nonspecific interactions of soluble lipid analogues

with sites on factor V_a. Indeed, only one site on factor X_a regulates activity; the second is an anomalous, *albeit* fairly specific, site located in the substrate binding region (*46*). To test whether any of these sites would have specific affects on the cofactor activity of factor V_a, we devised the activity assay described under Materials and Methods and illustrated in Figure 4. The results (Table 1) make it clear that the effect of soluble lipids on the cofactor activity of factor V_a is specific for PS or for lipid species that we and others have shown allow for assembly of an active prothrombinase complex (*11*, *14*, *18*, *40*). The three lipids that showed activity in our assay [C_6PS, C_6PA, and C_6P(h)S] all had equivalent and maximal effects on the activity of factor X_a toward prothrombin in the absence of factor V_a (*3*). However, the effect of these three lipids summarized in Table 1 is due to an effect on factor V_a and not factor X_a. Thus, within experimental uncertainty, all three of these lipids produced identical effects on factor X_a (*3*), whereas there was a difference between their effects on factor V_a (Table 1). In addition, we specifically removed the effect of lipid on factor X_a by taking the ratio of ratios described by eq 3 under Materials and Methods. Thus, our observations suggest that occupancy of at least one of the four lipid binding sites on factor V_a enhances cofactor activity with a specificity similar to that seen for the lipid-induced enhancement of factor X_a activity. This certainly points to a specific role of platelet membrane PS in regulating prothrombinase activity in vivo.

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