The Phosphatidylserine Binding Site of the Factor V_a C2 Domain Accounts for Membrane Binding but Does Not Contribute to the Assembly or Activity of a Human Factor X_a−Factor V_a Complex†

Rinku Majumder,‡ Mary Ann Quinn-Allen,§ William H. Kane,§ and Barry R. Lentz*,‡

Department of Biochemistry and Biophysics, CB #7260, University of North Carolina, Chapel Hill, North Carolina 27599-7260, and Division of Hematology, Departments of Medicine and Pathology, Duke University Medical Center, Durham, North Carolina 27710

Received September 21, 2004; Revised Manuscript Received October 27, 2004

ABSTRACT: Factors V_a and X_a (FV_a and FX_a, respectively) assemble on phosphatidylserine (PS)-containing platelet membranes to form the essential "prothrombinase" complex of blood coagulation. The C-terminal domain (C2) of FV_a (residues 2037−2196 in human FV_a) contains a soluble phosphatidylserine (C6PS) binding pocket flanked by a pair of tryptophan residues, Trp^{2063} and Trp^{2064}. Mutating these tryptophans abolishes FV_a membrane binding. To address both the roles of these tryptophans in C6PS or membrane binding and the role of the C2 domain lipid binding site in regulation of FV_a cofactor activity, we expressed W(2063,2064)A mutants of the recombinant C2 domain (rFV_{a2}-C2) and of a B domain-deleted factor V light isoform (rFV_{a2}) in Hi-5 and COS cells, respectively. Intrinsic fluorescence showed that wild-type rFV_{a2}-C2 binds to C6PS and to 20% PS/PC membranes with apparent K_d values of 2.8 μM and 9 nM, respectively, while mutant rFV_{a2}-C2 does not. Equilibrium dialysis confirmed that mutant rFV_{a2}-C2 does not bind to C6PS. Mutant rFV_{a2} binds to C6PS ($K_d \sim 37$ μM) with an affinity comparable to that of wild-type rFV_{a2} ($K_d \sim 20$ μM), although it does not bind to PS/PC membranes to which wild-type rFV_{a2} binds with native affinity ($K_d \sim 3$ nM). Both wild-type and mutant rFV_{a2} bind to active site-labeled FX_a (DEGR-X_a) in the presence of 400 μM C6PS with native affinity ($K_d \sim 3-4$ nM) to produce a solution rFV_{a2}−FX_a complex of native activity. We conclude that (1) the C2 domain PS site provides all but ~1 kT of the free energy of FV_a membrane binding, (2) tryptophans lining the C2 lipid binding pocket are critical to C6PS and membrane binding and insert into the bilayer interface during membrane binding, (3) occupancy of the C2 lipid binding pocket is not necessary for C6PS-induced formation of the FX_a−FV_a complex or its activity, but (4) another PS site on FV_a does have a regulatory role.

Thrombin-activated coagulation factor V is an essential component in the "prothrombinase" complex, which activates the zymogen prothrombin to thrombin. This complex consists of enzyme factor X_a (FX_a), factor V_a (FV_a), calcium ions, and a phosphatidylserine-containing membrane surface (*1*, *2*). Factor V_a is a heterodimer (*3*) composed of a heavy chain (FV_a-HC; A1 and A2 domains; $M_r = 94\,000$ in bovine; $M_r = 105\,000$ in human) and a light chain (FV_a-LC; A3, C1, and C2 domains; $M_r = 74\,000$ or $71\,000$) (*4*, *5*). Heterogeneity of the light chain is seen in both bovine and human factor V_a (*4*−*6*) and is due to alternative glycosylation at Asn^{2181} in the C2 domain (*7*). The species that is glycosylated at Asn^{2181} is termed factor V_{a1} (FV_{a1}), while the species lacking glycosylation at that position is factor V_{a2} (FV_{a2}).[1] FV_{a2} binds somewhat more tightly than FV_{a1} to PS-containing membranes (*8*, *9*). In addition, FV_{a2}, but not FV_{a1}, binds with high affinity to FX_a in the presence of a soluble form of PS (C6PS) (*10*). For this reason, all work reported here was performed with FV_{a2}.

FV_a and FX_a interact weakly in solution (*11*) but somehow bind with a K_d of ~3 nM on a PS-containing membrane (*12*). Because protein interactions are difficult to characterize on a membrane surface, it is difficult to know whether the increased affinity seen in the presence of membranes is due to a reduction in reaction dimensionality (two versus three dimensions) or to a surface- or lipid-induced change in either FV_a, FX_a, or both. Use of C6PS has allowed us to circumvent the complication of examining proteins on a membrane surface. C6PS binds to two sites on FX_a (*13*) and to four sites on FV_a (*14*). One of the sites on FX_a allosterically regulates this enzyme's conformation, aggregation state, and activity (*15*−*17*). It may also be that C6PS binding to one or more sites on FV_a regulates either cofactor activity or the interaction of FV_a with FX_a (*10*, *14*). Support for this

† Supported by U.S. Public Health Service Grants GM32707 (B.R.L.) and HL43106 (W.H.K.).

* To whom correspondence should be addressed. E-mail: uncbrl@med.unc.edu. Phone: (919) 966-5384. Fax: (919) 966-2852.

‡ University of North Carolina.

§ Duke University Medical Center.

[1] Abbreviations: rFV_{a2}, expressed recombinant light isoform of B domain-deleted factor V_a; rFV_2-C2, expressed recombinant, nonglycosylated C2 domain of human factor V_a; C6PS, 1,2-dicaproyl-*sn*-glycero-3-phospho-L-serine; PS, phosphatidylserine; DOPS, 1,2-dioleoyl-*sn*-glycero-3-phospho-L-serine; DOPC, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine; 6−7 bromo-PC, 1-palmitoyl-2-stearoyl-(6−7-dibromo)-*sn*-glycero-3-phosphocholine; 11−12 bromo-PC, 1-palmitoyl-2-stearoyl-(6−7-dibromo)-*sn*-glycero-3-phosphocholine; SUVs, small unilamellar vesicles; DAPA, dansylarginine *N*-(3-ethyl-1,5-pentanediyl)amide; DEGR-CK, [5-(dimethylamino)-1-naphthalenesulfonyl]glutamyl-glycyl-arginyl chloromethyl ketone; CMC, critical micelle concentration.

10.1021/bi047962t CCC: $30.25
Published on Web 12/22/2004

hypothesis is derived from the observation that C6PS triggers tight binding of FV_{a2} to FX_a ($K_d \sim 2$ nM) in solution to yield a fully active complex (*10*). We do not know whether this regulation is accomplished solely by binding of C6PS to FX_a or if binding to FV_a is also required.

One of the four C6PS sites on FV_a has been identified with a binding pocket in the C2 domain (*18*). Two Trp residues located at the mouth of this lipid binding pocket, Trp^{2063} and Trp^{2064}, are required for binding of factor V_a to immobilized PS (*19*) and PS-containing membranes (*20*). This supports a model in which the indole side chains of Trp^{2063} and Trp^{2064} occupy the binding pocket, but are forced out and into a membrane by occupancy of the binding pocket by PS (*18*). It could be that PS occupancy of this pocket triggers conformational changes that lead to altered cofactor activity or altered interaction with FX_a. Here we address several questions inspired by this model and by these possible roles of Trp^{2063} and Trp^{2064} in membrane binding.

(1) *Are the Trp^{2063} and Trp^{2064} residues of the C2 domain necessary for occupancy of the C2 binding pocket by short-chain, soluble C6PS?* In other words, while mutation of Trp^{2063} or Trp^{2064} to Ala did not alter significantly the rFV_{a2} or rFV_{a2}-C2 domain conformation (*20*), *might there be a local, Trp-dependent conformation that is needed for creation of a lipid binding pocket?*

(2) Trp residues are known to strongly favor location at the interface region of a membrane bilayer and can provide considerable free energy for binding of Trp-containing proteins to a membrane (*21*). *Are these Trp residues in the C2 domain inserted into the membrane upon membrane binding?*

(3) It has been proposed that the C2 binding pocket could function as a PS-mediated switch that would trigger changes elsewhere in FV_a (*22*). *Is the lipid binding pocket identified in the C2 domain a specific PS regulatory site, occupancy of which is required for full cofactor activity or for assembly of the $FV_{a2}-FX_a$ complex, or might there be another site on FV_{a2} specific for PS that might serve as a regulatory site for assembly or activity of the $FV_{a2}-FX_a$ complex?*

EXPERIMENTAL PROCEDURES

Materials

1,2-Dicaproyl-*sn*-glycero-3-phospho-L-serine (C6PS), 1,2-dioleoyl-*sn*-glycero-3-phospho-L-serine (DOPS), 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC), and brain phosphatidylserine (PS) were purchased from Avanti Polar Lipids Inc. (Alabaster, AL). Dansylarginine-*N*-(3-ethyl-1,5-pentanediyl)amide (DAPA) was obtained from Haematologic Technologies Inc. (Essex Junction, VT). [5-(Dimethylamino)-1-naphthalenesulfonyl]glutamyl-glycyl-arginyl chloromethyl ketone (DEGR-CK) was purchased from Calbiochem (La Jolla, CA). All other chemicals were American Chemical Society (ACS) reagent grade or the best available grade.

Methods

Preparation of Soluble C6PS Stocks. C6PS stocks were prepared from measured quantities of 2.5 mg/mL stock solutions in chloroform. The chloroform was evaporated under a stream of nitrogen. The lipid was redissolved in cyclohexane, and then the frozen solution was lyophilized overnight to a white powder. The resulting dry powder was dispersed in the appropriate volume of buffer and vortexed thoroughly to reach the desired concentration. These stocks were used within 1 day of preparation.

Preparation of Small Unilamellar Vesicles. Small unilamellar vesicles (SUVs) composed of DOPC and DOPS in an 80:20 molar ratio were prepared by suspending lipids, dried as described for C6PS, in the desired buffer with vigorous vortexing and subjecting this suspension to sonic disruption in a Misonix (Farmingdale, NY) Sonicator 3000 (*23*). SUV preparations were fractionated by centrifugation at 70 000 rpm for 25 min in a Beckman (Palo Alto, CA) TL-100 ultracentrifuge (*24*). Phospholipid concentrations were determined by the analysis of inorganic phosphate (*25*). SUVs were used the day of preparation.

Isolation of Recombinant Proteins. Wild-type and mutant W(2063,2064)A rFV were expressed in COS cells using a B domain-deleted factor V construct and purified to produce rFV_{a2} as previously described (*7*). The isolation and purity of these recombinant proteins are described in detail elsewhere (*20*). The concentration of purified rFV_{a2} was estimated by measuring the absorbance at 280 nm using an extinction coefficient ($E^{1\%}_{280}$) of 1.74 (*26*). Native and mutant recombinant rFV_{a2}-C2 domain constructs were expressed in Hi-5 cells, and recombinant proteins were isolated using established methods (*18*). The concentration of purified rFV_{a2}-C2 was determined by measuring the absorbance at 280 nm using an extinction coefficient of 2.85 that was calculated on the basis of the amino acid composition of rFV_{a2}-C2 (*18*). Alternative glycosylation of recombinant B domain-deleted factor V at Asn^{2181} results in two glycoforms, rFV_{a1} and rFV_{a2}, that were separated by chromatography on a Mono S column (*7*). The recombinant proteins utilized in this study, rFV_{a2} and rFV_{a2}-C2, are not glycosylated at Asn^{2181}. The mutant form of rFV_{a2}, in which Trp^{2063} and Trp^{2064} are replaced with Ala residues, was still able to form an active rFX_a-FV_a complex when the mutant rFV_{a2} was added in excess to a membrane-based assay and was still recognized by a C2 domain specific monoclonal antibody (*20*). In addition, mutant rFV_{a2} added at a roughly 50-fold excess increased the activity of FX_a in the presence of PS-containing membranes to the same extent seen with wild-type rFVa2 (*20*). Finally, the expressed C2 domain with and without this mutation had similar secondary structures as revealed by CD spectroscopy. All this suggests that mutation of Trp^{2063} and Trp^{2064} to Ala did not alter significantly the conformation of rFV_{a2} or rFV_{a2}-C2.

Preparation of DEGR-X_a. DEGR-X_a was prepared by sequential addition of 5 μL aliquots of DEGR-CK [1 mg/mL in 0.02 M Tris and 0.15 M NaCl (pH 7.5)] to 1 mL of $\sim$17 μM purified factor X_a until there was a complete loss of enzymatic activity, as monitored by the S-2765 assay (*27*). DEGR-X_a was then dialyzed against 20 mM Tris and 0.15 M NaCl (pH 7.5) to remove the free reagent (*28*).

Binding Measurements. The intrinsic fluorescence intensity was recorded with an SLM 48000-MHF spectrophotometer (SLM Aminco, Urbana, IL) as described previously (*18*). The buffer consisted of 20 mM Tris, 150 mM NaCl, and 5 mM $CaCl_2$ (pH 7.4) filtered through a 0.8 m polycarbonate filter (white AAWP, 47 mm, Millipore Corp.). Equilibration between additions of lipids took place for at least 2 min with

continuous stirring. The total volume added to a sample during the titration was always <5% of the total sample volume. The buffer without proteins was titrated as a scattering control.

Titration of DEGR-X_a by rFV_{a2} was accomplished as described previously (*10*) in 1 nM DEGR-X_a with 400 μM C6PS, 20 mM Tris, 0.15 M NaCl, and 5 mM Ca^{2+} (pH 7.5), with a 4 min stirred equilibration between rFV_{a2} additions. Several intensity readings were averaged after each addition. Controls were performed to correct for dilution, buffer background, and any small amount of photobleaching. Slits were closed except during measurements to limit photodegradation.

The response of rFV_{a2} and rFV_{a2}-C2 intrinsic fluorescence to membrane addition was fitted to the site binding model described below using a Marquardt−Levenberg algorithm supplied with Sigmaplot (version 4.2, Jandel Scientific, Corte Madera, CA). The observed fluorescence response was taken to be proportional to the fraction of protein bound to membrane-located binding sites. The observed response (R_{obs}) is given as previously described (*9*) in terms of the total concentration of membrane phospholipid added, $[PL]_T$:

$$R_{obs} = R_0 + \frac{R_{sat} - R_0}{2[P]_T}\Gamma\sqrt{\Gamma^2 - 4[P]_T\frac{[PL]_T}{i}} \quad (1)$$

where

$$\Gamma = \frac{[PL]_T}{i} + [P]_T + k_d$$

where $[P]_T$ is the total protein concentration, R_0 is the observed fluorescence of the protein in the absence of lipid, ΔR_{sat} ($=R_{sat} - R_0$) is the observed change in fluorescence when all the membrane sites are occupied by protein, k_d is the effective site dissociation constant, and i is the stoichiometry (number of lipids associated with each membrane-located protein binding site). In the case of C6PS binding to a single site on FV_a, the concentration of ligand is much greater than the concentration of protein, and the analysis simplifies to (*13*)

$$R_{obs} = R_0 + \Delta R_{sat}\frac{[C6PS]}{k_d + [C6PS]} \quad (2)$$

where k_d is the apparent site dissociation constant.

Determination of the Stoichiometry of Binding of C6PS to Mutant rFV_{a2}-C2. The stoichiometry of soluble C6PS binding to mutant rFV_{a2}-C2 was determined by equilibrium dialysis measurements as described previously (*18*).

Assay for Prothrombin Activation in the Presence of C6PS. 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 mixing chamber (Spectronic Instruments, Inc., Rochester, NY) attached to the SLM 48000 spectrofluorometer as described previously (*29*, *30*). Reactions were initiated by rapidly mixing equal volumes (400 μL) of the contents of the two driving syringes to obtain final concentrations in the observation chamber of 1 μM prothrombin, 5 μM DAPA, 1 nM factor X_a, and 0.4 mM C6PS in the absence or presence of 5 nM FV_{a2}, in 50 mM Tris, 150 mM NaCl, and 5 mM $CaCl_2$ (pH 7.5). The fluorescence intensity at the completion of the reaction (infinite time) was considered to represent quantitative conversion of prothrombin to thrombin, so the initial rate of thrombin generation was determined from the initial rate of fluorescence intensity change normalized to the intensity once thrombin formation was complete (*30*).

Effect of Soluble Lipids on FVa2 Cofactor Activity. Enhancement of cofactor activity (E) by soluble lipids was measured as described in ref *14* using the following equation:

$$E = \frac{r(X_a{\cdot}V_a{\cdot}PL)/r(X_a{\cdot}PL)}{r(X_a{\cdot}V_a)/r(X_a)} = \frac{r(X_a{\cdot}V_a{\cdot}PL)/r(X_a{\cdot}V_a)}{r(X_a{\cdot}PL)/r(X_a)} \quad (3)$$

The ratio on the left is the ratio used experimentally to evaluate E. Here, $r(X_a{\cdot}V_a{\cdot}PL)$ is the rate of prothrombin activation by enzyme in the presence of factor V_a and lipid, as measured as the initial slope of the rate of change of the normalized DAPA fluorescence with time. Other terms are defined analogously. The $r(X_a{\cdot}V_a)/r(X_a)$ ratio was determined as the average from five independent experiments, while $r(X_a{\cdot}V_a{\cdot}PL)/r(X_a{\cdot}PL)$ was the average of three independent measurements. The physical meaning of this expression can be seen best in the rearranged right-most form. The $r(X_a{\cdot}V_a{\cdot}PL)/r(X_a{\cdot}V_a)$ ratio gives the effect of phospholipid binding to both X_a and V_a, while the $r(X_a{\cdot}PL)/r(X_a)$ ratio 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 FV_{a2}.

Depth of Tryptophan Residues of the C2 Domain in the Membrane. We have measured the depth of penetration of Trp^{2063} and Trp^{2064} inside the lipid bilayers using the parallax method (*31*). Lipids labeled with bromine at carbons 6, 7, 11, and 12 in the acyl chain were used as tryptophan quenchers (20 mol % brominated PCs in place of DOPC). Aliquots of wild-type rFV_{a2}-C2 (200 nM) were added to 100 μM vesicle suspensions and the mixtures incubated for 10 min before the tryptophan fluorescence intensity was recorded. The fluorescence intensity was measured at 22 °C with a 48000 SLM spectrofluorometer using an excitation wavelength of 280 nm. The signal from an identical sample with the vesicle itself without the protein was used as background and subtracted from the data.

The depth of the fluorophore (indole moiety of tryptophan) was calculated according to published methods (*31*, *32*):

$$Z_{cF} = L_{c1} + [(-1/\pi C)\ln(F_1/F_2) - L_{21}^2]/2L_{21} \quad (4)$$

where Z_{cF} is the depth of fluorophore from the center of the bilayer, L_{c1} is the distance of the center of the bilayer from shallow quencher 1, L_{21} is the difference in depth between the two quenchers, and C is the two-dimensional quencher concentration in the plane of the membrane [molecule per unit area, (mole fraction of quencher lipid in total lipid)/70 Å^2]. F_1 and F_2 are normalized fluorescence intensities of samples containing quenchers located at known depths in the bilayer. The area of a PC molecule was taken to be 70 Å^2 (*33*) for calculation of the quencher concentration, and the average bromine distances from the center of the bilayer were taken to be 10.8 and 6.3 Å for the 6−7 Br-PC and 11−12 Br-PC, respectively (*34*).

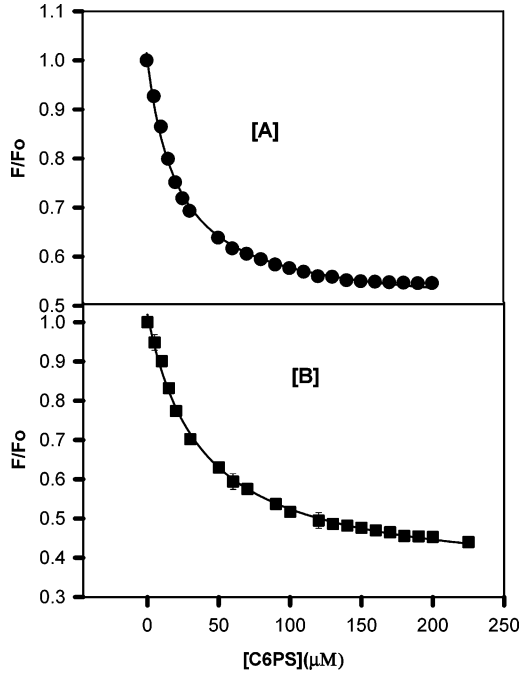

FIGURE 1: Binding of rFV_{a2} to soluble C6PS. The intrinsic fluorescence intensities of 0.2 μM FV_{a2} [wild type (A) and W(2063,2064)A mutant (B)] in 20 mM Tris, 150 mM NaCl, and 5 mM $CaCl_2$ (pH 7.5) were measured as a function of C6PS concentration at 24 °C to follow C6PS binding. The data were analyzed according to a simple stoichiometric binding model as described in eq 1 in Experimental Procedures, with parameters given in Table 1.

RESULTS

Binding of C6PS and Membranes to Mutant and Wild-Type rFV_{a2}. Figure 1 shows the change in the intrinsic fluorescence of wild-type (frame A) and W(2063,2064)A mutant (frame B) rFV_{a2} as a result of titrations with C6PS. The critical micelle concentration (CMC) of C6PS in the presence of 0.33 μM human FV_{a2}, which is close to the FV_{a2} concentration (0.2 μM) used in the experiment presented in Figure 1, was determined previously using quasi-elastic light scattering to be ca. 750 μM, well above the concentrations used in our titrations. Thus, the effect seen in Figure 1 was due to monomeric C6PS and not to this lipid in a micellar form. These data were described well by a simple independent binding site model assuming a stoichiometry of 1 (lines in Figure 1), yielding effective dissociation constants of 20 $\pm$ 1 and 37 $\pm$ 1 μM (average of three determinations) for wild-type and mutant rFV_{a2}, respectively. We note that native FV_a actually binds four C6PS molecules, not one (*14*). However, the data in Figure 1 do not permit an estimate of stoichiometry, so a value of 1 was assigned for consistency to both proteins. Assuming a stoichiometry of 4 for wild-type and 3 for mutant rFV_{a2} would yield numerically equivalent apparent site dissociation constants and would alter only the apparent fluorescence change per site. From these results, we see that elimination of the Trp^{2063} and Trp^{2064} residues from the rim of the putative binding pocket on the C2 domain did not have a large effect on binding of short chain C6PS. This is probably because binding to the C2 site is similar in affinity to binding to the three sites outside the C2 domain. Alternatively, or in addition, it could be because binding of C6PS to the C2 site is not dependent on these Trp residues.

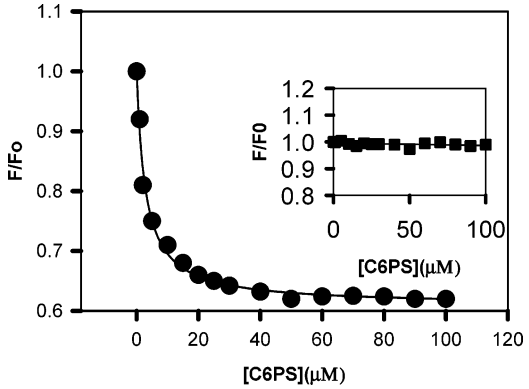

FIGURE 2: Binding of C6PS to rFV_{a2}-C2. The intrinsic fluorescence of 0.2 μM rFV_{a2}-C2 [wild type and W(2063,2064)A mutant (in the inset)] in the presence of increasing amounts of C6PS was recorded in a buffer containing 150 mM NaCl, 20 mM Tris, and 5 mM Ca^{2+} (pH 7.4) at 24 °C. The data were fit as described in the legend of Figure 1. The wild-type protein bound to C6PS with a K_d of 2.8 $\pm$ 0.3 μM [stoichiometry = 1 (*18*)], while the mutant did not bind (see the text).

The importance of these Trp residues to membrane binding has been previously documented using energy transfer from Trp to membrane-located dansylphosphatidylethanolamine (dansyl-PE) (*20*). Nonetheless, we determined the response of wild-type or Trp mutant rFV_{a2} intrinsic fluorescence to DOPC/DOPS (8:2) SUVs to document binding in a probe-free system (data not shown). As expected, wild-type rFV_{a2} bound tightly [K_d = 3 $\pm$ 1.5 nM; stoichiometry = 52 $\pm$ 5 lipids/protein (average of two determinations)], in reasonable agreement with our earlier results ($K_d \sim$ 1.6 nM) for native human FV_{a2} using the intrinsic fluorescence (*7*). However, both these K_d values are significantly higher than we reported for rFV_{a2} binding to 25% PS membranes using dansyl-PE to detect binding ($K_d \sim$ 0.3 nM) (*20*). The difference between these two results probably represents a probe effect; i.e., there could be a favorable interaction of the large dansyl probe with rFV_{a2}. By contrast to the wild-type protein, the W(2063,2064)A mutant protein showed no response of its intrinsic fluorescence to DOPC/DOPS membranes. This could be because the alanine substitutions for Trp^{2063} and Trp^{2064} interfered with binding, or because W(2063,2064)A rFV_{a2} does bind, but binding is invisible because insertion of the Trp residues into a membrane is responsible for the fluorescence response (increase) associated with addition of PS-containing membranes. The latter explanation can be eliminated since no energy transfer was observed between the remaining Trp residues in mutant rFV_{a2} and the dansyl group of headgroup-labeled dansyl-PE and by the lack of competition of W(2063,2064)A rFV_{a2} with wild-type rFV_{a2} binding (*20*).

Interaction of rFV_{a2}-C2 with Membranes and C6PS. To address the importance of the Trp residues for the occupancy of the C2 domain pocket by C6PS, we titrated wild-type and W(2063,2064)A mutant rFV_{a2}-C2 with C6PS, as shown in Figure 2 and its inset, respectively. The intrinsic fluorescence of the wild-type C2 domain decreased with addition of C6PS, as previously reported (*18*). With the stoichiometry fixed at 1, the effective K_d was 2.8 $\pm$ 0.3 μM (average of three determinations). The fluorescence of mutant rFV_{a2}-C2 did not change in response to addition of C6PS (inset of Figure 2). Again, this could be because the Trp residues are essential

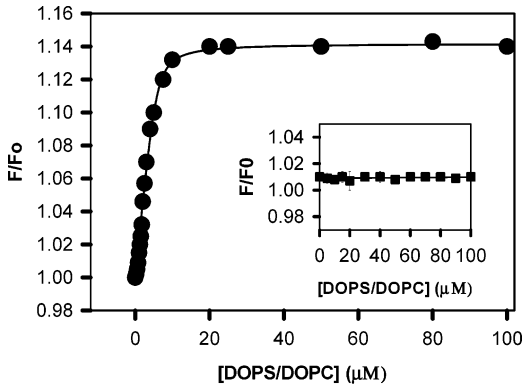

FIGURE 3: Binding of rFV_{a2}-C2 to DOPC/DOPS membranes. The intrinsic fluorescence of 0.2 μM rFV_{a2}-C2 was measured in a buffer containing 150 mM NaCl, 20 mM Tris, and 5 mM Ca^{2+} (pH 7.4) at 24 °C. Proteins were titrated with DOPC/DOPS (80:20) SUV membranes. Solid lines through the data show the results of fitting the data (see Figure 1) to estimate a K_d of 9 ± 2.5 nM, with a stoichiometry of 46 ± 5 lipids/site. The inset shows the titration of W(2063, 2064)A mutant rFV_{a2}-C2 with DOPC/DOPS membranes (80:20).

for binding of C6PS or because they are essential for the fluorescence response to binding. We turned to a direct measurement of binding, equilibrium dialysis (*13*, *14*, *18*), to resolve this ambiguity. With this method, we confirmed that <0.1 molecule of C6PS bound to mutant rFV_{a2}-C2 (variability of the measurement is <2%), while 1 molecule bound to the wild-type protein, as previously reported (*18*). These results unambiguously demonstrate that Trp^{2063} and Trp^{2064} are essential for binding of C6PS to the C2 domain binding pocket.

We asked next whether Trp^{2063} and Trp^{2064} are needed for binding of rFV_{a2}-C2 to a PS-containing membrane. Figure 3 shows that the wild-type rFV_{a2}-C2 domain binds with high affinity to PS-containing membranes. This agrees qualitatively with our earlier report (*18*). However, our current results indicate that rFV_{a2}-C2 binds more tightly to PS-containing membranes than we previously reported. Because of this discrepancy, we repeated this titration three times with two different protein preparations. All experiments were consistent and gave an average site dissociation constant k_d of 9 ± 2.5 nM, with a stoichiometry of 46 ± 5 lipids/site (the standard error is the average of the regression errors obtained from each experiment). Upon revisiting the original data from our earlier titration, we discovered that the background scattering had been inadequately taken into account, leading to overestimation of the K_d for this interaction (*18*). No change in the fluorescence of W(2063,2064)A mutant rFV_{a2}-C2 occurred upon addition of PS-containing membranes (inset of Figure 2). This means either that the deleted Trp residues are needed for binding of the C2 domain to PS-containing membranes or that they are needed for the change in fluorescence upon binding to membranes. This latter explanation was ruled out by the fact that a remaining Trp residue in rFV_{a2}-C2 located close to the lipid binding pocket (*18*) did not transfer energy to dansyl-labeled phosphatidylethanolamine in DOPC/DOPS SUVs (*20*).

Functional Significance of Binding of PS to the C2 Pocket. Having established the importance of Trp^{2063} and Trp^{2064} for the occupancy of the C2 binding pocket by C6PS (Figure

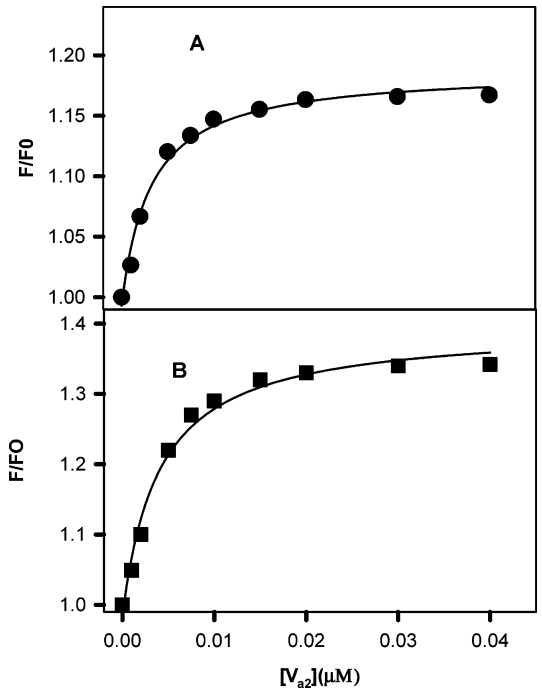

FIGURE 4: Binding of rFV_{a2} to DEGR-X_a in the presence of C6PS. Binding of rFV_{a2} to factor X_a was detected by the change in the fluorescence intensity of a fluorescence probe, DEGR, covalently bound to the active site of factor X_a. Small aliquots of rFV_{a2} [wild type (A) and mutant (B)] were added to DEGR-X_a [1 nM in 5 mM Ca^{2+}, 20 mM Tris, 150 mM NaCl, and 400 μM C6PS (pH 7.5)]. The lines through the data were obtained by least-squares regression to a single-site binding model with best fit K_d values given in Table 1.

2), we now asked whether occupancy of this site is required for assembly of the prothrombinase complex and/or cofactor activity. Clearly, binding of FV_{a2} to FX_a is essential for expression of cofactor activity. Figure 4 presents isotherms, obtained in the presence of 400 μM C6PS, for the binding of wild-type (frame A) and mutant (frame B) rFV_{a2} to FX_a. We have previously shown that the complex formed under these conditions has a stoichiometry of 1:1 (X_a:rFV_{a2}), contains <17 C6PS molecules, and activates prothrombin at nearly the same rate as the membrane-assembled complex (*10*). With the stoichiometry fixed at 1:1, we obtained a best fit description of the data in Figure 4 with K_d values of 3.1 ± 0.4 and 4.0 ± 0.6 nM for wild-type and mutant rFV_{a2}, respectively. Because C6PS does not occupy the C2 site on the mutant rFV_{a2}, these results establish that C6PS occupancy of the C2 site has no effect on the interaction between factors X_a and V_{a2}. Thus, the C2 site does not regulate association of rFV_{a2} and FX_a.

The final question posed in the introductory section was whether the W(2063,2064)A mutation in the C2 domain might alter the activity of the rFX_a−FV_a complex in the presence of C6PS. In the absence of membranes or C6PS, the prothrombinase activity of 1 nM FX_a was 0.25 nM II_a s^{-1} (nM FX_a)$^{-1}$, but in the presence of 50 nM wild-type or mutant rFV_{a2}, the prothrombinase activity of 1 nM X_a was 5.8 ± 0.4 and 6 ± 0.3 nM II_a s^{-1} (nM FX_a)$^{-1}$, respectively. Although activity is quite low in the absence of C6PS (compare to roughly 170 nM II_a/s in the presence of either wild-type or mutant rFV_{a2} and C6PS; Figure 5), this result clearly shows that the W(2063,2064)A mutation in the C2 domain does not impact cofactor activity in the absence of

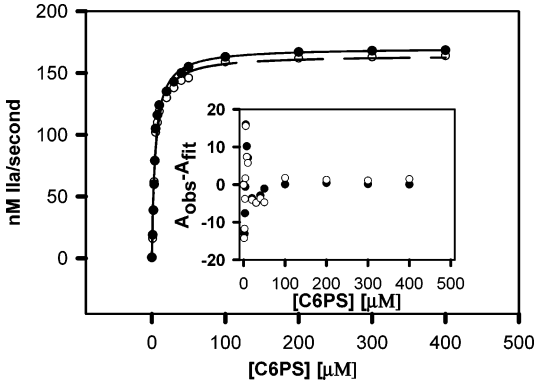

FIGURE 5: Effect of C6PS on the cofactor activity of wild-type and mutant W(2063,2064)A FV_{a2} during prothrombin activation. Initial rates of active site formation as monitored by DAPA fluorescence were determined as a function of added C6PS concentration. Stopped-flow fluorescence measurements were performed at 37 °C at various concentrations of C6PS in a buffer containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, and 5 mM $CaCl_2$. Reaction mixtures contained 1 μM prothrombin, 5 μM DAPA, and 1 nM FX_a, along with either 5 nM wild-type rFV_{a2} (●) or 5 nM mutant rFV_{a2} (○). Lines drawn through the data result from least-squares regression of the data to a hyperbola (solid line for the wild type and dashed line for the mutant). The inset shows the residuals from these regressions, using the same symbols.

lipid. Both wild-type and mutant FV_{a2} support the same prothrombin activating activity at saturating FV_a concentrations, but in the presence of 25% PS membranes, a 10-fold higher concentration of mutant rFV_{a2} is needed to elicit the same activity from FX_a as from wild-type rFV_{a2} (*20*). This could be because of a difference in the interaction between wild-type and mutant FV_a and factor X_a in the presence of the membrane or to the difference in membrane binding of these two forms of FV_{a2}. The presence of a membrane makes it difficult to distinguish between these two possibilities. C6PS triggers assembly of a fully active $FV_{a2}-FX_a$ complex in the absence of a membrane (*17*). Because of this, FV_a cofactor activity is best described in terms of the enhancement of FX_a activity due to the presence of the cofactor, in the presence of C6PS (*14*). Table 1 contains the cofactor activities of native, recombinant wild-type, or mutant FV_{a2} obtained by the assay described in Experimental Procedures, and these are all equal within experimental uncertainty. Clearly, cofactor activity was unaffected by the W(2063,2064)A mutation.

Next we consider the variation in prothrombinase activity in the presence of both wild-type (●) and W(2063,2064)A mutant (○) FV_{a2} with addition of increasing concentrations of C6PS (Figure 5). The results in Figure 5 show that both the wild-type and mutant W(2063,2064)A FVa2 behave nearly identically as a function of C6PS concentration. The lines through these data show the result of fitting them to a hyperbola (the expected response if the effect of C6PS on activity was caused by simple, single-site binding to either FX_a or FV_{a2}), yielding effective K_d values and saturation values for wild-type [4.6 ± 0.4 μM, 170 ± 3 nM IIa s^{-1} (nM FX_a)$^{-1}$] and mutant [4.5 ± 0.4 μM, 164 ± 3 nM IIa s^{-1} (nM FX_a)$^{-1}$] rFV_{a2}. Clearly, the mutant and wild-type proteins behaved identically, within experimental uncertainty, in their responses to C6PS. However, careful examination of the fits of the single-site binding model to these data reveals that this model is not an adequate description, since the observed responses are likely a combination of effects of C6PS on both rFV_{a2} and FX_a. This is quite evident in the systematic errors seen in the plot of residuals given in the inset of Figure 5. The moderate increase in activity between ~20 and 100 μM C6PS probably reflects relatively weak binding [$k_d \sim 70$ μM (*35*)] to FX_a. However, the sharp initial increase in activity at low C6PS concentrations means that binding of C6PS to rFV_{a2} ($K_d \sim 20$ μM; Figure 1) has a dramatic influence on activity. This clearly indicates that binding of C6PS to some site on FV_{a2} is involved in regulation of either FV_{a2} cofactor activity or binding of FV_{a2} to FX_a, although the data presented here indicate that this site is not the C2 domain site.

Penetration of C2 Tryptophans into the Membrane. The C2 domain has three Trp residues (Trp^{2063}, Trp^{2064}, and Trp^{2068} at the top of the lipid binding pocket). Since Trp^{2068} is roughly 22 Å above Trp^{2063} and Trp^{2064} (*22*), it is unlikely to penetrate the membrane interface. We have used the fluorescence of Trp^{2063} and Trp^{2064} to judge their location in the bilayer, using quenching of Trp fluorescence based on the parallax method (*31*). We used two dibromo lipids (6−7 and 11−12 Br-PC) as quenchers, with the results shown in Figure 6. Trp fluorescence was quenched more by the 6−7 Br-PC than by the 11−12 Br-PC, suggesting that the Trp^{2063} and Trp^{2064} residues are closer to C_6 and C_7 in the lipid acyl chain than to C_{11} and C_{12}. Using published equations (*31*) as described in Experimental Procedures (eq 4), we estimated from these results that the indole moiety of one or both of the Trp^{2063} and Trp^{2064} residues is located ~9 Å from the center of the bilayer, and thus penetrates the interface region of the bilayer.

DISCUSSION

In this paper, we set out to address three questions that relate to the mechanism and functional consequences of binding of FV_a to membranes. These questions have been answered in the Results, and here we comment on the insights these answers offer about the mechanism of membrane binding and regulation of FV_a.

Membrane Binding Mechanism. We pointed out previously that most of the free energy of binding of rFV_{a2} to a PS-containing membrane is provided by binding of the rFV_a-C2 domain (*20*). However, this initial analysis was made using a membrane-located fluorescent lipid probe, dansyl-PE. Because probe molecules can perturb the events being examined, we judged it wise to reexamine, using a probe-free measurement, the extent to which the rFV_a-C2 domain might contribute to binding of whole rFV_{a2}. The difference in the standard free energies (RT ln K_d) of rFV_{a2}-C2 and rFV_{a2} binding to 20% PS membranes, as determined here using intrinsic Trp fluorescence, is very small ($\Delta\Delta G_{bind} \sim 650 \pm 340$ cal/mol, i.e., ~1 ± 0.6 kT). Our previous estimate of this quantity was ~1800 ± 130 cal/mol, i.e., ~3 ± 0.2 kT (*20*), with the difference in the estimates apparently attributable to the dansyl-PE probe. Both estimates suggest that lipid binding sites outside of the C2 domain make little or no contribution to the thermodynamics of membrane binding. While the free energy of PS association with other sites may be insufficient for membrane binding, even 1−3 kT can suffice to trigger protein conformational shifts that are important for function.

Table 1: Summary of Results with Wild-Type and W(2063,2064)A rFV_{a2}-C2 and FV_{a2}[a]

protein	K_d(membrane) (nM)	stoichiometry, membrane (no. of lipids/site)	K_d (C6PS) (μM)	K_d (FX_a) (nM)	cofactor activity[b]
human plasma V_{a2}	1.6 (7)	26 (7)	14.5 ± 1 (3)	ND[c]	15 ± 1 (3)
$rHFV_{a2}$	3 ± 1.5 (2)	52 ± 5 (2)	20 ± 1 (3)	3.1 ± 0.4 (3)	17 ± 1 (3)
$rHFV_{a2}$ W(2063,2064)A	ND[c]	ND[c]	37 ± 1 (2)	4.0 ± 0.6 (3)	16 ± 1 (3)
rC2	9 ± 2.5 (3)	46 ± 5 (3)	2.8 ± 0.3 (3)	NM[d]	NA[e]
rC2 W(2063,2064)A	ND[c]	ND[c]	ND[c]	NM[d]	NA[e]

[a] Average values and average standard errors from two or three determinations (in parentheses) are given. [b] Determined as described by eq 3 in Experimental Procedures. [c] Binding not detectable. [d] This measurement was not performed. [e] Measurement not applicable.

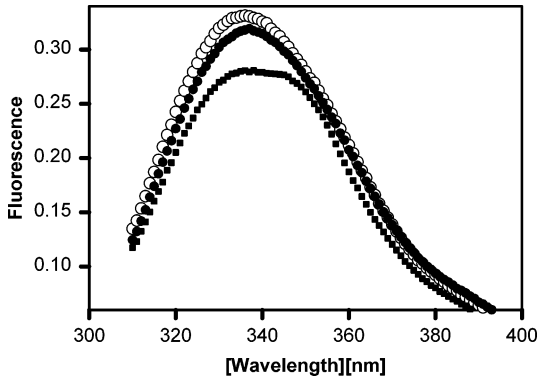

FIGURE 6: Depth of penetration of Trp^{2063} and Trp^{2064} into the membrane. Corrected intrinsic Trp fluorescence spectra at 22 °C of wild-type rFV_{a2}-C2 (200 nM) bound to PC/PS SUVs (100 μM) were collected in the absence (○) and presence of different quenchers [11−12 Br-PC (●) and 6−7 Br-PC (■)]. Samples were in buffers containing 20 mM Tris, 150 mM NaCl, and 5 mM Ca^{2+}, and spectra were collected and analyzed as described in Experimental Procedures.

We turn now to the mechanism by which the C2 domain lipid binding pocket mediates membrane binding. Using C6PS, we have been able to show that Trp^{2063} and Trp^{2064} are required for occupancy of the C2 domain lipid binding pocket. Loss of these Trps also obliterates membrane binding (*20*), which derives almost exclusively from the C2 domain. These observations suggest three things. First, while replacement of these Trp residues with Ala residues did not produce a measurable shift in rFV_{a2}-C2 domain secondary structure (*20*), clearly the loss of these tryptophans altered in a functionally significant way the local conformation in the neighborhood of the lipid binding pocket. We suspect that Trp^{2063} and Trp^{2064} stabilize this pocket by occluding its opening (*22*), and that their absence may lead to collapse of the pocket. Occupancy of this pocket by a lipid molecule [presumably but not necessarily PS (*18*)] would displace these tryptophans. When the binding pocket is occupied by C6PS, the tryptophans would be displaced into water, leading to the observed loss of intrinsic fluorescence intensity (*18*). If displaced by a membrane-located lipid, the Trp residues would penetrate the membrane, leading to the observed increase in fluorescence intensity (*18*). Using phosphatidylcholine molecules labeled in specific positions in their acyl chains, we have confirmed that Trp^{2063} and/or Trp^{2064} indole groups penetrate within 9 Å of the membrane center during association with a membrane. This places the Trp backbone just at the membrane interface, which would provide much of the free energy of FV_a membrane interaction (*21*), although occupancy of the C2 domain lipid binding pocket presumably makes a contribution as well. This model of C2 domain membrane binding presumes no large changes in protein structure, just small local changes. This is consistent with the observation that C6PS and membrane binding do not cause a secondary structure change in the C2 domain, although a small conformational shift is suggested by an observed change in thermal stability (*18*).

Role of C2 Domain Lipid Binding in Regulation of Prothrombinase. On the basis of the observations that rFV_{a2} and the W(2063,2064)A rFV_{a2} mutant are equally active at saturating C6PS concentrations (Figure 5) and that this mutation blocks binding of C6PS to the C2 domain (Figure 2), we conclude that C6PS occupancy of the C2 lipid binding site does not alter FX_a-FV_a complex activity. Because the W(2063,2064)A rFV_{a2} mutation had no effect on the assembly of the rFV_{a2}−DEGR-X_a complex (Figure 4), the C2 domain lipid binding site seems not to regulate either cofactor activity or complex assembly. Our previous observation that the W(2063,2064)A rFV_{a2} mutation alters the response of prothrombinase activity to either FX_a or rFV_{a2} (*20*) must therefore reflect only the defect in membrane binding associated with this mutation. This conclusion is made possible only by the results obtained here using C6PS to activate both FX_a and rFV_{a2} in solution, free of the complications of a membrane surface. This disconnect between the roles of PS in membrane binding and in prothrombinase activity is consistent with previous reports that acidic lipids other than PS support FV_a membrane binding (*36*) and that even basic lipid membranes support prothrombin activation as long as they contain a small amount of PS (*37*).

This leads to the question of whether there is a PS binding regulatory site elsewhere on FV_a. C6PS binds to three sites on FV_a outside of the C2 domain (*14*, *18*). Our results (Figure 5) suggest that one (or more) of these sites is a regulatory site for either X_a-V_a tight association, FV_a cofactor activity, or both. Additional work will be needed to locate and determine the function of this site, although recent results show that mutations in the C1 domain of rFV_{a2} have functional consequences (*38*) that may be worth exploring further.

REFERENCES

1. Kane, W. H., and Davie, E. W. (1988) Blood coagulation factors V and VIII: Structural and functional similarities and their relationship to hemorrhagic and thrombotic disorders, *Blood 71*, 539−555.
2. Mann, K. G., Nesheim, M. E., Church, W. R., Haley, P., and Krishnaswamy, S. (1990) Surface-dependent reactions of the vitamin K-dependent enzyme complexes, *Blood 76*, 1−16.
3. Esmon, C. T. (1979) The subunit structure of thrombin-activated factor V. Isolation of activated factor V, separation of subunits, and reconstitution of biological activity, *J. Biol. Chem. 254*, 964−973.
4. Dahlback, B. (1980) Human coagluation factor V purification and thrombin-catalyzed activation, *J. Clin. Invest. 66*, 583−591.

5. Kane, W. H., and Majerus, P. W. (1981) Purification and characterization of human coagulation factor V, *J. Biol. Chem. 256*, 1002−1007.
6. Ortel, T. L., Quinn-Allen, M. A., Keller, F. G., Peterson, J. A., Larocca, D., and Kane, W. H. (1994) Localization of functionally important epitopes within the second C-type domain of coagulation factor V using recombinant chimeras, *J. Biol. Chem. 269*, 15898−15905.
7. Kim, S. W., Ortel, T. L., Quinn-Allen, M. A., Yoo, L., Worfolk, L., et al. (1999) Partial glycosylation at asparagine-2181 of the second C-type domain of human factor V modulates assembly of the prothrombinase complex, *Biochemistry 38*, 11448−11454.
8. Rosing, J., Bakker, H. M., Christella, M., Thomassen, L. G. D., Hemker, H. C., and Tans, G. (1993) Characterisation of two forms of human factor Va with different cofactor activities, *J. Biol. Chem. 268*, 21130−21136.
9. Koppaka, V., Talbot, W. F., Zhai, X., and Lentz, B. R. (1997) Roles of factor Va heavy and light chains in protein and lipid rearrangements associated with the formation of a bovine factor Va-membrane complex, *Biophys. J. 73*, 2638−2652.
10. Majumder, R., Weinreb, G., Zhai, X., and Lentz, B. R. (2002) Soluble phosphatidylserine triggers assembly in solution of a prothrombin-activating complex in the absence of a membrane surface, *J. Biol. Chem. 277*, 29765−29773.
11. Boskovic, D. S., Giles, A. R., and Nesheim, M. E. (1990) Studies of the role of factor Va in the factor Xa-catalyzed activation of prothrombin, fragment 1.2-prethrombin-2, and dansyl-L-glutamyl-glycyl-L-arginine-meizothrombin in the absence of phospholipid, *J. Biol. Chem. 265*, 10497−10505.
12. Krishnaswamy, S. (1990) Prothrombinase complex assembly. Contributions of protein−protein and protein−membrane interactions toward complex formation, *J. Biol. Chem. 265*, 3708−3718.
13. Banerjee, M., Drummond, D. C., Srivastava, A., Daleke, D., and Lentz, B. R. (2002) Specificity of the phospholipid regulatory sites on human Factor Xa, *Biochemistry 41*, 7751−7762.
14. Zhai, X., Srivastava, A., Drummond, D. C., Daleke, D., and Lentz, B. R. (2002) Phosphatidylserine Binding Alters the Conformation and Specifically Enhances the Cofactor Activity of Bovine Factor V(a), *Biochemistry 41*, 5675−5684.
15. Banerjee, M., Majumder, R., Weinreb, G., Wang, J., and Lentz, B. R. (2002) Role of Procoagulant Lipids in Human Prothrombin Activation. 2. Soluble Phosphatidylserine Upregulates and Directs Factor X(a) to Appropriate Peptide Bonds in Prothrombin, *Biochemistry 41*, 950−957.
16. Srivastava, A., Wang, J., Majumder, R., Rezaie, A. R., Stenflo, J., et al. (2002) Localization of phosphatidylserine binding sites to structural domains of factor Xa, *J. Biol. Chem. 277*, 1855−1863.
17. Majumder, R., Wang, J., and Lentz, B. R. (2003) Effects of Water Soluble Phosphotidylserine on Bovine Factor X(a): Functional and Structural Changes Plus Dimerization, *Biophys. J. 84*, 1238−1251.
18. Srivastava, A., Quinn-Allen, M. A., Kim, S. W., Kane, W. H., and Lentz, B. R. (2001) Soluble phosphatidylserine binds to a single identified site in the C2 domain of human factor Va, *Biochemistry 40*, 8246−8255.
19. Kim, S. W., Quinn-Allen, M. A., Camp, J. T., Macedo-Ribeiro, S., Fuentes-Prior, P., et al. (2000) Identification of functionally important amino acid residues within the C2-domain of human factor V using alanine-scanning mutagenesis, *Biochemistry 39*, 1951−1958.
20. Peng, W., Quinn-Allen, M. A., Kim, S. W., Alexander, K. A., and Kane, W. H. (2004) Trp^{2063} and Trp^{2064} in the factor Va C2 domain are required for high-affinity binding to phospholipid membranes but not for assembly of the prothrombinase complex, *Biochemistry 43*, 4385−4393.
21. Yau, W. M., Wimley, W. C., Gawrisch, K., and White, S. H. (1998) The preference of tryptophan for membrane interfaces, *Biochemistry 37*, 14713−14718.
22. Macedo-Ribeiro, S., Bode, W., Huber, R., Quinn-Allen, M. A., Kim, S. W., et al. (1999) Crystal structures of the membrane-binding C2 domain of human coagulation factor V, *Nature 402*, 434−439.
23. Lee, J., and Lentz, B. R. (1997) Outer leaflet-packing defects promote poly(ethylene glycol)-mediated fusion of large unilamellar vesicles, *Biochemistry 36*, 421−431.
24. Barenholz, Y., Gibbes, D., Litman, B. J., Goll, J., Thompson, T. E., and Carlson, R. D. (1977) A simple method for the preparation of homogeneous phospholipid vesicles, *Biochemistry 16*, 2806−2810.
25. Chen, P. S., Jr., Toribara, T. Y., and Warner, H. (1956) Microdetermination of Phosphorus, *Anal. Chem. 28*, 1756−1758.
26. Krishnaswamy, S., and Mann, K. G. (1988) The binding of factor Va to phospholipid vesicles, *J. Biol. Chem. 263*, 5714−5723.
27. Jameson, G. W., Roberts, D. V., Adams, R. W., Kyle, W. S., and Elmore, D. T. (1973) Determination of the operational molarity of solutions of bovine α-chymotrypsin, trypsin, thrombin and factor Xa by spectrofluorimetric titration, *Biochem. J. 131*, 107−117.
28. Husten, E. J., Esmon, C. T., and Johnson, A. E. (1987) The active site of blood coagulation factor Xa. Its distance from the phospholipid surface and its conformational sensitivity to components of the prothrombinase complex, *J. Biol. Chem. 262*, 12953−12961.
29. Wu, J. R., Zhou, C., Majumder, R., Powers, D. D., Weinreb, G., and Lentz, B. R. (2002) Role of Procoagulant Lipids in Human Prothrombin Activation. 1. Prothrombin Activation by Factor X(a) in the Absence of Factor V(a) and in the Absence and Presence of Membranes, *Biochemistry 41*, 935−949.
30. Nesheim, M. E., and Mann, K. G. (1983) The kinetics and cofactor dependence of the two cleavages involved in prothrombin activation, *J. Biol. Chem. 258*, 5386−5391.
31. Chattopadhyay, A., and London, E. (1987) Parallax method for direct measurement of membrane penetration depth utilizing fluorescence quenching by spin-labeled phospholipids, *Biochemistry 26*, 39−45.
32. Chattopadhyay, A., and McNamee, M. G. (1991) Average membrane penetration depth of tryptophan residues of the nicotinic acetylcholine receptor by the parallax method, *Biochemistry 30*, 7159−7164.
33. Lewis, B. A., and Engelman, D. M. (1983) Lipid Bilayer Thickness Varies Linearly with Acyl Chain-Length in Fluid Phosphatidylcholine Vesicles, *J. Mol. Biol. 166*, 211−217.
34. McIntosh, T. J., and Holloway, P. W. (1987) Determination of the depth of bromine atoms in bilayers formed from bromolipid probes, *Biochemistry 26*, 1783−1788.
35. Koppaka, V., Wang, J., Banerjee, M., and Lentz, B. R. (1996) Soluble phospholipids enhance factor Xa-catalyzed prothrombin activation in solution, *Biochemistry 35*, 7482−7491.
36. Cutsforth, G. A., Koppaka, V., Krishnaswamy, S., Wu, J. R., Mann, K. G., and Lentz, B. R. (1996) Insights into the complex association of bovine factor Va with acidic-lipid-containing synthetic membranes, *Biophys. J. 70*, 2938−2949.
37. Gerads, I., Govers-Riemslag, J. W., Tans, G., Zwaal, R. F., and Rosing, J. (1990) Prothrombin activation on membranes with anionic lipids containing phosphate, sulfate, and/or carboxyl groups, *Biochemistry 29*, 7967−7974.
38. Saleh, M., Peng, W., Quinn-Allen, M. A., Macedo-Ribeiro, S., Fuentes-Prior, P., et al. (2004) The factor V C1 domain is involved in membrane binding: Identification of functionally important amino acid residues within the C1 domain of factor V using alanine scanning mutagenesis, *Thromb. Haemostasis 91*, 16−27.

BI047962T