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BRIEF REPORT

Stoichiometric analysis reveals a unique phosphatidylserine binding site in coagulation factor X

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Abstract

Background: Cellular trauma or activation exposes phosphatidylserine (PS) and the substantially more abundant phospholipid, phosphatidylethanolamine (PE), on the outer layer of the plasma membrane, thereby allowing binding of many blood clotting proteins. We previously proposed the Anything But Choline (ABC) hypothesis to explain how PS and PE synergize to support binding of clotting proteins with gamma-carboxyglutamate (Gla)-rich domains, which posited that each Gla domain binds to a limited number of PS molecules and multiple PE molecules. However, the minimal number of PS molecules required to stably bind a Gla-domain–containing blood clotting protein in the presence of excess PE was unknown.

Objective: To test the ABC hypothesis for factor X by determining the threshold binding requirement of PS molecules under conditions of PS–PE synergy.

Methods: We used surface plasmon resonance to investigate the stoichiometry of factor X binding to nanoscale membrane bilayers (Nanodiscs) of varying phospholipid composition.

Results and Conclusions: We quantified 1.05 ± 0.2 PS molecules per bound factor X molecule in Nanodiscs containing a mixture of 10% PS, 60% PE, and 30% phosphatidylcholine. Hence, there appears to be one truly PS-specific binding site per Gla domain, while the remaining membrane binding interactions can be satisfied by PE.

KEYWORDS
factor X, membranes, phosphatidylserines, phospholipids, surface plasmon resonance

1 | INTRODUCTION

The anionic phospholipid, phosphatidylserine (PS), is essential for reversible membrane binding of a number of clotting proteins.[1] Indeed, seven clotting proteins bind to PS-containing membranes via their γ-carboxyglutamate (Gla)-rich domains.[1] The surfaces of healthy, resting cells are anticoagulant because PS and phosphatidylethanolamine (PE) are sequestered to the inner leaflet of the plasma membrane, with phosphatidylcholine (PC) and sphingomyelin being the most abundant phospholipids in the outer leaflet.[2] Cellular injury or platelet activation leads to loss of membrane asymmetry and externalization of PS and PE, thus creating binding sites for clotting proteins.[1]

Artificial membranes containing PS dramatically enhance many clotting reactions, with maximal rates seen with 20%–40% PS.[3,4] However, mammalian plasma membranes typically have no more than about 10% PS, while the relatively more abundant glycerophospholipids, PC and PE, account for about 40% and 28%, respectively, of the phospholipid content.[5,6] Clotting reactions on liposomes with

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high PS content are therefore conducted under conditions far from the true physiological membrane composition. In 1994, Smirnov and Esmon[7] reported that PE synergizes with PS to promote factor Va inactivation by activated protein C, a finding that was subsequently extended by us and others to show that the presence of PE allows as little as 3%–5% PS to support maximal rates of factor X (FX) activation by the tissue factor/factor VIIa complex,[8] prothrombin activation by the FXa/factor Va complex,[9] and factor VIII binding to membranes.[10] These low PS percentages are much closer to those found in plasma membranes, suggesting that the ability of PS/PE synergy to promote membrane binding by clotting proteins has physiologic significance.

In quantitative binding experiments using Nanodiscs of varying PS/PC composition, we previously showed that a membrane binding site for FX consists of 6–8 PS molecules, presumably clustered together via contact with the Gla domain.[11] We examined the mechanism of PS/PE synergy and found that almost any phospholipid tested (except PC) synergizes with PS to promote FX activation by the tissue factor/factor VIIa complex.[12] This led us to propose what we termed the Anything But Choline (ABC) hypothesis, which states that Gla domains bind to membranes via a limited number of "PS-specific" interactions that are satisfied only by PS, plus multiple "phosphate-specific" interactions that are satisfied by the far more abundant PE.[12] The idea is that a total of about 6–8 PS + PE molecules collaborate to create a single FX binding site. Because structural details for Gla domain/membrane interactions are lacking, we felt it would be an important step forward to determine the minimum number of PS molecules that can constitute a FX binding site in the presence of excess PE. We hypothesized that this number would be an integer, and furthermore, that it could be as low as one PS-specific binding site per Gla domain. Accordingly, we now report the results of quantifying the stoichiometry of FX binding to Nanodiscs with a low number of PS molecules per disc and increasing PE content.

Essentials

- Plasma membranes have much more phosphatidylethanolamine (PE) than phosphatidylserine (PS).
- In membranes with excess PE, the minimal number of PS required to bind factor X was unknown.
- We analyzed factor X's membrane binding stoichiometry using surface plasmon resonance.
- One factor X was bound per 1.05 ± 0.2 PS molecule in membranes with excess PE.

2 | METHODS

Phospholipids were from Avanti Polar Lipids, as follows: PC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; PS, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phospho-L-serine; and PE, 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine. Human FX was from Haematologic Technologies. Membrane scaffold protein (MSP1D1) was expressed and purified as described.[13]

Nanodiscs were prepared by self-assembly reactions followed by purification as described,[14] using varying ratios of PC, PS, and PE with MSP1D1. Phospholipid concentrations in samples of purified Nanodiscs were quantified by measuring total phosphate after complete hydrolysis,[15] while MSP1D1 protein concentrations were determined by A_{280} (extinction coefficient: 21 000 M^{-1} cm^{-1}).

Assuming two copies of MSP1D1 per Nanodisc,[14] the ratio of the molar concentration of phospholipid to MSP1D1 represents the number of phospholipid molecules per leaflet. This ratio was used to calculate the molecular mass (MW) of the entire Nanodisc from the MW of MSP1D1 (24.6 KDa) and the component phospholipids. Table 1 gives calculations for a typical set of Nanodisc preparations in this study.

FX binding to Nanodiscs of varying phospholipid composition was quantified via surface plasmon resonance (SPR) as described,[11] using a Biacore T-200 instrument (Cytiva, formerly GE Healthcare). Briefly, Nanodiscs were immobilized by flowing in Loading Buffer (20 mM HEPES pH 7.4, 100 mM NaCl, 0.02% sodium azide) over Ni-NTA Series S Sensor chips, and recording the net change in response units (RU) due to Nanodisc immobilization (RU_{ND}). For most experiments, this was about 770–1390 RU. Increasing FX concentrations were then flowed over Nanodisc-containing sensorchips at 30 μL/min in Loading Buffer plus 5 mM $CaCl_2$ and 0.2% bovine serum albumin. The maximal steady-state RU level was recorded at each input FX concentration (RU_{FX}), then divided by RU_{ND} to yield the RU_{FX}/RU_{ND} ratio (from which background binding to a parallel flow cell with 100% PC Nanodiscs was subtracted). Binding isotherms were generated by plotting RU_{FX}/RU_{ND} versus FX concentration, to which the single-site ligand binding equation (Equation 1) was fitted using GraphPad Prism 8.0:

$$RU_{FX}/RU_{ND} = (B_{max} \times x) / (K_d + x) \quad (1)$$

where B_{max} is the calculated maximal RU_{FX}/RU_{ND} ratio at saturation, x is the FX molar concentration, and K_d is the equilibrium dissociation constant. Phospholipid bilayers and proteins have equivalent refractive indices in solution.[16,17] Therefore, by utilizing B_{max} from Equation 1, we calculated the number of FX molecules bound per membrane leaflet (N_{FX}) at saturation as shown in Equation 2:

$$N_{FX} = ((B_{max}) \times (MW_{ND}/MW_{FX}))/2 \quad (2)$$

in which MW_{ND} is the MW of the Nanodisc, MW_{FX} is the MW of human FX (58.9 kDa), and division by 2 reflects two membrane leaflets per Nanodisc.

3 | RESULTS AND DISCUSSION

Our goal was to determine the minimum number of PS molecules required to bind an FX molecule on nanoscale membrane bilayers

TABLE 1 Calculations for a typical set of Nanodisc preparations used in this study

Nanodisc lipid composition	Phospholipid [μM][a]	MSP1D1 [μM][a]	Phospholipid molecules/ leaflet[b]	N_{PS}[c]	MW_{ND} [kDa]
100% PC	550	9.59	57.4	0	136
50% PS, 50% PC	640	10.6	60.4	30.2	142
10% PS, 20% PE, 70% PC	680	11.9	57.1	5.71	136
10% PS, 40% PE, 60% PC	542	9.92	54.6	5.46	140
10% PS, 60% PE, 30% PC	451	9.31	48.4	4.84	122

Abbreviations: MSP1D1, membrane scaffold protein; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine.

[a]Phospholipid and MSP1D1 concentrations in purified Nanodisc preparations were quantified as described in the Methods section.

[b]Calculated as the ratio of the molar concentrations of phospholipid to MSP1D1.

[c]N_{PS} (the number of PS molecules per leaflet) is the number of phospholipid molecules per leaflet multiplied by the fraction of the input phospholipid that was PS (i.e., 0, 0.5, or 0.1).

in the presence of excess PE. We reasoned that limiting PS would minimize steric hindrance between bound FX molecules at saturation. We previously found that 100% PS Nanodiscs could bind 8.4 FX molecules per bilayer, which is close to the theoretical maximum number of Gla domains that could occupy the bilayer surface (about 10 FX Gla domains, based on cross-sectional area).[11] We therefore considered creating Nanodiscs with 10% PS. Given that Nanodiscs typically have 50–60 phospholipid molecules per leaflet, this should result in Nanodiscs with about 5–6 PS molecules per leaflet. Thus, if the minimum number of PS molecules that constitutes a FX binding site in the presence of excess PE turned out to be 1, we would expect approximately 5–6 FX molecules bound per leaflet at saturation, which is less than the maximal packing of bound FX molecules previously measured on Nanodiscs. If the minimum number of PS molecules necessary to constitute a FX binding site (in the presence of excess PE) were greater than 1, then we would expect even fewer FX molecules bound per leaflet at saturation.

Accordingly, we prepared Nanodiscs with 10% PS and increasing PE content (20%, 40%, or 60% PE, with the balance being PC). As a comparator, we prepared Nanodiscs with 50% PS but no PE (balance = PC). After carefully quantifying the phospholipid and MSP1D1 content of these preparations, we calculated the number of phospholipid molecules per leaflet and MW of each Nanodisc preparation (typical results in Table 1). The calculated numbers of PS molecules per Nanodisc were within the predicted range of 5–6 per leaflet. Note that these calculations assume that the PS content of Nanodiscs reflects the input PS content. We previously quantified this using ^{3}H-labeled PS and showed that the final PS content faithfully recapitulated the percent PS in self-assembly reactions, over a wide range of input PS contents.[11]

3.1 | SPR-based binding analyses of FX with Nanodiscs

We next quantified FX binding to this range of Nanodisc preparations using SPR, with the resulting binding isotherms shown in Figure 1. Saturable, steady-state binding of FX was observed, as shown in a representative raw sensorgram of FX binding to Nanodiscs containing 10% PS, 20% PE, 70% PC (Figure 1A). The binding affinities of FX to Nanodiscs containing 10% PS and varying PE (Figure 1B) were somewhat weaker than that observed with Nanodiscs containing 50% PS (Figure 1C). Thus, the K_d value for FX binding to 50% PS Nanodiscs was 0.31 ± 0.14 μM, in close agreement with our previously reported K_d of 0.38 ± 0.005 μM for FX binding to such Nanodiscs.[18] For Nanodiscs with 10% PS and varying PE, the K_d values were: 1.57 ± 0.19 μM for 10% PS, 20% PE; 1.51 ± 0.54 μM for 10% PS, 40% PE; and 1.28 ± 0.20 μM for 10% PS, 60% PE (compared graphically in Figure 1D).

3.2 | Stoichiometry of FX binding

Table 2 summarizes the results of FX binding experiments to the range of Nanodiscs listed in Table 1, using three separate Nanodisc preparations for each experiment. For discs containing 50% PS, 50% PC, we found one FX binding site per 7.53 ± 1.3 PS molecules, in good agreement with our previous determination of one FX binding site per 7.97 PS molecules using Nanodiscs composed of binary mixtures of PS and PC.[11] When we examined FX binding to Nanodiscs containing 10% PS and varying PE, we found that the number of PS molecules per FX binding site decreased from 1.73 ± 0.4 with 20% PE down to 1.05 ± 0.2 with 60% PE.

These results indicate that the FX Gla domain requires just a single PS molecule to bind to membranes in the presence of excess PE, consistent with the ABC hypothesis[12] and extending it to state that there is only one truly PS-specific binding site in the FX Gla domain. Membranes consisting of binary PE/PC mixtures do not appreciably support FX binding or FX activation by the tissue factor/factor VIIa complex,[8,12] which is also consistent with the notion that at least one PS molecule is required to create a Gla-domain binding site on the membrane, even in the presence of a large excess of PE.

Although there is weak but detectable binding of FX to PE/PC discs,[19] we chose to subtract background binding from 100% PC discs instead of PE/PC discs for the following reason. Nanodiscs

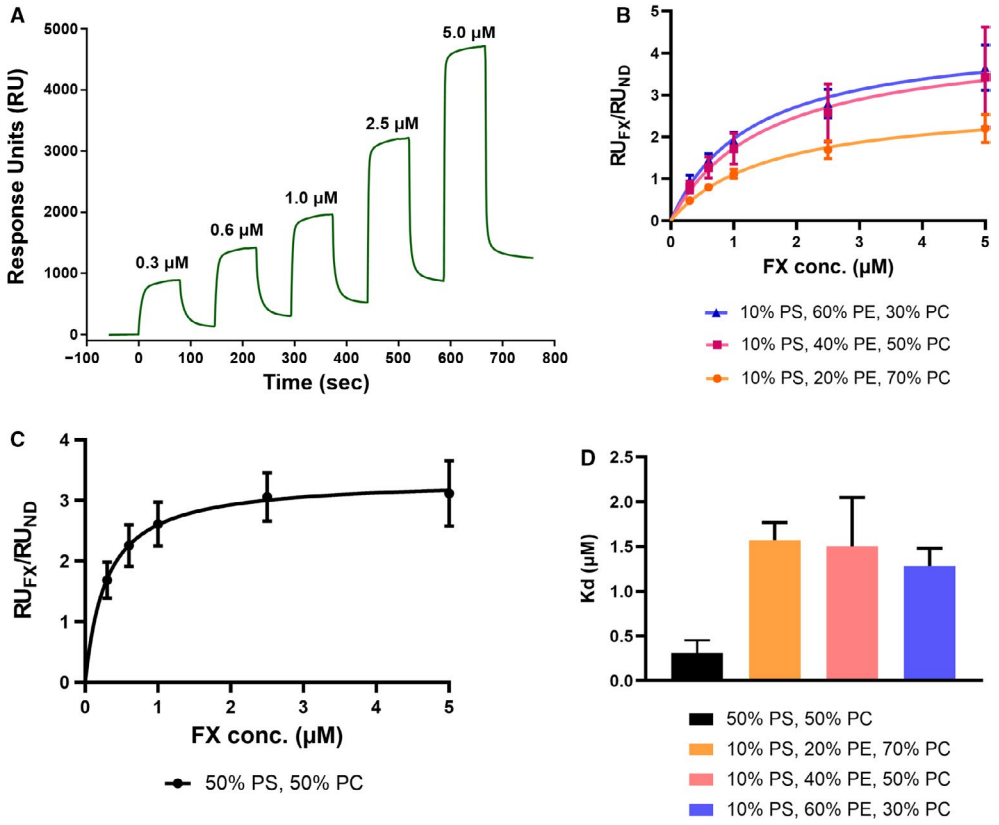

FIGURE 1 Binding of factor X (FX) to Nanodiscs of varying phospholipid composition, quantified by surface plasmon resonance. A, Raw sensorgram of FX binding to 10% phosphatidylserine (PS), 20% phosphatidylethanolamine (PE), 70% phosphatidylcholine (PC) Nanodiscs. B, Binding isotherms of the association of FX with Nanodiscs of the following lipid compositions: 10% PS, 20% PE; 10% PS, 40% PE; and 10% PS, 60% PE. C, Binding isotherm of the association of FX with Nanodiscs containing 50% PS. (In all cases, the balance is PC.) In (B) and (C), RU_{FX}/RU_{ND} is plotted on the *y*-axis, which represents saturating response units (RU) values for FX binding (RU_{FX}) divided by the RU values for Nanodisc immobilization (RU_{ND}), after subtraction of background binding to a reference cell with 100% PC Nanodiscs. D, Bar graphs of K_d values obtained by fitting Equation 1 to the data in (B) and (C). In (B–D), error bars are standard error from three independent experiments

TABLE 2 Stoichiometry of FX binding to Nanodiscs

Nanodisc lipid composition	N_{PS}[a]	N_{FX}[b]	N_{PS}/N_{FX}
50% PS, 50% PC	30.4 ± 2.7	4.09 ± 0.4	7.53 ± 1.3
10% PS, 20% PE, 70% PC	5.31 ± 0.6	3.13 ± 0.6	1.73 ± 0.4
10% PS, 40% PE, 50% PC	4.74 ± 0.6	4.60 ± 2.0	1.16 ± 0.4
10% PS, 60% PE, 30% PC	4.64 ± 0.5	4.50 ± 0.7	1.05 ± 0.2

Note: Data are mean ± standard error; $n = 3$.

Abbreviations: FX, factor X; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine.

[a]N_{PS}, number of PS per leaflet.

[b]N_{FX}, number of FX molecules bound per leaflet at saturation.

with 10% PS, 60% PE, 30% PC have 48.4 phospholipids per leaflet (Table 1), which translates to 4.84 PS molecules and 29.0 PE molecules per leaflet. For such discs, we quantified 5.19 FX molecules bound per leaflet at saturation, meaning 1 FX molecule bound per 0.93 PS + 5.60 PE molecules (= 6.53 non-PC phospholipid molecules). As discussed above, 1 FX molecule binds for every 6 to 8 non-PC lipids, so it follows that virtually all the PE molecules are engaged with FX when it binds at saturation to these discs. Because there should be insufficient free PE molecules left to create another FX binding site under such conditions, we did not subtract FX binding to 60% PE, 40% PC discs.

We currently lack direct structural information on how Gla domains bind to membranes, although molecular dynamics simulations of Gla domain binding to PS-containing bilayers have provided tantalizing clues.[12,20,21] These simulations show Gla domains interacting with PS headgroups in multiple ways, sometimes involving just one charged group on the PS headgroup (such as the carboxylate or the phosphate), and other times involving interactions between multiple atoms in the PS headgroup and a combination of amino acid residues and bound Ca^{2+} in the Gla domain. Interestingly, binding of Gla domain-containing proteins to membrane surfaces requires PS with a headgroup composed of L-serine, not D-serine.[1,12] This suggests that this one truly PS-specific binding site in the FX Gla domain is likely to bind the PS headgroup in a stereospecific manner, although this idea was not tested specifically in the present study. It will be interesting to further identify and characterize the unique PS binding site in the FX Gla domain, perhaps by mutagenesis or structural studies.

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CONFLICTS OF INTEREST

Both authors declare no relevant conflicts of interest.

AUTHOR CONTRIBUTIONS

D.P. performed the experiments, analyzed the results, and wrote the report. J.H.M conceptualized and designed the study, helped in data interpretation, and wrote the report.

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