Soluble Phosphatidylserine Triggers Assembly in Solution of a Prothrombin-activating Complex in the Absence of a Membrane Surface*

Received for publication, January 28, 2002, and in revised form, May 31, 2002
Published, JBC Papers in Press, June 3, 2002, DOI 10.1074/jbc.M200893200

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Factor Xa (FXa) binding to factor Va (FVa) on platelet-derived membranes containing surface-exposed phosphatidylserine (PS) forms the “prothrombinase complex” that is essential for efficient thrombin generation during blood coagulation. There are two naturally occurring isoforms of FVa1, FVa2, and FXa2. These two isoforms differ by a 3-kDa polysaccharide chain (at Asn218 of human FVa1; Kim, S. W., Ortel, T. L., Quinn-Allen, M. A., Yoo, L., Worfolk, L., Zhai, X., Lentz, B. R., and Kane, W. H. (1999) Biochemistry 38, 11448–11454) and have different coagulant activities. We examined the interaction of the two bovine isoforms with active site-labeled FXa, finding no significant difference. A soluble form of PS (C6PS) bound to FVa1 and FVa2 with comparable affinities ($K_d = 11–12 \mu M$) and changes in FVa1 intrinsic fluorescence. At concentrations well below its critical micelle concentration, C6PS binding to bovine FVa2 enhanced its affinity for FXa in solution by nearly 3 orders of magnitude ($K_{D,\text{off}} = 40–2 \text{ nM}$ over a C6PS range of 30–400 \text{ mM}) but had no effect on the affinity of FVa1 for FXa ($K_f = 1 \mu M$). This results in a soluble complex between FXa and FVa2, whose expected molecular weight was confirmed by calibrated native gel electrophoresis. This complex behaved as a normal Michaelis-Menten enzyme in its ability to produce thrombin from meizothrombin (apparent $k_{\text{cat}}/K_m = 10^8 \text{ M}^{-1} \text{s}^{-1}$). The ability of soluble PS to trigger formation of a soluble prothrombinase complex suggests that exposure of PS molecules during platelet activation is likely the key event responsible for the assembly of an active membrane-bound complex.

The final step in the blood coagulation cascade involves the activation of prothrombin to thrombin, which is the central enzyme of the coagulation system. This activation requires assembly of an enzyme complex, called prothrombinase (1), which consists of blood coagulation factors Xa (a serine protease) and Va (a cofactor), Ca$^{2+}$, and membranous vesicles derived from stimulated platelets (2). Several studies (3–5) have suggested that phosphatidylserine (PS)† might play a specific role in prothrombin activation. PS is asymmetrically distributed to the cytoplasmic surface of resting platelet membranes (6) but is exposed when human platelets are activated (7). It has become clear only very recently that PS regulates the structure and function of factors Xa and Va (8, 10).‡ Here we explore further the extent of this regulation.

Factor V exists in plasma as an inactive, single chain glycoprotein with a molecular mass of 330 kDa. The active form of factor V, FVa, has a central domain removed to yield a heterodimer composed of two chains, a heavy chain ($M_r = 94,000$ in the bovine species; 105,000 in human) and a heterogeneous light chain ($M_r = 74,000$ in FVa1 or 71,000 in FVa2). The heavy and light chains form a tight complex in the presence of a calcium ion (11). The heterogeneity in the light chain is seen in both the bovine and human molecules. In the human form, it appears to arise from glycosylation of Asn218 at the C-terminal end of the light chain (12). Prothrombinase complexes assembled from the two molecular species derived from human plasma are observed to have somewhat different cofactor activities (13, 14). This has been attributed to substantially different affinities for binding to membranes (13). However, our lab has reported that the two forms of both bovine and human FVa bind to membranes with only ~3-fold different affinities (12, 14). This suggests that differences in the ability to support prothrombinase activity must reflect either different binding between factors FXa and FVa1 versus FVa2 or different intrinsic activities of the FXa/FVa1 and FXa/FVa2 complexes. Although our results have favored the former possibility (14), it has been difficult to prove this unambiguously because it is difficult to measure precisely the interaction between FXa and FVa on a membrane surface (15).

The presence of FVa in a reaction mixture is critical to obtaining a maximal and physiologically significant rate of prothrombin activation (1). As a result, intense effort has been devoted to discerning the role of this cofactor in thrombin generation. The picture that emerges is that factor Va binds tightly to acidic lipid membranes ($K_D \sim 2 \text{ nM}$) (16) and then tightly binds factor Xa already bound to that surface ($K_D \sim 1 \text{ nM}$) (15), making it effectively an anchor to assemble the prothrombinase at the low concentrations of factor Xa and membranes expected to be found in vivo (17). Because the interaction between factors Va and Xa in solution is much weaker ($K_D \sim 1–3 \mu M$) (18) than that reported on a membrane (15), it has been
speculated that either the membrane alters these two proteins so as to enhance their interaction or the presence of both proteins in the reduced dimensionality of the membrane surface leads to tighter association than would be seen in three dimensions (15). Again, the difficulty of assessing interactions between proteins on a membrane surface makes it hard to distinguish between these two possibilities.

We have shown previously that a soluble form of PS, C6PS, binds to and FXn (8). Binding is accompanied by conformational changes in FXn that alter protein function (8). Four C6PS molecules also bind to bovine FVa to induce conformational and functional changes (10). We present here compelling evidence that PS binding to these two proteins regulates their assembly into an active complex and thus regulates the functioning of the prothrombinase complex. In doing so, we answer five questions. First, do the two isoforms of factor Vn (FVa1 or FVa2) form complexes of different affinities with FXn? Second, does soluble C6PS bind with similar affinities to these two isoforms? Third, does C6PS binding alter the affinities of the complexes between the two FVa isoforms and factor FXn? Fourth, can differential binding of the two FVa isoforms to FXn account for the apparently different cofactor activities of these two isoforms? Fifth, if C6PS induces tight association between FVa and FXn, in solution, is this sufficient to trigger formation of a prothrombinase complex in solution? If so, our ability to study the structure and function of this critical enzyme complex would be dramatically enhanced.

**EXPERIMENTAL PROCEDURES**

**Materials**

Ecarin from Echis carinatus snake venom, hepamin, and EGTA were purchased from Sigma. Dansylarginine-N-(3-ethyl-1,5-pentanediyi)lami-

ide (DAPA) was obtained from Hematologic Technologies Inc. (Essen,

Junction, VT). The FXn as well as the Vn-specific substrates S-2765 and

S-2238 were purchased from Ab Kabi Diagnostica (Molndal, Sweden).
The sodium salt of 1,2-dicaproyl-sn-glycero-3-phospho-l-serine (C6PS)

was purchased from Avanti Polar Lipids (Birmingham, AL). [5-(Di-
methylamino)-1-naphthalenesulfonyl]glutamylglycylarginyl chlorom-
edithyl ketone (DEGR-CK) was purchased from Calbiochem.

**Methods**

**Phospholipid and Protein Preparation—**C6PS stock solutions, bovine FVa, FVa1, and FVa2 were prepared as reported previously (8, 14).

Prothrombin, FXn, and Fxa were purified from bovine plasma (19). The final purity of the proteins was ascertained by SDS-PAGE to be greater than 90%. DEGR-Xa was analyzed by SDS-PAGE and visualized under UV of about 17

°/H11003 excitation and emission) were closed between measurements to avoid

any small amount of photobleaching.

Critical Micelle Concentration (CMC) Determination—The diameters of C6PS aggregates were measured by quasi-elastic light scattering (8, 10).

**Fluorescence Titration of DEGR-Xn and FVa1 and FVa2—**Changes in the fluorescence intensity of DEGR-Xn in response to FVa addition were measured at 37 °C using a SLM 48000™ spectrophluorometer (Spectro-

tronics Instruments, Inc., Rochester, NY). Slits (8 × 8 and 4 × 4 nm for excitation and emission) were closed between measurements to avoid photo-degradation of the sample. Buffer solutions were filtered using 0.2-μm filters (Nalgene Co., Rochester, NY). A stirred microcuvette (Hellma Cells, Jamaica, NY) was initially charged with a solution of 1 nM DEGR-Xn and 5/50 nM FVa/FVa2 and then rinsed with buffer. This procedure prevented the formation of aggregates on the cuvette walls. To 0.95 ml of DEGR-Xn solution (1 nM DEGR-Xn, in 30, 60, and 400 μM C6PS, 50 mM Tris, 0.1 mM NaCl, 5 mM CaCl2, pH 7.5), FVa or FVa2 were added from stock solutions with 4 min of equilibration, and fluorescence intensity was recorded using an excitation wavelength of 340 nm and an emission wavelength of 550 nm. Several intensity readings were averaged after each addition, and corrected, via controls, for dilution, buffer background, and any small amount of photobleaching.

Fluorescence Titration of DEGR-Xn and FVa1 and FVa2—Changes in FVa1/FVa2 intrinsic fluorescence in response to C6PS was measured similarly, except that samples were excited at 295 nm (slits: 8 nm), and emission was recorded at 345 nm (slits: 8 nm).

**Fluorescence Stopped-flow Measurements—**Rates of thrombin formation from MxiII or of thrombin plus MxiII, formation from prothrombin at 37 °C were estimated from time-dependent changes in the fluorescence of DAPA, an active site inhibitor of the activation products (1). Stopped-flow measurements were performed on the SLM 48000™ spectrophluorometer with 280 nm excitation (4-nm slits) and a 515-nm cut off filter and 8-nm slits in the emission path. Reactions were initiated by rapidly mixing equal volumes (200 μl) of the two solutions of a SLM-Amino Milliflow™ stopped-flow reactor. Syringe A contained substrate solution and DAPA in 50 mM Tris, 150 mM NaCl, 5 mM CaCl2, pH 7.5, and syringe B contained a mixture of factors Xn and Vn and C6PS pre-equilibrated 37 °C for 2–3 min in the same buffer. The final concentration of FXn in the reaction chamber was always maintained as 1 nM, and the concentrations of FVa and C6PS were varied. The substrate/DAPA ratio was 1.5. The initial fluorescence intensity (F0) was obtained from mixing experiments with substrate and DAPA in syringe A and only lipid in syringe B. All fluorescence intensities were corrected for background light scattering. Fluorescence intensity at the completion of the reaction (F) reflected complete conversion of a given substrate to thrombin. The initial rate of thrombin generation was then determined from the initial rate of fluorescence change (5–10% completion) normalized to the intensity at completion times the concentration of substrate in the reaction mixture (23).

**Native Gel Electrophoresis—**Polyacrylamide gels were prepared at five different percentages of cross-linking (5, 6, 6.5, 7, and 8% total acrylamide with bisacrylamide/acylamide being 1:29 in all cases). A mixture of 1 nM factor Xn and 1 nM factor Vn was incubated at 37 °C for 2 min in the presence and absence of 400 μM C6PS, and these two samples were run together with known marker proteins (270 to 14.2

kDa; Sigma) on these five gels in a Bio-Rad Mini-Protein II™ minigel apparatus (Bio-Rad) and stained with colloidal Coomassie Blue (24). Factor Xn and factor Vn at these same concentrations and in the presence of 400 μM C6PS, were run separately as controls. R values in each gel were measured relative to tracking dye. For each protein, a plot of [log(R100) against the percent gel concentration gave a straight line, the negative slope of which is the retardation coefficient (25). A log-log plot of the retardation coefficient against the molecular weights of the marker proteins produced a linear curve from which the molecular weight of the FVaFX complex was determined (26).

**Data Analysis**

FXn-FVa Interaction—There were two approaches to data analysis. The first recognized that FXn and FVa bind two and four C6PS molecules, respectively, and that their interaction with each other will depend on how many sites are occupied in each protein. This approach, described under the “Appendix” in its simplest form, requires knowl-
edge of the mechanism of C6PS binding to the two proteins as well as an adjustment of the values of some unknown parameters, but provides physical insight into our results if one is willing to accept some uncertainty in parameter values. Alternatively, one can simply acknowledge that the interaction between DEGR-Xn and FVa determines on C6PS but make no assumptions about the mechanistic details of this dependence. In this instance, several equilibria are replaced by a single effective DEGR-Xn + Vn binding equilibrium with an apparent KdXn that depends on C6PS concentration (Equation 1).

![Equation 1](\text{DEGR-Xn} + Vn \rightleftharpoons \text{DEGR-Xn} \cdot \text{Vn})

The total concentration of DEGR-Xn-FVa complex at any given C6PS concentration ([DEGR-Xn-Vn]tot) is then given by the familiar expression for two-species binding (Equation 2),

![Equation 2](\text{[DEGR-Xn-Vn]}_{\text{tot}} = \text{[DEGR-Xn]} \cdot \text{[Vn]} + \text{[DEGR-Xn]} \cdot \text{[Vn]} + \text{[DEGR-Xn]} \cdot \text{[Vn]} + \text{[DEGR-Xn]} \cdot \text{[Vn]})

where the concentration terms all are “total” concentrations of each species in a reaction mixture. The change in fluorescence signal of DEGR-Xn was taken as proportional to the fraction of DEGR-Xn bound to FVa (Equation 3)
Effect of C6PS on the cofactor activity of FVa1 and FVa2 during prothrombin activation. Initial rates of active site formation as monitored by DAPA fluorescence were determined as a function of added C6PS. Stopped-flow fluorescence measurements were performed at 37 °C at various concentrations of C6PS in a buffer containing 50 mM Tris-Cl, pH 7.5, 150 mM NaCl, and 5 mM CaCl2. Reaction mixtures contained 1 μM prothrombin, 5 μM DAPA, 1 μM factor Xa, along with either 50 μM factor Vα (circles), 5 μM FVa2 (squares), or 5 μM of the natural mixture of these two isoforms (triangles). The C6PS CMC under the condition of this experiment (except that prothrombin was omitted) was determined by QELS to detect C6PS aggregates, as indicated in the inset. Prothrombin has been shown previously to bind C6PS very weakly (Kd >5 mM) and thus to have no effect on its CMC at a concentration of 2 μM (8).

\[
\frac{F - F_0}{F_\text{sat} - F_0} = \frac{[\text{DEGR-Xa-Vα}]}{[\text{DEGR-Xa}]} \left(\frac{[C6PS]}{[\text{C6PS}]_0}\right)
\]

where the fluorescence parameter F0 was fixed as the DEGR-Xa fluorescence before titration began and Fsat is the fluorescence at saturation, which was obtained along with Kd by regression of the data to Equations 2 and 3.

The interaction between FXa and FVa1 or FVa2 was also detected by an increase in the rate of prothrombin activation. In this case, the same analysis was used except that the increase in initial rate of activation (V) and [Xa] replaced F and [DEGR-Xa] in Equation 3.

The kinetics of MzIIa activation were parameterized according to the normal Michaelis-Menten model (Equation 4),

\[
V_{\text{cat}} = \frac{[MzIIa]}{[MzIIa] + k_{\text{cat}}} \times \frac{[Xa-Vα]}{[C6PS]}
\]

where the enzyme concentration ([Xa-Vα]C6PS) was calculated using the Kd values and Equation 2 described above.

In all cases, regression of data sets to these models was performed using SigmaPlot 2000 (SPSS Inc., Chicago, IL).

RESULTS

Effect of C6PS on Prothrombin Activation by FXa in the Presence of FVa1/FVa2—Initial rates of FXa-catalyzed prothrombin activation (see “Methods”) were determined as a function of added soluble C6PS concentration (Fig. 1). In the presence of 5 μM FVa2, the initial rate saturated at about 100 μM C6PS (squares), but for 50 μM FVa1 (higher concentration needed to record rates comparable with those seen with FVa2) the initial rate increased rapidly up to a value larger than seen for FVa2 by 100 μM C6PS and then slowly increased well beyond this rate up to and beyond 400 μM C6PS (triangles). For 5 μM FVa1, where the enzyme concentration of these two isoforms, the initial rate saturated at about 100 μM C6PS (circles) at about 2/3 the rate seen with FVa2. Because FVa1 is roughly 2/3 FVa2, it seems that the cofactor activity of the natural mixture is dominated under our conditions by a complex containing the lighter FVa2 isoform.

It is important to know whether the effects recorded in Fig.
Fig. 3. Binding of FVa1 and FVa2 to DEGR-Xa as detected by changes in fluorescence. Binding of factor FVa to DEGR-FXa, in the absence (open symbols) and presence (closed symbols) of 400 μM C6PS was detected by the change in DEGR fluorescence intensity. Small aliquots of FVa1 (circles) or FVa2 (squares) were added at 2°C to a 2-ml solution of DEGR-Xa (1 nM in 5 mM CaCl2, 20 mM Tris, 100 mM NaCl, pH 7.5). The inset shows the DEGR-Xa response to FVa2 in the presence of C6PS as an expanded concentration scale. The lines through the data were obtained by least squares regression to a simple stoichiometric binding model (Equations 1–3 under “Experimental Procedures”) with best fit Kd values given in the text.

0.6 μM for FVa1 and 12 ± 0.7 μM for FVa2) and changes in intrinsic fluorescence.

We determined the CMC of C6PS (Fig. 2, inset) under exactly the conditions used for our titration (0.33 μM FVa) to be ~750 μM, which is consistent with what we have found elsewhere in the presence of FVa1 (10) and far above the lipid concentrations used for our C6PS titrations of FVa1/FVa2. This confirms that the responses we have seen are to a molecular rather than aggregated form of C6PS.

Binding of FVa1 and FVa2 to DEGR-Xa in the Presence and Absence of C6PS—Fig. 3 shows the increase in DEGR-Xa fluorescence with addition of FVa1 (squares) or FVa2 (circles) in the absence of C6PS (open symbols). Both FVa1 and FVa2 bind similarly to DEGR-Xa in the absence of C6PS (Kd = 1.0 ± 0.07 μM for FVa2 and 1.3 ± 0.07 μM for FVa1). These Kd values are in essential agreement with those derived from prothrombin activation kinetics, fluorescence experiments (18), affinity chromatography studies (27), and ultracentrifugation experiments (28). The presence of 400 μM soluble PS (filled symbols) dramatically enhanced the interaction of FVa2 with FXa (Kd = 2.0 ± 0.02 nM; inset) but had little effect on the interaction of FXa with FVa1 (Kd = 1.1 ± 0.07 μM). Thus, at least part of the different effects of C6PS on the activation of prothrombin by FXa in the presence of FVa2 or FVa1 (Fig. 1) reflects a dramatic difference in how C6PS influences the FVa1- or FVa2-enzyme interaction.

Binding of FVa1 and FVa2 to FXa Increases Proteolytic Activity—Because the presence of C6PS influenced the interactions of FXa and FVa2 with FXa, so differently, we could not compare the cofactor activities of FVa1 and FVa2 under a common set of conditions. For this reason, initial rates of active site formation from prothrombin were monitored by DAPA fluorescence in the presence of 400 μM C6PS and are shown in Fig. 4 and its inset on different cofactor concentration scales. The rate saturated upon addition of very low concentrations of FVa2 (inset) to yield an initial rate of catalysis by the FXa-FVa2 complex of 100 ± 2 nM/s. However, the initial rate of prothrombin activation did not saturate with addition of FVa1 (circles), and an estimate of the rate catalyzed by the FXa-FVa1 complex had to be made from the hyperbolic fit shown by the solid line in Fig. 4 (540 ± 5 nM/s). Just as for titration of DEGR-Xa fluorescence, the Kd of FVa2 interaction with FXa obtained from these hyperbolic fits (2.8 ± 0.3 nM) was ~350-fold smaller than that for FXa interaction with FXa (1.0 ± 0.15 μM). It seems from these results that FVa1 is the more effective cofactor but that it has limited ability to bind FXa in a PS-dependent fashion. This explains why the behavior of the FXa-FVa2 complex seemed to dominate the whole FVa experiment presented as open circles in Fig. 1.

Binding of DEGR-Xa to FVa2 in the Presence of Varying C6PS—Next, we titrated DEGR-Xa with FVa2 at fixed C6PS concentrations of 30, 60, 150, and 400 μM (circles, squares, triangles, and diamonds, respectively, in Fig. 5). Each titration curve was fitted as described under “Experimental Procedures” to obtain effective dissociation constant, Kd', for the Xa-FVa2 complex. This yielded the variation with C6PS concentration of Kd' shown in the inset in Fig. 5 as well as fluorescence parameters (F0 and Fsat, Equation 3) characterizing the complexes at different C6PS concentrations (not recorded in Fig. 5). Note that Kd' obtained in this way is not related to the Kd of interaction between FXa and FVa1 in solution that is known to be ~1–3 μM (18). This is an effective Kd of interaction between DEGR-Xa and FXa in which all C6PS-bound species of DEGR-Xa and FXa are in equilibrium with all possible C6PS-bound complex species (DEGR-FXa-FVa2-C6PS) (see Equation 1 and “Appendix”). Because the species present in this equilibrium vary with C6PS concentration, Kd' also varies with C6PS concentration.

Characterization of the FXa-FVa2 Complex in the Presence of C6PS by Native Gel Electrophoresis—Native PAGE was performed with FXa/FVa2 samples in the presence and absence of 400 μM C6PS (see “Methods”). The inset to Fig. 5 shows one such gel. FXa and FVa2 samples in the presence of C6PS were also run as controls. A log-log plot of the retardation coefficient against the molecular weights of the marker proteins (circles), FXa (inverted triangle), or FVa2 (4-pointed open star) in the absence of C6PS, and of the FXa-FVa2 complex (5-pointed star) in the presence of C6PS is shown in Fig. 6. From this, we estimated the molecular mass of the complex in the presence of C6PS as 217 ± 2.5 kDa. In the absence of C6PS, no complex appeared (Fig. 6, inset, lane 1), and the measured molecular masses were 45.5 ± 1.0 and 167 ± 2 kDa for FXa and FXa2, respectively. These compare with literature values of 46 kDa (29) for FXa and 168 kDa (30) for FVa2, making the expected molecular mass of the complex 214 kDa. Controls gave measured molecular masses of FXa and FVa2 individually in the presence of C6PS as 45.8 ± 1.2 and 167 ± 2 kDa. These results demonstrate unequivocally the complex that is implied by our DEGR-Xa fluorescence and FXa activity measurements and establish the stoichiometry of this complex as 1:1 as expected (1). An additional control (lane 3) confirmed that FVa1 (measured molecular mass 170 ± 1.92 kDa) did not form a complex with FXa even in the presence of C6PS.

Activation of Meizothrombin to Thrombin in the Presence of FXa—The results in Fig. 5 show that the ability to form a FXa-FVa2 complex clearly increased with C6PS concentration. We next asked whether the ability of that complex to function as a “prothrombinase” also increases with C6PS concentration. Activation of prothrombin to thrombin involves cleavage of two bonds. This means that activation proceeds via two possible intermediates and that four distinct proteolytic reactions must be characterized to define the process (22). We showed that, even in the absence of FVa, PS-containing membranes as well
as C6PS 1) alter the rates of all four reactions, 2) alter the preferred pathway of activation, and 3) cause some intermediate to be converted processively to thrombin without release from the prothrombinase complex (22, 31). In order to avoid these complications in characterizing the activity of the soluble FXa/H18528FVa2 complex, we focused here on only one of the four possible reactions, conversion of the intermediate MzIIa to thrombin. Because the activation of this intermediate to thrombin reflects the prothrombinase-catalyzed cleavage of only one peptide bond, it is reasonable to expect proteolysis to behave according to a Michaelis-Menten formalism.

Fig. 7 shows the initial rates of thrombin formation from MzIIa measured at different lipid concentrations (50, 100, and 200 μM) and as a function of increasing concentrations of MzIIa in the presence of C6PS. The initial rate of active site formation, monitored by DAPA fluorescence, was determined by stopped-flow fluorescence measurements, performed at 37 °C with final concentrations in the mixing chamber of 1 μM prothrombin, 5 μM DAPA, 1 nM factor Xa, 400 μM C6PS, and various concentrations of factor FVa1 or FVa2 (inset) in 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM CaCl2. The lines drawn through the data were obtained by fitting the data to a simple stoichiometric binding model (Equations 1–3) to determine $K_d^{eff}$ values given in the text.

FIG. 4. Initial rate of active site formation as a function of added FVa2 and FVa1 in the presence of C6PS. The initial rate of active site formation, monitored by DAPA fluorescence, was determined by stopped-flow fluorescence measurements, performed at 37 °C with final concentrations in the mixing chamber of 1 μM prothrombin, 5 μM DAPA, 1 nM factor Xa, 400 μM C6PS, and various concentrations of factor FVa1 or FVa2 (inset) in 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM CaCl2. The lines drawn through the data were obtained by fitting the data to a simple stoichiometric binding model (Equations 1–3) to determine $K_d^{eff}$ values given in the text.

**DISCUSSION**

We set out in this work by asking five questions posed in the Introduction, whose answers have led to the following significant new conclusions.

1) The two isoforms of factor $V_a$ (FVa1 or FVa2) bind with nearly identical affinities to FXa (Fig. 3).

2) Soluble C6PS binds with the same affinity to the two isoforms of $F_a$ (Fig. 2).

3) In the presence of 400 μM C6PS, the binding of FVa2 with factor FXa is 300–350 times tighter than is the binding of FVa1.
whose affinity for FX<sub>a</sub> was not significantly influenced by C6PS (Fig. 3).

4) C6PS stimulates the assembly of FVa<sub>2</sub>, as opposed to FVa<sub>1</sub>, into a V<sub>a2</sub>X<sub>a</sub> complex. This means that at least some of the difference in the cofactor activities of the two isoforms of FVa derives from their different abilities to bind FX<sub>a</sub>. However, it appears also that the FX<sub>a</sub>-FVa<sub>2</sub> complex is more active than the FX<sub>a</sub>-FVa<sub>1</sub> complex.

5) Perhaps the most significant conclusion from this study is that the prothrombinase complex assembled in the presence of soluble C6PS + FVa<sub>2</sub>, without a membrane surface, catalyzes MzII<sub>a</sub> activation to II<sub>a</sub> at a rate (apparent <i>k</i><sub>cat</sub>/<i>K</i><sub>m</sub> = 1 × 10<sup>6</sup> M<sup>-1</sup> s<sup>-1</sup>) that is comparable with the rate reported in the presence of a PS-membrane surface and FVa<sub>a</sub>.

The first three conclusions are directly evident from our data, as referenced above, but still deserve brief comment here. These three conclusions require that the reader be convinced that C6PS binds to individual sites on FX<sub>a</sub> and the FVa isoforms rather than condensing with them to form macromolecular complexes or micelles. That FX<sub>a</sub> bound to and was activated by C6PS well below the CMC of this short chained phospholipid has been thoroughly documented (8). Another paper from our lab (32) has confirmed this and shown by equilibrium dialysis that only two C6PS bind to FX<sub>a</sub> as follows: one to a site that regulates activity and is located in the epidermal growth factor domains linked to the Gla domain at the N terminus of the molecule, and one that competes with synthetic substrate binding and probably binds in a functionally insignificant fashion to the amine binding locus of the S2-S3 substrate recognition motif (33). Recent work from our lab has documented by equilibrium dialysis, intrinsic fluorescence, CD spectroscopy, and functional analysis that FVa binds four C6PS, also below the C6PS CMC, with resulting structural and C6PS-specific functional changes (10). One of these four C6PS binds to a single site in the C2 domain (34); the other three sites have not been located.

One might argue that some lipid complex smaller than a micelle could be responsible for the influence of C6PS on the structure and function of FVa<sub>a</sub> and FX<sub>a</sub>, i.e. that the effects of C6PS on FVa<sub>a</sub> and FX<sub>a</sub> might be due to locally organized surfaces rather than to individual C6PS molecules binding to individual sites on these two proteins. This is unlikely for two reasons. First, although it is well documented that amphipaths can form both small and large micelles of different shapes (35), the smallest micelle we could detect for C6PS under our conditions was roughly 11 nm in diameter and contained only
about 220 lipids (8). This is a very reasonable limiting micelle size for a two-chain amphiphil, because such amphipaths are unlikely to be able to form smaller, spherical micelles (35). Any smaller structure would leave hydrocarbon chains exposed to water, a situation that is thermodynamically very unfavorable (35). The smallest C6PS micelle in the presence of FXa was 26 nm in diameter, whereas the smallest we have detected in the presence of FV was about 70–90 nm in diameter (10). Our results indicate that the mix of FXa and FV considered here lowered the CMC a bit more than either FXa or FV did individually, but led to aggregates about the same size (70–90 nm) as those recorded previously in the presence of FXa alone. This means that many more lipid molecules were needed to accomplish efficient lipid packing in the presence of these proteins. This makes it very unlikely that very small and hydrodynamically undetectable C6PS aggregates could explain our observations. The second, and even stronger, argument against this possibility is provided by the observations that only two and four C6PS molecules were bound at saturation per FXa and FV, respectively (10, 32). This small amount of lipid could not form even a local surface for the binding of these proteins, and we must conclude that specific sites on both FXa and FV bind C6PS in a way that regulates the structure, function, and assembly of the components of the prothrombinase complex.

C6PS Promotes the Assembly of a FXa-FVa Complex, although It Is Less Active than the FXa-FVa Complex—The experiment that originally stimulated this investigation is shown in Fig. 1. The response of a mixture of FXa and either FVa isoform to C6PS is clearly biphasic, with the initial rapid rise in activity seen for both FVa isoforms up to about 20 μM C6PS probably due to the tight association of FVa with C6PS (Fig. 2). The affinity of FXa for C6PS (Kd −110 μM)2 is such that very little FXa can be activated by C6PS at such low C6PS concentrations, so it must be that this rise is due to C6PS binding to FVa to stimulate its association with FXa. This must mean that binding of FVa to FXa in the absence of the activation of FXa by binding of C6PS significantly enhanced the activity of the enzyme. The second phase of the rise in prothrombinase activity occurs over a C6PS concentration range of 10–100 μM, consistent with this being due to binding of C6PS to FXa (8).2 We conclude that C6PS binding to factor Xa still has a significant role in actively facilitating factor Xa even when it is already partially activated by binding to FVa.

It is evident from Fig. 1 that the rates of prothrombin activation in the presence of FVa (or FVa') are quite different. To observe good activity in the presence of FVa, we used the concentration of C6PS (50 nM) that is very close to the Kd of C6PS for FXa in the experiment shown in Fig. 1, and therefore, the rate of prothrombin activation did not saturate in the presence of 400 μM C6PS, whereas in the presence of FVa, saturation occurred at roughly 200 μM C6PS, a concentration for which both FVa and the tight site on FXa were saturated with C6PS. When bound to C6PS, the apparent affinity of FVa for FXa was 2 nM (Fig. 3), meaning that all FVa was bound to FXa under the conditions of our experiments. This means that the observed rate at saturation represents the activity of the FVa-FXa complex. However, because the interaction between FVa and FXa remained weak in the presence of 400 μM C6PS (Fig. 3), titration of the FXa/FVa mixture with C6PS in Fig. 1 did not result in complete incorporation of FXa into FVa-FXa complex, consistent with the observed failure of prothrombin activation to saturate. These observations imply that the activity of the FVa-FXa complex must be much more than 30% larger than that of the FVa-FXa complex. This is supported by the FVa titration presented in Fig. 4, which indicates that the prothrombin-activating ability of the FVa-FXa complex is at least twice that of the FVa-FXa complex. Why might both these isoforms, with different abilities to bind to and activate FXa, be present in plasma? At this point, an answer is not known.

The Solution Prothrombinase Complex Is Optimally Active with Binding of One C6PS to FXa—Under the “Results,” we treated the assembly of the prothrombinase complex in solution by an empirical approach that ignored the fact that several species exist in solution. Here we present a detailed treatment in terms of the actual prothrombinase species likely to be present (see “Appendix”). We have shown that C6PS binding to the tight site on factor Xa enhances the activity of the enzyme (8),2 and binding of C6PS to factor Va enhances its affinity for factor Xa (Fig. 3 and Ref. 10). Thus, the observed decrease of Vmax/Ebound for MzII activation with increasing C6PS concentration (Fig. 7) suggests that binding of a second C6PS to factor Xa might actually inhibit binding to VaPS4 and, therefore, inhibit the activity of the assembled prothrombinase complex. In an effort to test quantitatively this prediction, we adopted a second approach to analyzing the data in Figs. 5 and 7. This approach acknowledges that FXa can bind two molecules of C6PS (32), the first with a Kd of 110 μM. To a reasonable approximation, the second site is occupied sequentially, i.e., only after the first site is occupied,2 with a site dissociation constant Kd(PS4) of 150–1500 μM. The value of this constant is uncertain because it depends on the aggregate state of FXa, being roughly 150–250 μM when FXa exists as a dimer and roughly 1500 μM when FXa is at low concentration,2 as it is in our studies. Because of the existence of two C6PS sites on FXa, it can bind to VaPS4 in any of three putative forms, FXa, FXa, or FXa, with stoichiometric dissociation constants that we cannot determine independently. We estimated these dissociation constants and the activities of the putative species as described under the “Appendix.” This analysis predicts that the presumed species XaPS2VaPS4 (Kd = 2360 ± 66 s−1 or 711 ± 20 s−1) formed with a Kd of 22 ± 6.7 ± 0.1 nM, depending on whether we used Kd(PS4) = 150 or 1500 μM, respectively. The putative VaPS2VaPS4 complex (Kd = 333 ± 13 or 167 ± 7 ± 1 nM) formed with a Kd of 2.2 ± 0.1 or 1.6 ± 0.04 nM, again depending on the value assigned to Kd(PS4). A third species (VaPS4 VaPS4) was present in our model but appeared to be formed with a weak affinity (Kd = 82 ± 13 or 13 ± 0.2 μM) that our activity data (Fig. 7) were adequately described without accounting for it. For comparison to the literature, we calculated pseudo-second order rate constants, kcat/Km, for the species XaPS2PS4 (1.5 to 5.0 × 108 M−1 s−1) or for the species XaPS2VaPS4 (3.5 to 7.1 × 108 M−1 s−1) at 37°C. These rate constants can be compared with a rate constant (9.2 ± 8 × 107 M−1 s−1) reported for the proteolysis of bovine MzII, at 22°C by a FXaFXa complex assembled on a membrane containing PS plus phosphatidylcholine (23). The difference between these pseudo-second order rate constants could derive from a variety of differences between our experiments and earlier kinetic studies. For instance, most previously published kinetic data are for a natural mixture (~2:1) of FVa and FVa, whereas our solution prothrombinase contains only FVa. Very little is known about the abilities of these two isoforms to form a productive prothrombinase on a PS-containing membrane, although there seems not to be as dramatic a difference (13, 14) as we have observed in solution. Until these and other issues are addressed, it would be premature to conclude that the prothrombinase assembled in solution is more active than that assembled on a membrane. To make this comparison properly, the rates on a membrane and in the presence of C6PS would need to be compared in the same lab under the same experimental conditions. A detailed kinetic analysis of the solution-assem-
bled human prothrombinase is underway and will accomplish this comparison.

Whereas the uncertainty in $K_d^{XaPS4}$ leads to considerable uncertainty in $k_{cat}$ estimates for the $X_aPSVaPS_4$ or $X_aPSVaPS_4$ species and in the $K_d$ values for forming these species, the results still clearly indicate that occupancy of the second C6PS site on FX$_a$ promotes formation of the prothrombinase complex but inhibits its proteolytic activity. At first glance, this is quite surprising, but it is actually not an unreasonable possibility, because we have shown that binding of C6PS to the second FX$_a$ site competes with synthetic substrate binding to the active site of FX$_a$ (32). Although this does not seem to interfere with the FX$_a$ proteolytic activity in the absence of FX$_a$ (8), our results indicate that C6PS binding to the second site on FX$_a$ bound to FX$_a$$_{as}$ may interfere with formation of a productive enzyme-substrate complex.

Implications for the Role of Platelet PS in Regulating Blood Coagulation—An ATP-dependent, amine-phosphatide-specific pump maintains PS asymmetry across the resting platelet membrane (36). However, PS is exposed on membrane vesicles that appear when human platelets are activated (2, 7, 37). It has been widely believed that membrane vesicles, either those released from activated platelets or synthetically produced membranes that model these (1), were essential for assembly of an active prothrombin-activating complex. Our results show that a soluble form of PS can assemble a fully active prothrombinase in solution. This implies that it is the exposure of PS on the surface of activated platelet membranes, not the membrane surface itself, that is crucial to assembly of the prothrombinase complex. It also suggests that PS exposure acts not only to locate factor X$_a$ to the membrane surface but also to activate this enzyme (8, 31). Similarly, PS exposure both locates factor V$_a$$_{as}$ to the membrane and activates it (10) (Fig. 3). Because the factor VIII-dependent factor X-activating complex is highly homologous to the prothrombinase (38, 39), it seems likely that platelet PS also triggers factor X$_a$ formation at the platelet plug. Thus, a single upstream event (platelet activation by thrombin or collagen) can trigger activation of key downstream steps in blood coagulation via exposure of otherwise buried PS. In this sense, platelet membrane PS can be thought of as a second messenger in regulating blood coagulation.

APPENDIX

C6PS binds to two different binding sites of FX$_a$ in roughly a sequential fashion to elicit different structural responses from the two sites$^2$ (Equation A1),

$$X_a + \text{C6PS} \rightleftharpoons X_a\text{PS}$$

$$X_a\text{PS} + \text{C6PS} \rightleftharpoons X_a\text{PS}_4$$  \text{(Eq. A1)}

The first of these sites seems to regulate activity and has a reasonably well defined site-dissociation constant of $K_d^{XaPS}$ $\approx$ 110 $\mu$m, whereas the site-dissociation constant of the second site ($K_d^{XaPS4}$) is less well defined.$^2$ Because binding of four C6PS to FX$_a$$_{as}$ is quite tight ($K_d^{XaPS4}$ = 12 $\mu$m) and follows a simple hyperbolic functionality (Fig. 2), we can treat this in terms of a simple stoichiometric binding equilibrium that is close to saturation under almost any C6PS concentration that we have considered (Equation A2):

$$X_a + 4 \text{C6PS} \rightleftharpoons X_a\text{PS}_4$$  \text{(Eq. A2)}

This means that, at a very low C6PS concentration, we must consider FX$_a$ binding to FX$_a$$_{as}$ ($K_d,XaVa = 1.0 \pm 0.07 \mu$m; Fig. 3). But, at any finite C6PS concentration, almost all FX$_a$$_{as}$ is in the lipid-bound form ($V_a$PS$_i$), and we need consider only binding of three possible FX$_a$ species to this form of FX$_a$$_{as}$. By this reasoning, we simplified the problem from 15 possible to three likely binding equilibria (Equation A3),

$$X_a + V_a\text{PS} \rightleftharpoons X_aV_a\text{PS}_i$$

$$X_a + V_a\text{PS} \rightleftharpoons X_aV_a\text{PS}_i$$

$$X_a + V_a\text{PS} \rightleftharpoons X_aV_a\text{PS}_i$$  \text{(Eq. A3)}

From the inset to Fig. 5, it seems that binding of FX$_a$ to FX$_a$$_{as}$ is not saturated even at 400 $\mu$m C6PS, the maximum concentration considered so as to remain well below the critical micelle concentrations of C6PS in the presence of these proteins. Thus, we could not perform experiments under conditions where only the fully C6PS-saturated form of FX$_a$ bound to the saturated form of FX$_a$. To circumvent this difficulty, all four observed fluorescence titrations in Fig. 5 were fit globally (using ScopP 3.5.1, Simulation Resources, Inc., Berrien Springs, MI) to obtain the dissociation constants $K_{d1}$, $K_{d2}$, and $K_{d3}$ as well as the fluorescence of species $i$ at 1 $\mu$m concentration ($F_i$ values in Equation A4). In doing so, the concentrations of five enzyme species ($X_aPS, X_aPS_VaPS, X_aPSVaPS_4, X_aPSVaPS_4$, and $X_aPS_VaPS_4$) were calculated from the $K_{d}$ values defined by the equilibria defined by Equations A1–A3 and used to calculate $F$ in Equation A4.

$$F = F_{XPS} \times [X_aPS] + F_{XPS} \times [X_aPSVaPS] + F_{XPS} \times [X_aPSVaPS] + F_{XPS} \times [PS_XVaPS] + F_{XPS} \times [PS_XVaPS]$$  \text{(Eq. A4)}

The procedure used to accomplish this took into account the conservation of all species ($X_a$, $V_a$, and PS) using an iterative procedure to deal with the linkage between equilibria A1–A3 (40). Of the five enzyme species considered in Equation A4, only three ($X_aPSVaPS_4, X_aPSVaPS_4$, and $X_aPSVaPS_4$) were presumed to contribute significantly to activity as measured by the initial rate of activation of MzII$_a$ to thrombin. To estimate the activities of these species, we assumed that each enzyme species ($E_i$) processed MzII$_a$ ($S$) according to the Michaelis-Menten mechanism ($E_i + S = ES \rightarrow E_i + P$). Then, we set up differential equations for the rate of appearance of thrombin (P) in terms of the concentrations of substrate (MzII$_a$) and three enzyme species, and we solved these by making the standard assumptions of the Michaelis-Menten model, yielding Equation A5,

$$V_{mi} = \sum_{i=1}^{3} [E_i] \times k_{cat} \times \frac{[MzII_a]}{[MzII_a] + K_i}$$  \text{(Eq. A5)}

Because fitting individual substrate-response curves in Fig. 7 to the Michaelis-Menten model gave a roughly constant value of $K_{d}$ = 0.47 ± 0.03 over a range of C6PS concentration (Fig. 7), we assumed that $K_{d}$ was the same for each prothrombinase species present, and we fixed this value and adjusted $k_{cat1}$, $k_{cat2}$, and $k_{cat3}$ to fit simultaneously, using SigmaPlot 2000, all data sets in Fig. 7 according to Equation A6.

$$V_{mi} = ([X_aPSVaPS_4] \times k_{cat1} + [X_aPSVaPS_4] \times k_{cat2} + [X_aPSVaPS_4] \times k_{cat3} \times \frac{[MzII_a]}{[MzII_a] + K_{d}}$$  \text{(Eq. A6)}

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