BIOCHEMISTRY
including biophysical chemistry & molecular biology

Article

pubs.acs.org/biochemistry

Phosphatidylserine-Induced Factor Xa Dimerization and Binding to Factor Va Are Competing Processes in Solution

Rinku Majumder,[†] Tilen Koklic,[‡] Alireza R. Rezaie,[§] and Barry R. Lentz*,[†]

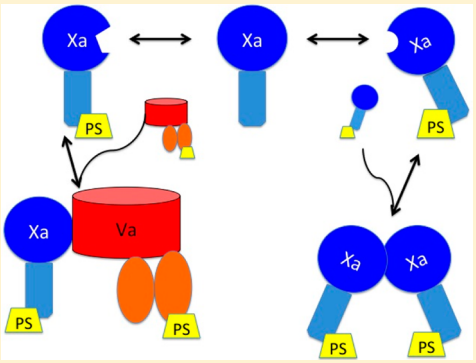
[†]Department of Biochemistry and Biophysics and Program in Molecular and Cellular Biophysics, CB #7260, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina 27599-7260, United States

[‡]Laboratory of Biophysics (EPR center), Jozef Stefan Institute, Jamova 39, 1000 Ljubljana, Slovenia

[§]Department of Biochemistry and Molecular Biology, Saint Louis University School of Medicine, Saint Louis, Missouri 63104, United States

Supporting Information

ABSTRACT: A soluble, short chain phosphatidylserine, 1,2-dicaproyl-*sn*-glycero-3-phospho-L-serine (C6PS), binds to discrete sites on FXa, FVa, and prothrombin to alter their conformations, to promote FXa dimerization (K_d ~ 14 nM), and to enhance both the catalytic activity of FXa and the cofactor activity of FVa. In the presence of calcium, C6PS binds to two sites on FXa, one in the epidermal growth factor-like (EGF) domain and one in the catalytic domain; the latter interaction is sensitive to Na^+ binding and probably represents a protein recognition site. Here we ask whether dimerization of FXa and its binding to FVa in the presence of C6PS are competitive processes. We monitored FXa activity at 5, 20, and 50 nM FXa while titrating with FVa in the presence of 400 μM C6PS and 3 or 5 mM Ca^{2+} to show that the apparent K_d of FVa−FXa interaction increased with an increase in FXa concentration at 5 mM Ca^{2+}, but the K_d was only slightly affected at 3 mM Ca^{2+}. A mixture of 50 nM FXa and 50 nM FVa in the presence of 400 μM C6PS yielded both Xa homodimers and Xa·Va heterodimers, but no FXa dimers bound to FVa. A mutant FXa (R165A) that has reduced prothrombinase activity showed both weakened dimerization (K_d ~ 147 nM) and weakened FVa binding (apparent K_d values of 58, 92, and 128 nM for 5, 20, and 50 nM R165A FXa, respectively). Native gel electrophoresis showed that the GLA-EGF_{NC} fragment of FXa (lacking the catalytic domain) neither dimerized nor formed a complex with FVa in the presence of 400 μM C6PS and 5 mM Ca^{2+}. Our results demonstrate that the dimerization site and FVa-binding site are both located in the catalytic domain of FXa and that these sites are linked thermodynamically.



Thrombin production, located at the convergence of the three legs of the traditional blood coagulation cascade, is arguably the central reaction of blood coagulation, and thrombin is the central metabolite of blood coagulation.[1] A complex between two soluble clotting factors, FXa and FVa, catalyzes the conversion of prothrombin to thrombin. This prothrombin-activating complex (or prothrombinase) assembles and functions in vivo on activated platelet membranes.[2,3] Phosphatidylserine (PS), which, along with phosphatidylethanolamine, is exposed on activated platelet membranes,[4] appears to be the platelet lipid required for efficient blood coagulation.[5] To examine the molecular details of regulation of FXa and FVa by PS, we introduced a soluble (CMC ~ 650−800 μM depending on exact conditions) form of PS [1,2-dicaproyl-*sn*-glycero-3-phospho-L-serine (C6PS)] that has allowed us to locate the PS regulatory sites on FXa[6] and FVa[7] and to establish the regions of these molecules affected by occupancy of these sites by PS. While C6PS does not exist in vivo, it has proven to be a very important tool in our efforts to reveal the molecular details of PS regulation of these two coagulation factors and of blood coagulation in general.

FXa has an N-terminal GLA module essential for membrane binding and two highly Cys-rich domains (epidermal growth factor-like cassettes, EGF_N and EGF_C) that link the Gla module to the C-terminal catalytic domain containing the catalytic triad (H/D/S) and FVa interaction sites.[8−11] The EGF cassette pair binds a molecule of C6PS, which is soluble up to ~700−800 μM under our conditions, to form in solution an inactive dimer[12,13] or a 60-fold activated monomer.[14,15] Binding of C6PS to the EGF pair is specific for L-phosphatidylserine,[16] triggers binding of a second molecule of C6PS to a Ca^{2+}- and Na^+-dependent site in the catalytic domain, alters the active site,[6,14] and elicits changes in FXa activity[15] only when the GLA and EGF domains are linked by C6PS binding.[6]

FVa is a heterodimer[17] composed of a heavy chain (FVa-HC; A1 and A2 domains; M_r = 105000 in humans) and a light chain (FVa-LC; A3, C1, and C2 domains; M_r = 74000 or 71000).[18,19] Heterogeneity of the light chain is seen in both bovine and human FVa[18−20] and is due to alternative glycosylation at

Received: September 11, 2012
Revised: October 30, 2012
Published: December 7, 2012

Asn^{2181} in the C2 domain.[21] The species that is glycosylated at Asn^{2181} is termed factor Va_1, while the species lacking glycosylation at this position is factor Va_2. FVa_2 differs from FVa_1 in its ability to bind tightly to FXa in the presence of phosphatidylserine (PS)-containing membranes or a short chain PS (C6PS).[22,23] For this reason, all of our studies are performed with FVa_2 that, for the sake of simplicity, will be termed FVa. FVa binds four molecules of C6PS, two molecules to the FVa-HC moiety and two molecules to the FVa-LC moiety.[24] Binding of PS to a Ca^{2+} site in FVa-LC is required for tight membrane association,[25] while binding to a site in the C1 domain is required for tight association between FXa and FVa on a membrane or in solution.[7] We have not identified a function for the C6PS-binding sites in FVa-HC.

The FVa-binding site in FXa is thought to be located in the catalytic domain (residues 162−169,[8,9] residues 185−189,[26] and residues 230−245[27,28]) and is thermodynamically linked to a Na^+-binding site and the active site[29] (numbering using the chymotrypsin system). Binding of C6PS to a regulatory site in the EGF_{NC} pair [ideally attached to the Gla (GLA) domain] is also linked to the Na^+-binding and active sites.[6] Binding of C6PS to the regulatory site also triggers formation of an inactive FXa dimer in which lysine 90 in the catalytic domain becomes buried.[13] Because binding of C6PS to the regulatory site is linked to both the FXa dimer and the Na^+/FVa-binding regions, we speculated that these binding regions might be directly linked within the catalytic domain. Here we test this hypothesis and show that FVa binding and dimerization of FXa are competitive processes. Next, we show that the GLA-EGF_{NC} fragment of FXa (lacking the catalytic domain) does not contribute to dimerization or to interaction with FVa, in the presence of C6PS, thus locating both interactions exclusively in the catalytic domain. Finally, we show that a FXa mutation in the catalytic domain that interferes with FVa binding (R165A[8]) also interferes with FXa dimer formation, thus locating the linkage between these binding regions in the catalytic domain. Because these regions are greatly separated from each other in current molecular models of FXa, our results imply either that a linkage path exists between them or that PS triggers formation of mutually exclusive FXa-binding or FVa-binding conformations of the FXa catalytic domain.

MATERIALS AND METHODS

Materials. The sodium salt of 1,2-dicaproyl-*sn*-glycero-3-phospho-L-serine (C6PS) was purchased from Avanti Polar Lipids (Birmingham, AL). Human prothrombin and FXa were obtained from Hematologic Technologies Inc. (Essex Junction, VT). FXa was assayed prior to each experiment using an active site titration standard[30] to measure its activity. The R165A mutant of FXa (chymotrypsin numbering[11]) was expressed and purified as described in ref 8, and the GLA-EGF_{NC} fragment of FXa was provided by J. Stenflo and prepared as described in ref 31. Purified human factor V was prepared from fresh frozen human plasma (American Red Cross Center, Durham, NC) and then activated to FVa as described previously.[18,32] The glycosylated form of human FVa (here termed simply FVa) was isolated as described previously.[21,23] The activity of FVa was assayed using either 25/75 PS/DOPC vesicles[33]or C6PS.[25] Dansylarginine-*N*-(3-ethyl-1,5-pentanediyl) amide (DAPA) was obtained from Hematologic Technologies Inc. All other chemicals were ACS reagent grade; all solvents were HPLC grade.

Short Chain Phospholipid Sample Preparation. Chloroform was removed from measured amounts of a C6PS stock using a stream of nitrogen, and the lipid sample was dissolved in cyclohexane and lyophilized overnight. The lyophilized lipid was then suspended in an appropriate amount of buffer to reach a desired concentration and used within 1−2 days.

Determination of the Critical Micelle Concentration (CMC). Changes in pyrene florescence were used to document the CMC of C6PS.[34] Pyrene fluorescence has been shown to detect micelles with aggregation numbers as small as 10, as validated by comparison to determination of the CMC by surface tension and conductivity measurements.[34,35]

Native Polyacrylamide Gel Electrophoresis. Separation of protein mixtures and protein standards on native gels of varying percentages allows the determination of both the charge and the mass of the sample proteins. The graphic analysis used is known as the Ferguson Plot.[36] The details of the procedures are described elsewhere.[12,23,30,36] Six marker proteins (myosin, BSA, trypsin inhibitor, α-lactalbumin, chicken albumin, and carbonic anhydrase) were subjected to native polyacrylamide gel electrophoresis at varying degrees of gel cross-linking, and a Ferguson plot (retardation coefficient vs molecular weight) was constructed on the basis of their mobilities. FXa, FVa, or their mixture (50 nM) in 50 mM Tris, 150 mM NaCl, 5 mM $CaCl_2$ (pH 7.5), and 0.6% PEG (buffer A) was incubated at 37 °C for 10 min in the presence and absence of 400 μM C6PS. These samples were run together with the marker proteins in 5 or 8% polyacrylamide gels in a Bio-Rad (Hercules, CA) Mini-Protean II minigel apparatus and stained with colloidal Coomassie blue.[37] The molecular masses of Xa, Va, and their complex were obtained by comparison with the marker proteins.

Prothrombin Activation in the Presence of C6PS. The rate of prothrombin activation was derived from the time-dependent change in DAPA fluorescence as it bound to the activation products.[30] Stopped-flow measurements were performed at 37 °C using an SLM-Aminco Milliflow stopped-flow reactor (Spectronic Instruments, Inc., Rochester, NY) attached to the Spex FluoroLog-3 spectrofluorometer (Jobin Yvon Inc., Edison, NJ), as described previously.[38] Reactions were initiated by rapidly mixing equal volumes (400 μL) of the contents of the two driving syringes. One syringe contained prothrombin and DAPA in buffer A (1:5 substrate:DAPA ratio), and the other syringe contained preassembled prothrombinase (FXa, FVa, and C6PS) in the same buffer. The FVa concentration was varied from 0 to 200 nM.

RESULTS

Dimerization of FXa Affects the Activity of the Prothrombinase Complex at 5 mM Ca^{2+}. The initial rates of thrombin formation by the prothrombinase complex formed at 5, 20, and 50 nM FXa in the presence of 400 μM C6PS and 5 mM Ca^{2+} are plotted in Figure 1B as a function of FVa concentration. Under these conditions, C6PS is present as a monomer (CMC ~ 650 μM) that binds to FXa to trigger dimer formation[12] and to FVa to trigger its tight association with FXa in solution.[30] Prothrombinase activity decreased with an increase in FXa concentration at a constant FVa concentration; i.e., FXa inhibited prothrombinase activity. The red hyperbolic curves (single-site binding model for FXa−FVa interaction) extrapolated to similar saturating activities $[k_{cat}/K_M \approx IR/[Xa_{tot}]/[S](1000\ nM) = (11 \pm 3) \times 10^7, (12 \pm 1) \times 10^7$, and

dx.doi.org/10.1021/bi301239z | *Biochemistry* 2013, 52, 143−151

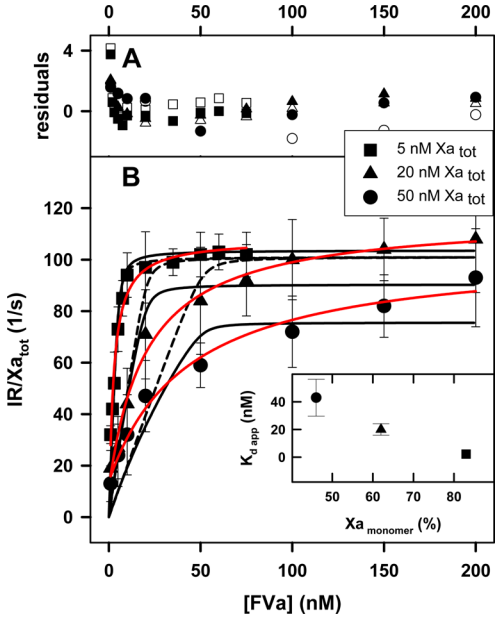


Figure 1. Initial rate of prothrombin activation by FXa-FVa at increasing concentrations of FVa under conditions favoring FXa dimers. The FXa−FVa interaction was monitored as an improvement in the ability of FXa at 5 (squares), 20 (triangles), and 50 (circles) nM to activate 1 μM prothrombin during titration with FVa in the presence of 400 μM C6PS at 5 mM Ca^{2+}. These conditions favor formation of both the FXa dimer and the FXa−FVa complex. (A) Deviation of experimental data from a value predicted by model 1 (empty symbols) and model 5a (filled symbols) relative to the average standard deviation $[(R_{exp} - R_{fit})/\sigma_{mean}]$. (B) The black curves drawn through the symbols indicate the global fit of the data to a simple competition model as described in the Supporting Information (---) or the $(XaVa)_2$ model [model 5a in the Supporting Information (—)]. Both models assume that the FXa dimer and XaVa dimer are inactive. Solid red lines represent a fit with a hyperbola. The inset shows the apparent K_d values of FXa−FVa dissociation were 2.2 ± 0.6, 20 ± 4.2, and 43 ± 13.3 nM for 5, 20, and 50 nM FXa, respectively. The inset shows a plot of the apparent K_d vs the percent of monomer of FXa obtained using eq 2 in the Supporting Information.

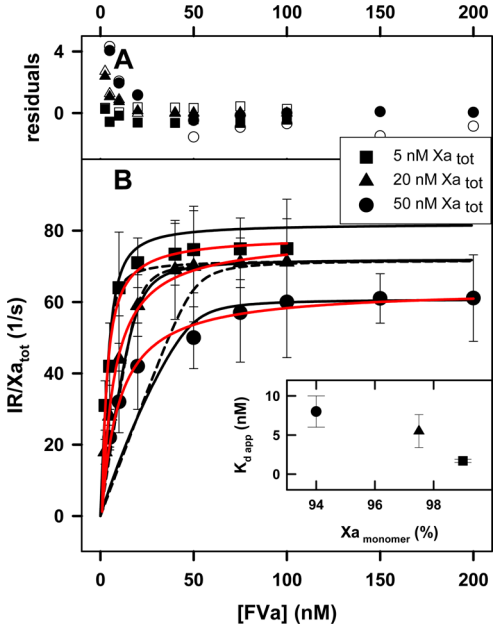


Figure 2. Initial rate of prothrombin activation by the FXa−FVa complex at increasing concentrations of FVa under conditions favoring monomers. Conditions and symbols are the same as in Figure 1 except that the Ca^{2+} concentration was 3 mM. (A) The deviation of experimental data from a value predicted by model 1 (empty symbols) and model 5a (filled symbols) relative to the average standard deviation $[(R_{exp} - R_{fit})/\sigma_{mean}]$. (B) The black curves drawn through the symbols indicate the global fit of the data to a simple dimer competition model as described in section A of the Supporting Information (---) or to an aggregate model as described in section B of the Supporting Information (—); both models predict that a dimer or aggregates are inactive. Solid red lines represent a fit with a hyperbola. The inset shows the apparent equilibrium constants (K_d) for dissociation of the FXa−FVa complex were 1.7 ± 0.4, 5.8 ± 0.8, and 8.6 ± 1.2 nM for 5, 20, and 50 nM FXa, respectively. The inset shows a plot of the apparent K_d vs the percent of monomer obtained using eq 2 of the Supporting Information.

(10 ± 1) × 10^7 M^{-1} s^{-1} for 5, 20, and 50 nM FXa, respectively], meaning that inhibition was overcome at saturating FVa concentrations. The $K_{d,app}^{XaVa}$ of FXa−FVa interaction, as estimated from a single-site binding model (solid red lines in Figure 1B), increased with FXa concentration: 2.2 ± 0.6, 20 ± 4.2, and 43 ± 13.3 nM for 5, 20, and 50 nM FXa, respectively. These results suggest that FXa interferes with formation of the prothrombinase complex. Because we know that human FXa forms a dimer in solution in the presence of C6PS,[12,13] it is reasonable to think that this inhibition is due to formation of inactive FXa dimers and, thus, that dimer formation competes with prothrombinase formation. Using a previously reported K_d for FXa dimerization ($K_d^{Xa_2}$ = 14 ± 1 nM),[13] we calculated the potential percent of FXa present as a monomer at 5 mM Ca^{2+} to be 46, 62, and 83% at $[Xa_{tot}]$ values of 50, 20, and 5 nM, respectively (Figure 1B inset), whereas at 3 mM Ca^{2+}, the potential percent of FXa present as a monomer is 94, 97.5, and 99%, at $[Xa_{tot}]$ values of 50, 20, and 5 nM, respectively (Figure 2B, inset), under the assumption that all FXa is free to dimerize (i.e., none is tied up in the FVa−FXa complex). Because FXa dimerization is highly sensitive to Ca^{2+} concentration,[12,13] and 5 mM Ca^{2+} is the optimal concentration for FXa dimerization,[12,13] we observed a smaller amount of FXa monomer at 5 mM Ca^{2+}. In the presence of FXa dimers, FVa is required to compete with FXa to form the prothrombinse complex. The inset of Figure 1B shows that the apparent K_d of FXa−FVa interaction ($K_{d,app}^{XaVa}$) decreased as the percent of FXa potentially present as a monomer increased. This result is consistent with the hypothesis that FXa dimerization and binding to FVa are competitive processes. Nonetheless, this result is an insufficient test of competition.

To test the competition hypothesis more quantitatively, we used MatLab to test the ability of a dimer competition model to account for our observations. Data at all three FXa concentrations were fit globally as described in detail in the Supporting Information, under model 1, and the results are shown in Figure 1B as dashed black curves [(▲) 5 nM FXa, (■) 20 nM FXa, and (●) 50 nM FXa]. The k_{cat}/K_M that yielded the best fit [(9.8 ± 0.9) × 10^7 M^{-1} s^{-1} (Table 1)] was in reasonable agreement with a previous estimate (22 × 10^7 M^{-1} s^{-1}).[30] Activity measurements at 37 °C have estimated the K_d of complex formation (K_d^{XaVa}) as ~3 nM,[23] while measurements with active site-labeled human FXa (DEGR-Xa) have estimated this K_d^{XaVa} as ~0.6 nM,[7] in good agreement with the value obtained from our fit, 0.32 nM (Table 1). Nonetheless, it is evident that the simple competition model significantly underestimated the degree of observed inhibition

dx.doi.org/10.1021/bi301239z | *Biochemistry* 2013, 52, 143−151

Table 1. Goodness of Fit Reduced χ^2 ($\bar{\chi}^2$) and Best Fit Parameters Obtained by Simultaneously Fitting All Three Data Sets ([Xa_{tot}] values of 5, 20, and 50 nM) by either the Simple Xa dimer−Prothrombinase Competition Model (model 1 of the Supporting Information) or the $(XaVa)_2$ plus Xa Dimer Model (model 5a of the Supporting Information)[a]

model (α)	$\bar{\chi}^2$	K_d (nM)		k_{cat}/K_M (10^7 M^{-1} s^{-1})
		K_d^{XaVa}	$K_d^{(XaVa)_2}$	$(k_{cat}/K_M)_{XaVa}$
5 mM Calcium (Figure 1)				
(1) simple Xa_2 XaVa competition (0.1)	1.38	0.32 (+0.73−0.31)		9.8 ± 0.9
(5a) $(XaVa)_2$ aggregate from XaVa (0.29) [2XaVa ↔ $(XaVa)_2$]	1.14	0.43 ± 0.3	150 (+80−50)	11 ± 0.6
3 mM Calcium (Figure 2)				
(1) simple Xa_2 XaVa competition (0.09)	1.41	0.76 (2−0.74)		7.2 ± 0.8
(5a) $(XaVa)_2$ aggregate from XaVa (0.17) [2XaVa ↔ $(XaVa)_2$]	1.25	1.5 (2−1.1)	166 (230−80)	8.7 ± 0.9

[a]Fixed parameters at 5 mM Ca^{2+} for both models: K_{dXa2} = 14 nM, and k_{cat}/K_{MXa} = 1.36 × 10^4 M^{-1} s^{-1} as measured previously.[12] Fixed parameters at 3 mM Ca^{2+} for both models: K_{dXa2} = 600 nM, and k_{cat}/K_{MXa} = 1.36 × 10^4 M^{-1} s^{-1} as determined in this paper.

of formation of the complex at low to intermediate FVa concentrations and 50 nM FXa and thus reached an asymptote well before the experimental data. Hyperbolic fits to our data (red lines) also suggest this asymptotic behavior. The reduced χ^2, $\bar{\chi}^2$, of the fit was large [1.38 (Table 1)], while its significance was low (0.1). While the simple dimer competition model confirmed that Xa dimer formation could result in inhibition of formation of the FXa−FVa complex, it fell short in its ability to account quantitatively for our data, leading us to consider alternative models that might provide better quantitative descriptions of our data.

Because dimer formation underestimated the extent of inhibition, we considered the possibility that FXa might form a higher-order aggregate, e.g., a tetramer (model 2 in section B of the Supporting Information). Model 2 provided no improvement in the description of data despite requiring an additional adjustable parameter, $K_d^{Xa_4}$. As a control, we tried an alternative form of the "FXa tetramer" model (model 2a in section C of the Supporting Information) and found, as anticipated if our analysis methods were robust, that the results of our fitting were independent of the thermodynamic path assumed in forming a tetramer. The inability of the "FXa tetramer" model to fit the data better than the simple dimer competition model (model 1) allows us to reject the "higher-order Xa aggregate" (as embodied to a first approximation in a tetramer) model to explain inhibition observed at higher concentrations of FXa. Next, we considered whether aggregation of FVa (model 3 in section D of the Supporting Information) and subsequent formation of an aggregated FXa−FVa complex (model 4) might contribute to the less than satisfactory quantitative description we had obtained using the dimer competition model (model 1). Both models provided better fits to the data; however, the parameter values were quite unreasonable relative to other reports (K_d^{XaVa} = 0.016 nM), relative to the reported value of 0.6 nM,[7] or relative to common sense ($K_d^{Va_2}$ = 1 pM), which means that FVa binds to FVa 100 times more tightly than it does to FXa. Because we also did not see any evidence of the FVa dimer in native gels of mixtures of FVa and FXa in the presence of C6PS (Figure 3), we must conclude that aggregation of FVa cannot offer an explanation for the poor quantitative agreement between the FXa dimer competition model (model 1) and our data.

Finally, we considered whether dimerization of the XaVa complex might help account for our data. The description of our data by this model is better than that of any other model considered [i.e., lowest $\bar{\chi}^2$ for physically reasonable parameters (Table S1 of the Supporting Information)]. The parameters for this model [K_d^{XaVa} and $(k_{cat}/K_M)_{XaVa}$] were also in reasonable agreement with literature estimates,[30] while the predicted $K_d^{(XaVa)_2}$ (150 nM) is consistent with our failure to observe this species in native gels (Figure 3). However, Figure 1B reveals that the predicted curves do not approach a common asymptotic $(k_{cat}/K_M)_{XaVa}$ at saturating FVa concentrations, which hyperbola through our data appear to do (see red hyperbolic curves in Figure 1B). Thus, allowing for a dimer of the FXa−FVa complex considerably improves the description of our high FXa data considerably at low and intermediate FVa concentrations but still falls somewhat short of accounting for the apparent asymptotic behavior of our data at very high FVa concentrations and 5 mM Ca^{2+}, which favors FXa dimerization.

Dimerization of FXa Affects the Activity of the Prothrombinase Complex at 3 mM Ca^{2+}. We showed previously that FXa dimerization is sensitive to Ca^{2+} concentration, with $K_d^{Xa_2}$ at 3 mM Ca^{2+} being roughly 40-fold larger than that at 5 mM Ca^{2+}.[12,13] Thus, to further test whether FXa dimerization might be a reasonable explanation for the variation with FXa concentration of $K_{d,app}^{XaVa}$ for the FXa−FVa complex, we repeated the experiments of Figure 1B at 3 mM Ca^{2+}. Surprisingly, Figure 2B still shows inhibition of prothrombinase assembly at increasing concentrations of FXa, but the inset of Figure 2B shows a 10-fold increase in FXa concentration produced a 3−4-fold increase in $K_{d,app}^{XaVa}$ at 3 mM Ca^{2+} (1.7 ± 0.2, 5.5 ± 2, and 8 ± 2 nM for 5, 20, and 50 nM FXa, respectively) compared to the 20−25-fold increase at 5 mM Ca^{2+} (Figure 1B, inset), suggesting that FXa dimer formation is less important at 3 mM Ca^{2+}, as expected.

To explore quantitatively the reasons for this unanticipated result, we again used the procedures described in the Supporting Information to test the compatibility of our data with the two most successful models we identified for 5 mM Ca^{2+} (model 1, the dimer competition model, and model 5a, the XaVa dimerization model). Because thermodynamic constants for the formation of the XaVa complex and the human FXa dimer are not reported at 3 mM Ca^{2+}, we measured these constants at 37 °C using previously documented methods that use active site-labeled human DEGR-FXa[12,30] to obtain a $K_d^{Xa_2}$ of 600 ± 10 nM and a K_d^{XaVa} of 1.7 ± 0.4 nM. This value of $K_d^{Xa_2}$ was fixed in our calculations, while K_d^{XaVa} was adjustable to remain consistent with our computations for 5 mM Ca^{2+}. The parameters providing this fit are summarized in Table 1. The best fit value for K_d^{XaVa} was somewhat lower than we measured but still well within the error range of these two estimates. The effect of Ca^{2+} on K_d^{XaVa} seen in the context of the dimer competition model is thus small. The same was also true for

dx.doi.org/10.1021/bi301239z | Biochemistry 2013, 52, 143−151

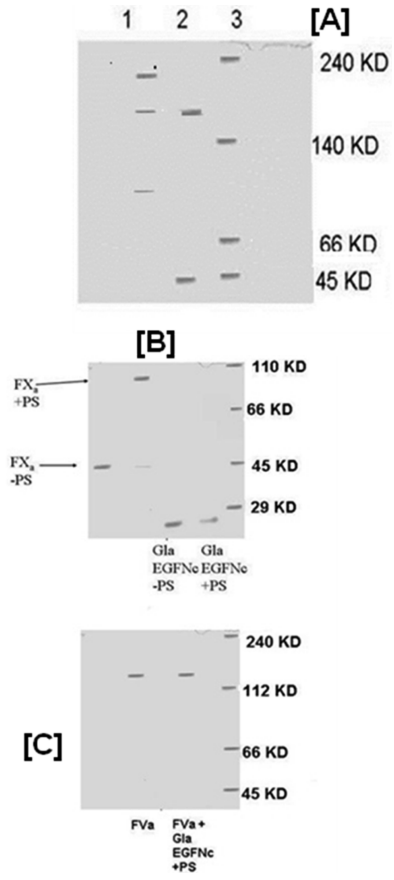


Figure 3. (A) Native gel electrophoresis of a FXa/FVa mixture in the presence and absence of C6PS. A 5% polyacrylamide gel of a mixture of 50 nM FXa and 50 nM FVa in the presence of 400 μM C6PS and 5 mM Ca^{2+} (lane 1) shows the coexistence of homodimers (FXa·FXa, 90 kDa), heterodimers (FXa·FVa, 224 kDa), and FVa (178 kDa), showing that the FXa monomer interacts with both FVa and FXa, which is consistent with the hypothesis that FXa and FVa compete to bind FXa. Lane 2 shows that the same reaction mixture in the absence of C6PS contains only monomers of both FVa and FXa, showing that this competition is regulated by phosphatidylserine (in this case C6PS). Lane 3 shows molecular mass markers. (B) Native gel electrophoresis of FXa and its GLA-EGF_{NC} fragment. An 8% native polyacrylamide gel electrophoresis of FXa and the GLA-EGF_{NC} fragment of FXa in the presence and absence of 400 μM C6PS and 5 mM Ca^{2+} shows that the GLA-EGF_{NC} fragment does not dimerize in the presence of C6PS and 5 mM Ca^{2+}. (C) A 5% native polyacrylamide gel electrophoresis of 50 nM FVa with and without 50 nM GLA-EGF_{NC} domain in the presence of 400 μM C6PS and 5 mM Ca^{2+}. The results show that the GLA-EGF_{NC} fragment of FXa does not form a complex with FVa.

$(k_{cat}/K_M)_{XaVa}$ [9.8 ± 0.8 and (7.2 ± 0.8) × 10^7 M^{-1} s^{-1} for 5 and 3 mM Ca^{2+}, respectively]. Apparently, within the context of this model, only FXa dimer formation was significantly affected by a change in Ca^{2+} concentration from 5 to 3 mM.

As for 5 mM Ca^{2+}, model 5a (XaVa dimerization) also offered a better description of the data at 3 mM Ca^{2+} than did the dimer competition model (model 1). The description was better because the data at 3 mM Ca^{2+} showed a difference in asymptotic behavior between 5 and 50 nM FXa, a feature predicted by the XaVa dimerization model. The parameters defined by this fit are also listed in Table 1, where we see that the value for $(k_{cat}/K_M)_{XaVa}$ for the FXa−FVa complex within the context of this model is also not significantly Ca^{2+}-dependent [11.0 ± 0.9 and (8.7 ± 0.7) × 10^7 M^{-1} s^{-1} for 5 and 3 mM Ca^{2+}, respectively]. In addition, K_d^{XaVa} was also not significantly Ca^{2+}-dependent (0.43 ± 0.3 nM versus 1.5 + 2 − 1.1 nM for 5 and 3 mM Ca^{2+}, respectively), nor was formation of the FXa−FVa complex significantly Ca^{2+}-dependent (150 and 166 nM for 5 and 3 mM Ca^{2+}, respectively). Thus, within the context of both models, Ca^{2+} significantly affected only FXa dimer formation ($K_d^{Xa_2}$ = 14 and 600 nM for 5 and 3 mM Ca^{2+}, respectively) in this concentration range.

Coexistence of the FXa−FVa Complex with the Dimer at 5 mM Ca^{2+}. Native polyacrylamide gel electrophoresis, in the presence of C6PS, detects both the human FXa−FVa complex and human FXa dimers with the expected molecular masses [measured values of 224 ± 3.7 kDa for the FXa−FVa complex[30] and ~90 kDa for $(FXa)_2$].[13] For formation of the FXa dimer and formation of the FXa−FVa complex to be competitive processes, FVa, FXa, FXa−FVa, and $(FXa)_2$ should all be able to coexist in equilibrium under a condition under which both FXa dimers and the prothrombinase complex can form (400 μM C6PS, 5 mM Ca^{2+}, 50 nM FXa, and 50 nM FVa). Figure 3A (lane 1) shows FVa (~178 kDa), FXa_2 (~90 kDa), and FXa−FVa complex (~224 kDa) bands, thus confirming that the FVa monomer, the FXa dimer, and the FXa−FVa complex can all coexist under appropriate conditions. Previous hydrodynamic studies showed that the solution prothrombinase formed in the presence of C6PS is a 1:1 complex at low FXa concentrations.[30] Current results show that the C6PS-induced complex has a 1:1 ratio even at a FXa concentration at which FXa dimers form. A complex of the FXa dimer with FVa (FXa·FXa·FVa) should enter the gel (270 kDa) and be located at the top of the gel, but it was not detected. This result is consistent with the hypothesis that only monomer FXa binds to FVa and that both FXa and FVa compete to bind FXa. Note that the FXa monomer does not appear in the gel, because, under these conditions, most FXa is incorporated into Xa_2 or the FXa−FVa complex. We note also that the $(FXa–FVa)_2$ complex (~450 kDa) would not have entered this 5% gel, but we did not detect any staining at the running gel−loading gel interface even at 200 nM FVa. Thus, if this species is present, it must be present in a small quantity. We note that the data Lane 1 in Figure 3A is consistent with our activity data in Figure 1B and indicate that FXa should be more than half incorporated into FXa−FVa complex under experimental conditions but also significantly present in FXa dimer. This means that FXa−FVa complex and FXa dimer coexist in solution, as we set out to demonstrate.

An R165A FXa Mutation in the FVa-Binding Region Interferes with Dimerization. Arg 165 in the human FXa catalytic domain is part of a basic region involved in FVa binding.[8,9,26,27] We also obtained $K_{d,app}^{XaVa}$ values for R165A FXa−FVa interaction from plots of initial rates of prothrombin activation (400 μM C6PS and 5 mM Ca^{2+}) catalyzed by 5, 20, and 50 nM R165A mutant human FXa in the presence of increasing concentrations of FVa. These apparent K_d values along with the $K_{d,app}^{XaVa}$ values of the wild-type FXa−FVa (Figure 1B) association are listed in Table 2. The results show that the apparent affinity of the R165A mutant FXa for FVa was lowered by 3−28-fold relative to that for native FXa, depending on the concentrations of mutant and wild-type FXa. This finding is consistent with a previous report of a 6-fold effect of this mutation on the interaction with FXa.[39]

The initial rates of prothrombin activation by different concentrations of wild-type and R165A FXa in the presence of

dx.doi.org/10.1021/bi301239z | Biochemistry 2013, 52, 143−151

Table 2. Apparent Dissociation Constants for Interactions of Wild-Type and R165A Mutant FXa with FVa[a]

	$K_{d,app}$ for FXa−FVa (nM)	
[FXa] (nM)	wild-type FXa	mutant (R165A) FXa
5	2.2 ± 0.6	58 ± 7
20	20 ± 4.2	92 ± 12
50	43 ± 13.3	128 ± 27
	K_d^{dimer} (nM)	
	wild-type FXa	mutant (R165A) FXa
	16 ± 4	147 ± 25

[a]Dissociation constants obtained from the data plotted in Figure 1B and from similar experiments with R165A rHFXa show that the R165A mutation in FXa inhibits both the interaction with FVa and formation of the FXa dimer. The value of $K_d^{Xa_2}$ was obtained from fitting the data in the figure. We previously reported a value of 14 ± 1 nM obtained from more extensive sets of data in the absence of FVa.[13]

400 μM C6PS versus FXa concentration are plotted in Figure 4. The curves were fit to a dimerization model[12] as described in

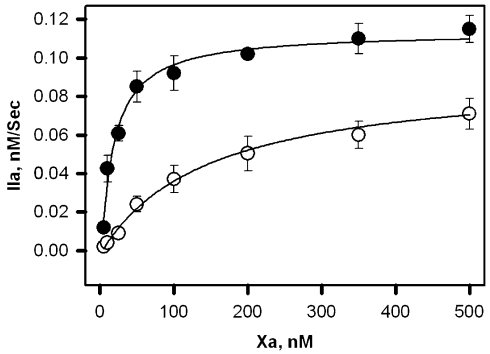


Figure 4. Prothrombin activation by increasing concentrations of wild-type and mutant (R165A) FXa in the presence of C6PS and 5 mM Ca^{2+}. The initial rates of prothrombin activation by FXa in the presence of FVa and 400 μM C6PS at different FXa concentrations are plotted as a function of FXa concentration. The appearance of thrombin was determined by the rate of hydrolysis of DAPA (as described in Materials and Methods) at 37 °C. The reaction was conducted at increasing concentrations of wild-type FXa (●) and mutant R165A FXa (○). The reaction mixture contained 1 μM prothrombin, FXa in 50 mM Tris, 175 nM NaCl, 0.6% poly(ethylene glycol), 5 mM Ca^{2+}, and 400 μM C6PS. The dimerization K_d values obtained in the presence of wild-type and mutant FXa were 16 and 147 nM, respectively.

Materials and Methods. The $K_d^{Xa_2}$ for R165A FXa was 9-fold larger than for wild-type FXa (Table 2). We conclude that the R165A mutation alters dimerization as well as binding to FVa in the presence of C6PS.

Role of the Catalytic Domain of FXa in the Dimerization of FXa and Its Interaction with FVa. FXa is generally thought to interact with Va through sites in the FXa catalytic domain.[8,10,27,40] We wanted to test whether there might be other regions of FXa required for FVa interaction and dimerization of FXa in the presence of 400 μM C6PS. Figure 3B shows an 8% native polyacrylamide gel of a GLA-EGF_{NC} fragment of FXa (lacking the catalytic domain) along with intact FXa in the absence and presence of C6PS, showing that FXa dimerizes but that the GLA-EGF_{NC} fragment does not dimerize in the presence of C6PS. Figure 3C shows a 5% native polyacrylamide gel of FVa in the absence and presence of C6PS and the GLA-EGF_{NC} fragment, showing that FVa cannot form a complex with the GLA-EGF_{NC} fragment of FXa. Because we have shown previously that native polyacrylamide gel electrophoresis can detect both the FXa dimer and FXa−FVa complexes,[12,30] these results demonstrate that, although C6PS binds to a regulatory site in the EGF_{NC} pair,[6] interactions between this region of FXa do not contribute directly either to FXa dimerization or to binding to FVa.

DISCUSSION

Our results support the following conclusions.

(1) FXa Dimer Formation and Prothrombinase Complex Formation Are Thermodynamically Competing Processes in Solution That Are Both Triggered by Binding of C6PS to a Regulatory Site in the EGF_{NC} Pair. Because of this competition, estimates of the $K_{d,eff}^{XaVa}$ for prothrombinase complex formation will depend on the reaction conditions (FXa, FVa, and Ca^{2+} concentrations), at least if activity is used to monitor prothrombinase formation. This dependence is demonstrated in Figures 1 and 2 but is also surmised from the dimer competition model (model 1 of the Supporting Information) we used to interpret our results. Because DEGR-FXa dimerizes weakly in solution,[41] estimates of K_d^{XaVa} using active site-labeled FXa (0.6 ± 0.09 nM[30]) are lower than estimates made using activity to follow complex assembly (2.8 ± 0.3 nM[23]). Estimates of K_d^{XaVa} using either active site-labeled FXa or activity may not differ for complex assembly on a membrane because DEGR-FXa dimerizes reasonably well on a membrane surface.[41]

(2) Other Interactions Appear To Complicate Further Complex Formation in Solution. This conclusion is implied by the observation that the dimer competition model (model 1 of the Supporting Information) was unable to account for our data at either 5 mM Ca^{2+} (where dimerization should be significant) or 3 mM Ca^{2+} (where dimer formation should be minimal). In both instances, less FXa−FVa complex was predicted to form than was suggested by observed prothrombin activation rates. We considered what other species might be limiting formation of the prothrombinase complex. Inclusion of higher-order aggregates of FXa (tetramer) produced no reduction in $\bar{\chi}^2$ and produced an estimate of the tetramer dissociation constants that resulted in this species not being present (Table S1 of the Supporting Information). The possibility that FVa formed a dimer reduced the $\bar{\chi}^2$ but predicted that the FVa dimer should be the dominant species, in contradiction with results (Figure 3A). Next, the assumption that a dimer of the FXa−FVa complex might form produced an improved description of observations in Figure 1 and a significant reduction in $\bar{\chi}^2$ as well as a considerable increase in the goodness of fit [α (Table 1)]. FXa−FVa dimer formation (model 5a of the Supporting Information) also improved our description of the data collected at 3 mM Ca^{2+} (Table 1 and Figure 2) and is thought to provide some insight into why the simple dimer competition model does not account well for our data. Within the context of this model, only the FXa dimerization constant ($K_d^{Xa_2}$) is significantly dependent on Ca^{2+} concentration; neither K_d^{XaVa} nor $K_d^{(XaVa)_2}$ was dependent on Ca^{2+} concentration, suggesting that formation of $(XaVa)_2$ involves a binding region other than that involved in Xa_2 formation. Because FVa seems not to form a dimer (model 3 of the Supporting Information), the most likely interaction region to support formation of the $(XaVa)_2$ species would be the dimer interaction region. If so, we would have to surmise that

dx.doi.org/10.1021/bi301239z | Biochemistry 2013, 52, 143−151

binding of FVa to the FVa-binding region of FXa, which is distant from the FXa dimerization region,[13] must alter the dimerization region so that a FXa−FXa interaction becomes more likely at low Ca^{2+} concentrations. If so, our hypothesis that these two binding regions are linked is more credible.

Despite the success of the XaVa dimer model (model 5a) relative to the Xa dimer competition model (model 1), the former model at 5 mM Ca^{2+} still had shortcomings in its ability to predict observed asymptotic behavior (Figure 1). Reference to simulations presented in Figure S3 of the Supporting Information reveals that the concentration of the prothrombinase complex does not approach its maximal possible value at high FVa and FXa concentrations because a stable amount of the FXa−FVa dimer species $[(XaVa)_2]$ is predicted to form under these circumstances. At 3 mM Ca^{2+}, when very little FXa_2 species is expected, incomplete formation of the prothrombinase complex at high FXa and FVa concentrations is both predicted (Figure S3 of the Supporting Information) and observed (Figure 2). This result led us to suspect that the value of $K_d^{Xa_2}$ that we used might be in error. To test this suspicion, we fixed best fit parameters [K_d^{XaVa}, $K_d^{(XaVa)_2}$, and $(k_{cat}/K_M)_{XaVa}$] and varied $K_d^{Xa_2}$ without producing a better description of asymptotic behavior. In this regard, we note the large uncertainty in K_d^{XaVa} and $K_d^{(XaVa)_2}$ (Table 1) that likely reflects the need to rectify different behaviors of the data at high and intermediate FVa concentrations. The reasons for this discrepancy between the data and XaVa dimer model (model 5a) at high FVa concentrations could be (1) the uneven distribution of data points, (2) large uncertainties in the rate data at high FVa concentrations, and (3) the inadequacy of the model. Indeed, use of an average 12% error for all rate data led to a somewhat larger value of 1.3×10^8 M^{-1} s^{-1} for $(k_{cat}/K_M)_{XaVa}$, a somewhat improved appearance of the fit at high FVa concentrations, and K_d^{XaVa} and $K_d^{(XaVa)_2}$ parameters still within the range of uncertainties listed in Table 1, but also a somewhat larger $\overline{\chi}^2$. Of course, we must consider that a XaVa dimer may not explain our observations, but if not this model, then what model? We know that FXa forms a dimer in solution under the influence of C6PS,[12,13] and the data in Figures 1 and 2 make it clear that both FXa and FVa concentrations must be taken into account to model the formation of the active FXa−FVa complex in solution at high FXa and FVa concentrations. We have eliminated a model that posits a FVa dimer (model 3 of the Supporting Information) and models that posit higher-order aggregates of FXa (models 2 and 2a of the Supporting Information). While we could still imagine other species (e.g., FXa·FXa·FVa), each such species added to the model adds a new adjustable parameter, meaning that any resulting improvement in $\overline{\chi}^2$ is less likely to be significant. We conclude that some higher-order aggregated species are required to explain the dependence of soluble prothrombinase formation on both FXa and FVa concentrations and that the most likely species at this point is a dimer of the FXa−FVa prothrombinase complex.

(2) FVa- and FXa-Binding Regions Are Distinct Linked Sites within the Catalytic Domain. Binding of C6PS to the regulatory site in the EGF_{NC} region[6] of human FXa is linked to binding sites in the catalytic domain responsible for interaction with FVa and for dimer formation.[13,30] Because the EGF_N domain is near the membrane-binding Gla domain, linkage of these sites must occur via the FXa light chain. Because FXa dimer formation and prothrombinase complex formation are not mediated by C6PS-triggered interactions between FXa light chains or between the FXa light chain and FVa (Figure 3C), the rules of a thermodynamic cycle require linkage between the catalytic domain-binding regions. Here we provide additional evidence of this linkage. We know that K90 in the catalytic domain becomes buried when C6PS triggers FXa dimer formation in the presence of 5 mM Ca^{2+} and is on the opposite face of a FXa structural model[42] from the most likely FVa-binding regions (a 185−189 loop,[26] a 162−169 helix containing R165,[8,9,27] or both). Both of these regions were identified by mutational analysis. There are two general views of linkage.[43] One asserts that binding of one ligand favors one of many conformational states of a protein that favors binding of another ligand. In the second view, inter-residue or interdomain interactions along a path convey information between regions of a protein. Mutational analysis has been proposed to identify such paths.[44] Some note that these views are not mutually exclusive but that "path" residues are residues that are crucial for protein conformational state equilibration. We show here that a mutation (R165A) that interferes with the interaction of FXa with FVa[8] also interferes with dimer formation (Figure 4). If R165 is in the FVa-binding region, this observation supports linkage between the FVa-binding and FXa-binding regions. It is noteworthy that another Lys (K230) becomes exposed upon dimer formation[13] and is spatially close to R165 in the proposed FXa structural model. These observations suggest that K230 and R165 may be residues that are part of a linkage path or key to switching between dimer-forming or FVa-binding conformational states, a possibility that also supports linkage between these two binding regions. Further supporting our hypothesis is the observation that K90 is present in two peptides that mimic regions of FXa sequence that interfere with or block binding of FXa to FVa.[10,40] The fact that K90 is far from the FVa-binding region supports our hypothesis that these two sites are linked and that binding of synthetic peptides to the FXa dimer interface interferes with binding of FXa to FVa.

(4) Linkage between the Dimer Interface and FVa-Binding Regions of FXa May Have Physiological Significance in Blood Coagulation. Both FXa dimer formation and assembly of the prothrombinase complex are triggered by C6PS or by binding of these proteins to PS-containing membranes.[13,30,41,45] Thus, the competition between FXa dimer formation and prothrombinase assembly could well occur on a membrane in vivo. If so, its sensitivity to Ca^{2+} concentration could be significant during the early stages of platelet plug formation when the calcium concentration in the local wounded area decreases much below the normal plasma concentration and increases back to normal levels within 3 h of the injury.[46] Thus, we speculate that FXa dimerization is a mechanism that limits prothrombinase formation when blood clotting is undesirable. We are investigating this possibility using model membranes.

■ ASSOCIATED CONTENT

Supporting Information

Demonstration of the ability of different binding models to account for observed binding data as well as the method used to obtain fit parameters and to estimate their uncertainties. This material is available free of charge via the Internet at http://pubs.acs.org.

AUTHOR INFORMATION

Corresponding Author

*Program in Molecular and Cellular Biology 120 Mason Farm Rd., Genetic Medicine Building, University of North Carolina at Chapel Hill, Chapel Hill, NC 27599-7260. Telephone: (919) 966-5384. Fax: (919) 966-2852. E-mail: uncbrl@med.unc.edu.

Author Contributions

R.M. and T.K. contributed equally to this work.

Funding

This work was supported by Grants GM32707 (B.R.L.) and HL 101917 (A.R.R.) from the National Institutes of Health.

Notes

The authors declare no competing financial interest.

ABBREVIATIONS

DAPA, dansylarginine-*N*-(3-ethyl-1,5-pentanediyl) amide; S765, *N*-α-benzyloxycarbonyl-D-arginyl-L-glycyl-L-arginine-*p*-nitroaniline dihydrochloride; C6PS, 1,2-dicaproyl-*sn*-glycero-3-phospho-L-serine; PS, phosphatidylserine; FXa, factor Xa; FVa, factor Va; II_a, thrombin; GLA, N-terminal γ-carboxyglutamic acid-rich region; EGF_C, epidermal growth factor nearest the C-terminus; PEG, polyethylene glycol; DEGR-FXa, [5-(dimethylamino)-1-naphthalenesulfonyl]glutamylglycylarginyl chloromethyl ketone.

REFERENCES

(1) Fenton, J. N. (1986) Thrombin. *Ann. N.Y. Acad. Sci. 485*, 5−15.

(2) Jackson, C. M., and Nemerson, Y. (1980) Blood coagulation. *Annu. Rev. Biochem. 49*, 765−811.

(3) Mann, K. G., Jenny, R. J., and Krishnaswamy, S. (1988) Cofactor proteins in the assembly and expression of blood clotting enzyme complexes. *Annu. Rev. Biochem. 57*, 915−956.

(4) Bevers, E. M., Comfurius, P., and Zwaal, R. F. (1983) Changes in membrane phospholipid distribution during platelet activation. *Biochim. Biophys. Acta 736*, 57−66.

(5) Jones, M. E., Lentz, B. R., Dombrose, F. A., and Sandberg, H. (1985) Comparison of the abilities of synthetic and platelet-derived membranes to enhance thrombin formation. *Thromb. Res. 39*, 711−724.

(6) Srivastava, A., Wang, J.-F., Majumder, R., Stenflo, J., Rezaie, A. R., Esmon, C. T., and Lentz, B. R. (2002) Locations of phosphatidylserine binding sites on Factor Xa. *J. Biol. Chem. 277*, 1855−1863.

(7) Majumder, R., Quinn-Allen, M. A., Kane, W. H., and Lentz, B. R. (2008) A phosphatidylserine binding site in factor Va C1 domain regulates both assembly and activity of the prothrombinase complex. *Blood 112*, 2795−2802.

(8) Rezaie, A. R. (2000) Identification of basic residues in the heparin-binding exosite of factor Xa critical for heparin and factor Va binding. *J. Biol. Chem. 275*, 3320−3327.

(9) Rudolph, A. E., Porche-Sorbet, R., and Miletich, J. P. (2000) Substitution of asparagine for arginine 347 of recombinant factor Xa markedly reduces factor Va binding. *Biochemistry 39*, 2861−2867.

(10) Sabharwal, A. K., Padmanabhan, K., Tulinsky, A., Mathur, A., Gorka, J., and Bajaj, S. P. (1997) Interaction of calcium with native and decarboxylated human factor X. Effect of proteolysis in the autolysis loop on catalytic efficiency and factor Va binding. *J. Biol. Chem. 272*, 22037−22045.

(11) Padmanabhan, K., Padmanabhan, K. P., Tulinsky, A., Park, C. H., Bode, W., Huber, R., Blankenship, D. T., Cardin, A. D., and Kisiel, W. (1993) Structure of human des(1−45) factor Xa at 2.2 Å resolution. *J. Mol. Biol. 232*, 947−966.

(12) 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.

(13) Chattopadhyay, R., Iacob, R., Majumder, R., Sen, S., Tomer, K., and Lentz, B. R. (2009) Functional and Structural Characterization of Factor Xa Dimer In Solution. *Biophys. J. 96*, 974−986.

(14) 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.

(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) Banerjee, M., Drummond, D. C., Srivastava, A., Daleke, D., and Lentz, B. R. (2002) Specificity of soluble phospholipid binding sites on human factor Xa. *Biochemistry 41*, 7751−7762.

(17) 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.

(18) Kane, W. H., and Majerus, P. W. (1981) Purification and characterization of human coagulation factor V. *J. Biol. Chem. 256*, 1002−1007.

(19) Dahlback, B., and Stenflo, J. (1980) The activation of prothrombin by platelet-bound factor Xa. *Eur. J. Biochem. 104*, 549−557.

(20) 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.

(21) Kim, S. W., Ortel, T. L., Quinn-Allen, M. A., Yoo, L., Worfolk, L., Zhai, X., Lentz, B. R., and Kane, W. H. (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.

(22) 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.

(23) 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.

(24) 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.

(25) Majumder, R., Quinn-Allen, M. A., Kane, W. H., and Lentz, B. R. (2005) The phosphatidylserine binding site of the factor Va C2 domain accounts for membrane binding but does not contribute to the assembly or activity of a human factor Xa-factor Va complex. *Biochemistry 44*, 711−718.

(26) Rezaie, A. R., and Kittur, F. S. (2004) The critical role of the 185−189-loop in the factor Xa interaction with Na^+ and factor Va in the prothrombinase complex. *J. Biol. Chem. 279*, 48262−48269.

(27) Rudolph, A. E., Porche-Sorbet, R., and Miletich, J. P. (2001) Definition of a factor Va binding site in factor Xa. *J. Biol. Chem. 276*, 5123−5128.

(28) Yegneswaran, S., Mesters, R. M., and Griffin, J. H. (2003) Identification of distinct sequences in human blood coagulation factor Xa and prothrombin essential for substrate and cofactor recognition in the prothrombinase complex. *J. Biol. Chem. 278*, 33312−33318.

(29) Camire, R. M. (2002) Prothrombinase Assembly and S1 Site Occupation Restore the Catalytic Activity of FXa Impaired by Mutation at the Sodium-binding Site. *J. Biol. Chem. 277*, 37863−37870.

(30) Majumder, R., Weinreb, G., and Lentz, B. R. (2005) Efficient thrombin generation requires molecular phosphatidylserine, not a membrane surface. *Biochemistry 44*, 16998−17006.

(31) Valcarce, C., Persson, E., Astermark, J., Ohlin, A. K., and Stenflo, J. (1993) Isolation of intact modules from noncatalytic parts of vitamin K-dependent coagulation factors IX and X and protein C. *Methods Enzymol. 222*, 416−435.
(32) 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.
(33) Krishnaswamy, S., Russell, G. D., and Mann, K. G. (1989) The reassociation of factor Va from its isolated subunits. *J. Biol. Chem. 264*, 3160−3168.
(34) Haque, M. E., Das, A. R., and Moulik, S. P. (1995) Behaviors of Sodium Deoxycholate (NaDC) and Polyoxyethylene *tert*-Octylphenyl Ether (Triton X-100) at the Air/Water Interface and in the Bulk. *J. Phys. Chem. 99*, 14032−14038.
(35) Haque, M. E., Das, A. R., and Moulik, S. P. (1999) Mixed micelles of sodium deoxycholate and polyoxyethylene sorbitan monooleate (Tween 80). *J. Colloid Interface Sci. 217*, 1−7.
(36) Alexander, E., Jr., Mitchell, O. C., Ferguson, K. G., and Leinbach, L. B. (1964) Excretory Urography Urography by-Product of Cerebral Angiography. *J. S. C. Med. Assoc. 60*, 237−239.
(37) Mitra, P., Pal, A. K., Basu, D., and Hati, R. N. (1994) A staining procedure using Coomassie brilliant blue G-250 in phosphoric acid for detection of protein bands with high resolution in polyacrylamide gel and nitrocellulose membrane. *Anal. Biochem. 223*, 327−329.
(38) Weinreb, G. E., Mukhopadhyay, K., Majumder, R., and Lentz, B. R. (2003) Cooperative roles of factor V(a) and phosphatidylserine-containing membranes as cofactors in prothrombin activation. *J. Biol. Chem. 278*, 5679−5684.
(39) Rezaie, A. R., and He, X. (2000) Sodium binding site of factor Xa: Role of sodium in the prothrombinase complex. *Biochemistry 39*, 1817−1825.
(40) Chattopadhyay, A., James, H. L., and Fair, D. S. (1992) Molecular recognition sites on factor Xa which participate in the prothrombinase complex. *J. Biol. Chem. 267*, 12323−12329.
(41) Koklic, T., Majumder, R., Weinreb, G. E., and Lentz, B. R. (2009) Factor XA binding to phosphatidylserine-containing membranes produces an inactive membrane-bound dimer. *Biophys. J. 97*, 2232−2241.
(42) Venkateswarlu, D., Perera, L., Darden, T., and Pedersen, L. G. (2002) Structure and dynamics of zymogen human blood coagulation factor x. *Biophys. J. 82*, 1190−1206.
(43) Whitley, M. J., and Lee, A. L. (2009) Frameworks for understanding long-range intra-protein communication. *Curr. Protein Pept. Sci. 10*, 116−127.
(44) Horovitz, A., and Fersht, A. R. (1990) Strategy for analysing the co-operativity of intramolecular interactions in peptides and proteins. *J. Mol. Biol. 214*, 613−617.
(45) Krishnaswamy, S. (1990) Prothrombinase complex assembly. Contributions of protein-protein and protein-membrane interactions toward complex formation. *J. Biol. Chem. 265*, 3708−3718.
(46) Stokes, B. T., Fox, P., and Hollinden, G. (1983) Extracellular calcium activity in the injured spinal cord. *Exp. Neurol. 80*, 561−572.