CONTRIBUTION OF DIFFERENT TYPES OF PHOSPHOLIPIDS IN BLOOD COAGULATION

BY

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DISSERTATION

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Abstract

In case of an injury platelets become activated and proper interaction of blood clotting proteins with membranes of activated platelets is essential for maintaining a balanced hemostasis. Hemophilia and thrombosis are the two extremes of an imbalanced hemostasis where patients with hemophilia suffer from severe bleeding and those with thrombosis from unnecessary blood clots that block normal flow of blood. There are several therapeutics available for treating patients suffering from different types of hemophilia and thrombosis, but lack of specific and efficient medications requires scientist to look for new and more specific activators or inhibitors of the blood clotting cascade. This will not be possible unless we have a detailed knowledge of the nature of the interactions between blood clotting proteins and phospholipid membranes.

In this study, we benefitted from combining the biochemical and biophysical techniques with Nanodisc technology (Sligar lab), molecular dynamics (MD) simulations (Tajkhorshid lab) and solid state-NMR (SSNMR) (Rienstra lab), as a part of a collaborative project at university of Illinois. This approach enabled us to look into the protein-phospholipid interactions in blood clotting at a molecular scale.

The first part of my thesis focuses mainly on γ-carboxyglutamate-rich (GLA) domains of factor X (fX) and prothrombin as representative GLA domains of clotting factors, which have been the subject of several protein-phospholipid interaction studies. Based on the results of this project, we proposed a new mechanism for the binding of GLA domains of clotting proteins to phospholipid membranes. The blood clotting reactions take place on membranes with exposed phosphatidylserine (PS). GLA domains are the most common PS-binding motifs in blood coagulation. A further intriguing aspect of the membrane’s contributions to clotting is that phosphatidylethanolamine (PE) supports little or no clotting activity in the absence of PS, but strongly synergizes with small amounts of PS to enhance many clotting reactions. The mechanism of PE/PS synergy is poorly understood, although several hypotheses have been proposed which rely on specific properties of PE’s phosphoethanolamine headgroup. We proposed a novel, general model for GLA domain binding to membranes; the ABC (Anything But Choline) hypothesis, supported by biochemical studies, solid-state NMR analyses and molecular dynamics simulations. This hypothesis invokes two types of protein-phospholipid interactions: “phospho-L-serine-specific” and “phosphate-specific.” In the latter, the accessible phosphate groups in phospholipids interact with tightly bound Ca\(^{2+}\) in GLA domains. We proposed that based on this model, phospholipids that can satisfy the phosphate-specific interactions should be able to synergize with PS to support fX activation. We showed that almost any glycerophospholipid other than phosphatidylcholine (PC) synergizes strongly with PS to enhance factor X activation by factor VIIa/tissue factor. We proposed that PC and sphingomyelin (the major external phospholipids of healthy cells) provide an anticoagulant surface to healthy cells because their bulky, highly hydrated choline headgroups sterically hinder access to their phosphates. Following cell activation, lysis, or damage, PE and PS are exposed on the outer leaflet where they can collaborate to create binding sites for GLA domains, by providing phosphate-specific and phospho-L-serine-specific interactions, respectively.
The ABC hypothesis study is mainly focused on the GLA domains of \( \text{fX} \) and prothrombin. There are seven proteins in blood clotting that bind reversibly to phospholipid membranes through \( \gamma \)-carboxyglutamate-rich (GLA) domains. Although the GLA domains of these blood clotting proteins are very similar structurally, their membrane-binding affinities vary by almost three orders of magnitude. To our knowledge, no study has compared the membrane binding affinities of all seven human GLA domain-containing blood clotting proteins side by side under the same conditions. Furthermore, the PS stereospecificity of GLA domain binding (i.e., whether they preferentially recognize PS containing L-serine versus D-serine) has not been evaluated for all of these clotting proteins. As the second project of my thesis, we employed surface plasmon resonance binding studies and enzymatic assays to systematically investigate the phospholipid specificity of these seven GLA domain-containing proteins. It has long been thought that GLA domains of blood clotting proteins bind preferentially to bilayers containing PS; but we found, surprisingly, that two of the GLA domain-containing blood clotting proteins (factor VII and protein C) actually bound preferentially to membranes containing phosphatidic acid (PA) or phosphatidylinositol phosphate (PIP), compared to membranes containing PS. Furthermore, PA and PIP strongly enhanced the enzymatic activities of factor VIIa and activated protein C. Incidentally, of the seven blood clotting proteins with GLA domains, factor VII and protein C are known to have the lowest binding affinities for PS-containing membranes. The results of our experiments provide new insights into the membrane binding mechanism for these two GLA domain-containing clotting proteins, through PA- and PIP-specific binding interactions.

Beside these projects, there are two other ongoing projects that were studied briefly as part my thesis. The first one was to investigate the effect of mixtures of \( \text{Ca}^{2+} \) and \( \text{Mg}^{2+} \) on blood clotting reactions. We usually use 2.5 mM calcium in our experiments which is an optimal concentration for supporting blood clotting reactions. Physiologically there are 1.25 mM free calcium and 0.5 mM magnesium in blood, and the crystal structures of GLA domains of fVIIa and fX prepared in mixtures of these ions show occupation of two of these calcium binding sites with magnesium ions. Comparing rates of fX activation by cell surface complexes of tissue factor (TF) and factor VIIa (fVIIa) (or TF:fVIIa enzyme complex) on liposomes prepared with mixtures of PS/PE/PC showed that at lower concentrations of calcium, and on membranes with lower PS contents, the effect of magnesium ions is higher. Increasing the calcium concentration diminishes the effects of magnesium but does not overcome it. These results might be due to the effect of magnesium on phospholipid clustering in phospholipid membranes and also to changes in GLA domain structure and binding affinity for phospholipid membranes. These could be further tested by SS-NMR and MD simulations.

The second ongoing project focuses on testing the ABC hypothesis in the prothrombinase complex (the complex of factors Va and Xa, which are responsible for converting prothrombin to thrombin). For fX activation by TF:fVIIa, it was shown that effects of PE and phosphatidylycerol (PG) are basically similar and that there is a linear enhancement in rates of fX activation when PE is added to PG. Similar experiments with the prothrombinase complex showed that PE is not able to synergize with PG and PA as well as it does with the PS, but that it still shows significant synergy with non-PS phospholipids. This is mainly due to the fact that prothrombinase binds to anionic
phospholipids well, but binds to PS better than the other anionic phospholipids. The phospho-L-seine specific interactions for the prothrombinase complex might not be as stringent as the tenase complex (the complex of TF and fVIIa, which are responsible for converting fX to fXa).

In conclusion, we proposed a new hypothesis for binding of GLA domains of clotting factors to phospholipid membranes and showed a new phospholipid specificity for two blood clotting proteins.
To my father, Saeed, Mehrzad, Mehrafarin, and Amin, for their unconditional love.
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Chapter 1

Introduction

Blood clotting cascade

Blood coagulation is the result of a cascade of enzymatic reactions in which the product of a previous step is the enzyme or cofactor for catalyzing the next step reaction. At the end, a blood clot develops at the site of injury which stops further bleeding.

There are two main pathways forming the blood clotting cascade that eventually merge and result in the formation of blood clots: the intrinsic (contact) and extrinsic (tissue factor). Physiologically, the blood clotting cascade is triggered with the cell surface complexes of tissue factor (TF) and factor VIIa (fVIIa) (or TF:fVIIa enzyme complex) through the extrinsic (tissue factor) pathway (Fig. 1.1).

Fig. 1.1- The intrinsic and extrinsic pathways of the blood clotting cascade. The intrinsic pathway is shown in blue, the extrinsic pathway in magenta and the common pathway in black.
In the case of an injury, blood encounters tissue factor, which together with fVIIa forms the TF:fVIIa enzyme complex. TF:fVIIa (sometimes called “extrinsic tenase”) catalyzes factor X (fX) and factor IX (fIX) activation. Activated fIX(fIXa) along with activated factor VIII (fVIIIa) form the fIXa:fVIIIa (intrinsic tenase) enzyme complex, which catalyzes activation of more fX molecules. Activated fX (fXa) forms an enzyme complex with activated factor V (fVa), fXa:fVa (or prothrombinase enzyme complex), which catalyzes prothrombin (PT) activation to thrombin. Thrombin catalyzes the conversion of fibrinogen to fibrin and also makes an enzyme complex with thrombomodulin that activates protein C. Activated protein C (APC) catalyzes fVa and fVIIIa inactivation via proteolysis.

As is shown in Fig.1.1, several steps of the blood clotting cascade happen on the surface of phospholipid membranes. Phospholipid membranes are an essential part of the cascade and can alter rates of clotting reactions depending upon their phospholipid composition.

To interact with phospholipid membranes, blood clotting proteins (also called clotting factors) are either integrated within cell membranes as integral membrane proteins (e.g., TF and thrombomodulin) or reversibly bind to phospholipid membranes through a discoidin type C2-domain (factor V and factor VIII) or a gamma-carboxyglutamate- rich (GLA) domain (factor VII, factor IX, factor X, prothrombin, protein S, protein Z and protein C). Both GLA domains and C2 domains bind preferentially to negatively charged areas of phospholipid membranes, especially to phosphatidylserine (PS) phospholipids.

The focus of my thesis is the study of GLA domains’ interaction with phospholipid membranes in the tissue factor pathway of the blood clotting cascade.

Fig. 1.2- Front (A) and back (B) view of the TF:fVIIa enzyme complex. Tissue factor is in white and factor Vlla is in grey. Based on the x-ray crystal structure of soluble tissue factor and active site inhibited factor Vlla (Fig. from \textsuperscript{1}).
Blood clotting factors

Tissue Factor

TF is an integral membrane protein with its amino-terminus located outside the cell and its carboxy-terminus located inside the cell. The extracellular part of tissue factor is composed of two fibronectin type III domains that, based on x-ray crystal structures, are joined at an angle of about 120 degrees (Fig.1.2). (The x-ray crystal structures were obtained from soluble tissue factor (sTF) molecules which lack the membrane spanning domain and intracellular domain of tissue factor).

Tissue factor is the cofactor in the first enzyme complex, TF:fVIIa, that triggers the blood clotting cascade. It is present in a variety of cell types, and is particularly found on the surface of adventitial cells, which surround all blood vessels. This guarantees that at the sites of injury, tissue factor is present to trigger the blood clotting cascade.

Tissue factor is also an inducible gene, and its synthesis can be induced in monocytes and smooth muscle cells by a variety of cytokines and inflammatory mediators. Atherosclerotic plaques are rich in TF, which is associated with monocytes and smooth muscle cells. Since the atherosclerotic plaques are separated from blood by a thin layer of endothelial cells, even minor damage to these endothelial cells results in TF exposure to blood which triggers formation of an unwanted thrombus.

Vitamin K dependent coagulation proteins

Some clotting factors have GLA domains permitting reversible interaction with phospholipid membranes. GLA domains are rich in gamma-carboxylated glutamate (Gla) residues. The gamma-carboxylation occurs in the endoplasmic reticulum of liver cells. This gamma-carboxylation requires vitamin K, and as a result these proteins are referred to as vitamin K-dependent coagulation proteins. The enzyme gamma-glutamyl carboxylase catalyzes the addition of a molecule of carbon dioxide to the gamma-carbon of glutamic acid. This reaction requires reduced vitamin K, which is reduced by vitamin K epoxide reductase (VKOR). When the glutamyl residue is gamma carboxylated, vitamin K is returned to the oxidized form (Fig.1.3) The clotting factors, fVII, factor IX (fIX), factor X (fX), prothrombin (PT), protein C (PrC), protein S (PrS) and protein Z (PrZ), have GLA domains for interaction with phospholipid membranes. The interaction
between GLA domains and phospholipid membranes is calcium dependent and is essential for activity of these proteins.

**Factor VII**

Factor VII is the zymogen form of the activated factor VII (fVIIa). FVIIa is a serine protease and one of the clotting factors that reversibly bind to phospholipid membranes through their N-terminal GLA domain. The structure of this protein consists of a GLA domain at the N-terminal followed by two epidermal growth factor (EGF)-like domains and a serine protease domain at the C-terminal (Fig. 1.4). The serine protease domain of fVIIa is homologous to that of trypsin and chymotrypsin and has a catalytic triad. Factor VII is activated by cleavage of the peptide bond
between Arg152 and Ile 153 by fIXa, fXa, thrombin and plasmin. The resulting two peptide chains are held together by a disulfide bond. FVII can also become activated through auto-activation\(^3\). Patients with hemophilia B have very low levels of fVIIa which suggests that fIX plays a significant role in fVII activation\(^4\). Factor VII has the shortest half-life of procoagulant clotting factors and is the only one that circulates in blood in both activated and non-activated forms. Circulating human fVIIa (hfVIIa) is about 1% of the total fVII circulating in blood \(^15,16\).

FVIIa is the catalytic subunit in the TF:fVIIa enzyme complex triggering the blood clotting cascade (Fig.s 1 and 2). The substrates for this enzyme complex are factor IX and factor X, which will be converted to factor Xa and factor IXa\(^3,10\).

**Assembly of TF:fVIIa on PS-membranes**

The first all-atom molecular dynamics (MD) simulation of the TF:fVIIa complex assembled on PS-containing membranes showed that in solution, fVIIa undergoes large hinge motions between the boundaries of the first EGF domain, while sTF is more rigid. When fVIIa is bound to sTF, its motions are significantly restricted, resulting in a rigid sTF:fVIIa complex that sits almost perpendicular to the membrane surface\(^17\). The results of these MD simulations are in agreement with FRET (fluorescence resonance energy transfer) studies that showed that when fVIIa binds TF, elongated fVIIa binds essentially perpendicularly to membrane surface, and its active site is reoriented relative to the membrane, \(^18-20\). Repositioning of fVIIa’s active site promotes optimal attack of its substrates, fX and fIX, on the scissile bond\(^21\).

**Factor X**

Factor X is the zymogen form of activated factor X (fXa) which is another serine protease in the blood clotting cascade with a GLA domain permitting interaction with phospholipid membranes. The building-blocks of factor X are basically very similar to those of factor VIIa: a GLA domain at the N-terminus followed by 2 EGF domains and a serine protease domain at the C-terminus. Factor X is converted to factor Xa when enzymatic activity by TF:fVIIa cleaves the Arg194-Ile195 peptide bond.
(Fig.1.4). Factor Xa along with its cofactor, factor Va, form the prothrombinase complex, which converts prothrombin to thrombin\textsuperscript{10,22,23}.

**Prothrombin**

Prothrombin is the zymogen form of thrombin, another clotting factor with a GLA domain and a serine protease domain. The structure of prothrombin consists of a GLA domain, and two Kringle like domains followed by the serine protease domain. Cleavage of two peptide bonds in prothrombin at Arg284 and Arg320 results in the formation of thrombin, an active serine protease that catalyzes conversion of fibrinogen to fibrin (Fig.1.4)\textsuperscript{10,22,23}.

![Diagram of vitamin-K dependent clotting factors](image-url)
Protein C

Protein C is another clotting factor that has a GLA domain. The structure of protein C consists of a GLA domain at the N-terminus, followed by 2 EGF domains and a serine protease domain at the C-terminus. Protein C is activated by thrombin bound to its cofactor, thrombomodulin, by a cleavage at Arg169 (Fig.1.4). Activated protein C, along with its cofactor, protein S, catalyzes factor Va inactivation via proteolysis 10.

The cell-based model of blood coagulation

The waterfall model (Fig.1.1) of blood clotting divides blood coagulation into two separate and redundant pathways, the intrinsic and extrinsic. This model does not explain some of the in vivo events (reviewed by Smith 6), in which deficiency in certain parts of the extrinsic or intrinsic pathway results in bleeding problems, though the other pathway is still intact. This suggests that the intrinsic and extrinsic pathways are not independent generators of fXa.

The cell-based model of coagulation24 newly takes into account the role of cells in blood coagulation and can explain the interaction between the intrinsic and extrinsic pathways. This model is divided into initiation, amplification and propagation phases (Fig.1.5).

Initiation: The initiation phase occurs on TF-bearing cells where they are exposed to blood at the site of injury. TF is the physiologic trigger of blood clotting, forming complexes with the 1% of fVII that circulates in form of fVIIa. TF:fVIIa activates small amounts of fIX and fX. FXa can activate fV, although the rate of this reaction is very slow. Small amounts of fXa make complexes with small amounts of fVa to generate small amounts of thrombin. This fXa is restricted to the surface of TF-bearing cells, and if it is dissociated from these membranes, becomes inhibited by Tissue Factor Pathway Inhibitor (TFPI) or antithrombin (AT). FIXa is not inhibited by TFPI, and its inhibition by AT is much slower than that of fXa. So it can dissociate from TF-bearing cells and move to platelet membranes6.

Amplification: The thrombin generated in the initiation phase diffuses away from TF-bearing cells and activates platelets, which results in changes in the shapes of platelets, changes in phospholipid distribution in their membranes, and makes them secrete the
contents of their granules. Thrombin also activates fXI, fV and fVIII on platelet membranes.

Propagation: Once platelets are activated, the small amounts of fIXa generated in the initiation phase, along with more fIXa generated by fXIa, combine with fVIIIa to make the intrinsic tenase complex on membranes of activated platelets and generate a large amount of fXa. This along with fVa, generates more thrombin molecules. Thrombin converts fibrinogen to fibrin 6.

**Phospholipid membranes**

**General properties of phospholipids and phospholipid membranes**

Phospholipids are amphipathic molecules, with hydrophilic headgroups and hydrophobic hydrocarbon chains. As amphipathic molecules, phospholipids tend to self-assemble in polar solvents with their hydrocarbon chains facing each other (entropy driven) and their headgroups facing outward, forming phospholipid membranes 25. The structure and ionization constants of principal phospholipids in cell membranes are shown in Fig.1.6.

Phospholipid membranes are more than just barriers between the inner and outer environment, and more even than just a surface that can support enzymatic reactions. Membranes actively affect biological events, depending upon their phospholipid composition. Phospholipids provide cell membranes with different charges, structures, and different binding sites for proteins, depending on the nature of their headgroups. Some examples are mentioned below.
As is known by the ionization constants, phosphatidylcholine (PC) and phosphatidylethanolamine (PE) are neutral, while PS, phosphatidic acid (PA) and phosphatidylinositol (PI) are negatively charged, at physiologic pH. The anionic phospholipids can provide binding sites for membrane-binding proteins, which can either bind to these phospholipids as general anionic phospholipids or bind specifically to either one of them as the PA- or PS- or PIP-binding proteins.

Since in PE and PA the diameters of headgroups are less than that of their hydrocarbon chains, these phospholipids are considered non-lamellar phospholipids and can introduce curvature into phospholipid membranes. Additionally, PA is a key precursor in phospholipid metabolism from which PS, PE and PC and PI can be synthesized. This leads to constant changes in PA concentration in cell membranes, which affects the structure of membrane and the signaling pathways involving PA.

<table>
<thead>
<tr>
<th>Phospholipid</th>
<th>Ionization Constant (pKa)</th>
</tr>
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<tbody>
<tr>
<td>POPC</td>
<td>1.0</td>
</tr>
<tr>
<td>POPE</td>
<td>1.7 11.25</td>
</tr>
<tr>
<td>POPS</td>
<td>2.6 11.55 5.5</td>
</tr>
<tr>
<td>POPA</td>
<td>3.0 8.0</td>
</tr>
<tr>
<td>POPI</td>
<td>2.5</td>
</tr>
</tbody>
</table>

Fig. 1.6- The structure and ionization constants of main phospholipids in cell membranes (from the Avanti polar lipids website).
Importance of phospholipid membranes for blood clotting reactions

Phospholipid membranes are an important part of the blood clotting cascade and can alter rates of clotting reactions depending upon their phospholipid composition. Beside that, blood coagulation is meant to be localized to the site of injury and the fact that most of the steps of the blood-clotting cascade happen on phospholipid surfaces ensures this. Blood clotting reactions mainly happen on the surface of injured endothelial cell and activated platelets. The membranes of activated platelets particularly support the tenase and prothrombinase reactions.

On phospholipid membranes, the apparent $K_m$ of the reaction substrates decreases from above to below these proteins’ plasma concentrations, which is mainly due to higher local concentrations around the phospholipid membrane. Moreover, the cofactors increase the $V_{max}$ of the enzymatic reaction, and together with $K_m$ improvements, the catalytic efficiency ($k_{cat}/K_m$) of the enzymatic reactions is significantly enhanced on phospholipid membranes. Additionally, on phospholipid membranes coagulation factors can transfer to another enzyme complex on the same phospholipid membrane through two-dimensional transfer between complexes. This further enhances the rate of clotting reaction on phospholipid membranes.

Phospholipid asymmetry

The concept of phospholipid asymmetry was first described in erythrocytes and platelet membranes. This lipid asymmetry has been studied most in erythrocyte membranes with their outer leaflet containing about 80% PC and SM and 20% PE, PA, PI and PIP2 and no PS or PIP.

Generally, phospholipids have choline headgroups (phosphatidylcholine, PC, and sphingomyelin, SM) in the outer leaflet, and the aminophospholipids (PS and PE) are in
the inner leaflet of cell membranes (Fig. 1.7). This asymmetry is a result of activity of two ATP-dependent translocases, flippase and floppase. The flippase is an aminophospholipid translocase now known as P4 ATPase, and transfers aminophospholipids, PS and PE, from the outer leaflet of cell membranes to the inner leaflet. Floppase transfers phospholipids from the inner leaflet to the outer leaflet. The coordinated action of these two transporters results in a dynamic, asymmetric steady-state distribution of phospholipids in cell membranes in which all phospholipids are slowly and continuously transferred to the outer leaflet while aminophospholipids are rapidly returned to the cytoplasmic leaflet.

In case of cell stimulation and µM enhancement in concentrations of free calcium in the cytoplasm, the latter two enzymes become inhibited; and scramblase, which is a calcium dependent lipid transporter, becomes activated. Scramblase catalyzes fast bidirectional transfer of phospholipids which disrupts the asymmetry of outer and inner leaflets of cell membranes and results in exposure of PS and PE to the outer leaflet (Fig.1.8). This phenomenon can be induced in vitro by using calcium ionophores and happens physiologically upon stimulation of cells with thrombin and collagen^{10,38-40}.

**Procoagulant membranes**

Based on the cell-based model of coagulation, the initiation phase of blood clotting happens on the surface of TF-bearing cells, usually the adventitial tissue cells. The amplification and propagation steps happen on membranes of platelets. When platelets are activated, the phospholipid asymmetry in their membranes is disrupted and anionic phospholipids and PE become exposed to the outer leaflet of their membranes. This supports binding of blood-clotting factors.

Microparticles (MPs) are vesicles derived from activated cells. Thrombin is one of the molecules that can induce formation of MPs. MPs are mainly derived from endothelial
cells (with ultra large vWF on their surface), platelets (with P-selectin on their surface) and monocytes (with TF on their surface). MPs can participate in blood clotting reactions, especially if their membrane surface is procoagulant⁶.

**Phospholipids in blood clotting**

As indicated, phospholipid membranes can alter rates of clotting reactions depending upon their phospholipid compositions. The membrane-binding domains of clotting factors -- the GLA and C2 domain -- require mixtures of anionic and neutral phospholipids to properly interact with the phospholipid membrane in a physiologically relevant manner.

Among all the anionic phospholipids, PS is the most effective ⁴¹. Other anionic phospholipids result in maximal rates of fX activation by fIXa:fVIIIa or prothrombin activation by fXa:fVa, at phospholipid concentrations higher than those of PS containing membranes. Moreover, upon increase in ionic strength of the solution, the activity of these enzyme complexes are significantly decreased when non-PS anionic phospholipids are incorporated into phospholipid membranes. Comparing phosphatidyl-L-serine vs. phosphatidyl-D-serine shows that interactions of prothrombinase and fIXa:fVIIIa with PS molecules are stereospecific for phosphatidyl-L-serine⁴¹-⁴⁶. PS-membranes can enhance the rates of blood clotting reactions by increasing the local concentrations of reactants, inducing conformational changes in reactants and making the reaction a 2D reaction. Without PS membranes, enzymatic reactions are thousands of times less efficient in solution⁴⁰.

The enzymatic and binding studies using liposomes and Nanodisc membranes ⁴⁷ have shown that increasing the PS content of phospholipid membranes enhances binding of GLA domains to membranes, until the maximum catalytic activity is reached⁴⁸. Using Nanodiscs, which provide control of the local phospholipid mixture for binding of clotting factors, it was shown that high concentrations of PS are required to reach the maximum activity of the TF:fVIIa enzyme complex⁴⁸. Formation of PS-rich membrane microdomains induced by calcium ions and the PS-binding domains of clotting factors provides the high local concentration of PS required for maximum activity of TF:fVIIa.

PE is also an important phospholipid in cell membranes. This phospholipid has a neutral charge at physiologic pH and like PS, is an aminophospholipid that is kept in
the inner leaflet of cell membranes. Upon cell activation, it becomes exposed to the outer leaflet, where clotting factors can bind to phospholipid membranes. Previous studies have shown that although PE on its own cannot support blood clotting reactions, when incorporated into PS/PC membranes, it enhances the catalytic activity of the enzyme complex. Without PE, concentrations of PS higher than those found in cell membranes are required to achieve the maximal enzymatic activity, which are mostly higher than physiologic concentrations of PS in cell membranes. But when 30-40% PE is incorporated into membranes, the maximal activity of the enzyme complex is reached at concentrations of PS which are in the physiologic range for this phospholipid in cell membranes. This phenomenon is called the “synergy” between PE and PS phospholipids. In the next chapter the current hypotheses for explaining this synergy will be discussed.

PA and phosphorylated derivatives of phosphatidylinositol (PIP) make up a few percent of cell membrane phospholipids. These phospholipids are involved in cell signaling pathways, and PA is also the intermediate for synthesis of several phospholipid molecules. Low and constantly changing concentrations of these phospholipids make determining their concentration in the inner and outer leaflet of cell membranes difficult. It is generally accepted that these phospholipids are primarily localized in the inner leaflet of cell membranes. However some studies have found a few percent of these phospholipids in the outer leaflet.

Calcium-dependent binding of GLA domains to phospholipid membranes

Calcium induces clustering of PS phospholipids

Calcium ions induce anionic phospholipids, especially PS molecules, to form negatively charged areas on activated phospholipid membranes. In the presence of calcium ions, PS molecules are distributed in two distinct, equally populated conformations, in close spatial proximity.
showing PS clustering induced by calcium ions (Fig. 1.9). Moreover, PS binding domains promote formation of PS-rich membrane microdomains in phospholipid membranes\textsuperscript{55}. Formation of PS-rich microdomains results in enhanced local concentration of PS molecules which satisfies the PS requirement for membrane binding of PS-binding domains.

**Cations induce folding of GLA domain**

GLA domains of vitamin-K dependent clotting factors also require calcium ions to fold properly and to bind to negatively-charged areas of phospholipid membranes, which are essential for their activity\textsuperscript{56-58}. The amino acid sequence of GLA domains of clotting factors are very similar (Fig. 1.10) and highly conserved. The first 9 Gla and two cysteine residues are conserved in all GLA domains.

![Pro-coagulant](image)

![Anti-coagulant](image)

Fig. 1.10- The amino acid sequence alignment of GLA domains of clotting factors. γ : gamma-carboxy glutamate (Gla) residue. The “h-”-prefix (as in hPT) denote the human proteins and the “b-”-prefix (as in bPT) denotes the bovine proteins.

Focusing on the GLA domains of fVII, fIX and fX, which have very similar structures\textsuperscript{1,59-61}, there is an Asn in the second position and two hydrophobic residues adjacent to the first Gla residue: Phe4 and Leu 5 in fVII and fX and Leu 6 and Phe9 in fIX. The N-terminal loop of GLA domain (residues 1-11 in fVIIa) is called the ω-loop, and is responsible for GLA domain-membrane interaction. The hydrophobic residues 4,5 (in...
fVII and fX or 6,9 in fIX) along with the hydrophobic residue 8 (Leu8 in fVII and Met8 in fX or the basic residue Lys5 of fIX) form the “keel” part of the ω-loop where the side chains of these residues point to the exterior of GLA domain and is responsible for insertion of GLA domain in the membrane \(^5,59,60,62\).

There are also three alpha-helices in GLA domain. The first two are held parallel by a disulfide bridge between the two cysteine residues mentioned above. The third alpha-helix connects the GLA domain to the rest of the protein (Fig.1.11).

There are 8 calcium ions bound to the GLA domain. One of these calcium ions is located between the third alpha helix and the EGF domain, and the other 7 calcium ions are aligned between the two first helices and above the ω-loop (Fig.1.11). Since the GLA domain is highly negatively charged, due to the presence of 10-11 Gla residues, its binding to negatively charged areas on phospholipid membranes is mediated by calcium ions. Recent studies have shown that when the GLA domain binds to the phospholipid membranes, calcium ions play two distinct roles: a structural role (Ca\(^{2+}\)-3, Ca\(^{2+}\)-4, Ca\(^{2+}\)-5, Ca\(^{2+}\)-6) and a membrane-anchoring role (Ca\(^{2+}\)-7, Ca\(^{2+}\)-8, Ca\(^{2+}\)-9) \(^5\). GLA domain conformation is conserved in solution when the inner 4 calcium ions (Ca\(^{2+}\)-3, Ca\(^{2+}\)-4, Ca\(^{2+}\)-5, Ca\(^{2+}\)-6) are present. These calcium ions are chelated between the inner Gla residues 6, 7, 16, 20, 25, and 25 and maintain the connection between the helical part of GLA domain and the ω-loop, while pushing the side-chains of the Phe4, Leu5 and Leu8 residues (keel) into the solvent, holding the keel of the GLA domain properly folded. If these calcium ions are removed, Phe4, Leu5 and Leu8 form a hydrophobic cluster in the interior of the GLA domain, which will lack the keel part for interaction with and penetration into phospholipid membranes \(^5,56\).

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Fig. 1.11- The GLA domain of fVIIa based on PDB file: 1DAN (from \(^5\)). The GLA domain backbone is a blue tube, the Gla residues are yellow, tightly bound Ca\(^{2+}\) are magenta spheres, and the Arg and Lys residues are shown as green sticks.
On the other hand, the calcium ions performing an anchoring role (Ca\(^{2+}\)-7, Ca\(^{2+}\)-8, Ca\(^{2+}\)-9) are bound only with Gla residues from the helical part of the GLA domain and are mainly important for anchoring the GLA domain into phospholipid membranes, rather than for sustaining the GLA domain’s proper conformation for membrane binding.

Magnesium ions maybe substituted for the two external calcium binding sites with anchoring role, Ca\(^{2+}\)-7 and Ca\(^{2+}\)-9, depending on the solution the GLA domain is prepared in \(^{1,59-61}\). From the crystal structures, 1DAN, 2A2Q, 1J34, 1J35, 1IOD and 1P0S, we know if the GLA domain crystals are prepared in solutions with mixtures of CaCl\(_2\) and MgCl\(_2\), at physiological concentrations, these calcium binding sites become occupied by magnesium ions, while the inner calcium binding sites stay bound to calcium ions, except for the calcium 5 binding site, which can either stay bound to calcium ions or become occupied with magnesium ions (2A2Q, 1P0S).

**GLA domain binding to phospholipid membranes**

Several models have been proposed for describing GLA domain binding to phospholipid membranes (Fig.1.12). The Nelsestuen model (Fig.1.12A) suggests that the GLA domain does not penetrate into the phospholipid membrane and is lying on the membrane surface\(^{63}\). The Furie model (Fig.1.12B) shows penetration of the GLA domain into the phospholipid membrane through the keel residues\(^{9}\). The Tajkhorsid model (Fig.1.12C) shows that the GLA domain penetrates deeply into phospholipid membranes, consistent with the Furie model, except that in the Tajkhorsid model, the GLA domain is inserted deeper than the Furie model in phospholipid membranes, where the calcium ions bound to the GLA domain are close to the phosphate groups of PS phospholipids and the omega loop is deeply inserted into the phospholipid membrane into the acyl-chain layer. Based on the Tajkhorsid model, the Nelsestuen model is an intermediate in GLA domain insertion into the phospholipid membrane\(^5\).
The MD simulations of fVIIa GLA domain interactions with PS phospholipid molecules revealed two distinct classes of interaction between GLA domains and phospholipid molecules\(^5\) (Fig.1.13). These interactions include one unique binding interaction between GLA domain and a PS molecule, where the PS molecule interacts with Arg9 in the GLA domain through its phosphate part, and its carboxylate coordinates calcium ions that are tightly bound to the GLA domain. There were also extensive contacts between the GLA domain and the glycerol moiety of this PS molecule (Fig.1.13 C). This PS-binding site was also shown in the x-ray crystal structure of bovine prothrombin GLA domain that was co-crystalized with a lyso-PS molecule (Fig.1.13 B)\(^9\). The second class of interactions between GLA domain and PS molecules was observed in the MD simulations of fVIIa bound to PS membranes. In these interactions, the PS headgroups folded away from the GLA domain and the tightly bound calcium ions of the GLA domain participated in coordination complexes with the phosphate moieties of PS molecules (Fig.1.13 D).

**Objectives**

As described above, almost every step of the blood clotting cascade is tied to the presence of membrane surfaces with a proper mixture of phospholipid molecules. Although phospholipid membranes play an important role in the blood clotting cascade, we still lack detailed knowledge of the nature of the interactions between blood clotting proteins and phospholipid membranes.
I will first discuss our new findings, showing the “molecular determinants of phospholipid synergy in blood clotting”, which mainly emphasize understanding how phospholipid membranes accelerate fX activation by TF:fVIIa, using biochemical techniques, nanoscale membrane bilayers (Nanodiscs), solid-state-NMR and molecular dynamics (MD) simulations.

Afterwards, I will discuss my next project, which compares the binding affinities of all GLA domains of clotting factors for phospholipid membranes using surface plasmon resonance (SPR) technique. At the end, I will discuss our new findings about phospholipid specificity of fVIIa and APC (activated protein C).

In the last two chapters, I will discuss two other ongoing projects. The first project focuses on PE synergy with other phospholipid molecule in the prothrombinase complex. The second project studies the effect of mixtures of physiologic concentrations of calcium and magnesium on clotting reactions.

This project is a part of a collaborative work between four labs at University of Illinois: the Morrissey lab, the Sligar lab, the Tajkhorshid lab and the Rienstra lab.
Membrane systems used in our experiments

Liposomes and Nanodiscs are the two membrane systems used in my studies.

Liposomes are prepared using the Bio-Bead technique\textsuperscript{66}, and depending on the type of the detergent used, liposomes of different sizes can be prepared. These liposomes mimic the phospholipid membranes of cells, with the advantage that the mixtures of phospholipids and proteins incorporated into the membrane are in our control.

![Figure 1.14](image-url)

Fig. 1.14- (A) The cartoon representation of a Nanodisc prepared using MSP1D1, which is about 10nM in diameter. (B) The cartoon representation of TF:VIIa:FXa complex on a Nanodisc membrane. Since Nanodiscs are prepared based on the self-assembly of phospholipids and MSP molecules, integral membrane proteins, such as TF, can also be integrated into Nanodiscs, while Nanodiscs are being formed.

On cell membranes specific proteins and specific lipids are sequestered into different types of microdomains. Even on liposomes, anionic phospholipids tend to form microdomains in the presence of calcium ions and phospholipid binding proteins. This shows that the microenvironment in liposomes is dramatically different from the average composition of membrane phospholipids.

To study the interaction of GLA domains with phospholipid membranes and their binding to phospholipid microdomains, nanometer-scale phospholipid membranes known as Nanodiscs were used in our studies.
Nanodiscs are water soluble, nanometer-scale bilayers ringed by an amphipathic helical protein called the membrane scaffold protein (MSP)\textsuperscript{57-70}(Fig. 1.14A). Nanodiscs self-assemble from mixtures of phospholipid and MSP molecules and provide strict control over the local composition of the membrane surface.

Depending on the type of MSP used, Nanodiscs with various diameters, ranging from 8 to 15 nm, can be prepared\textsuperscript{70}. The smallest Nanodiscs have about 67 phospholipids per leaflet. Nanodiscs self-assemble and integral membrane proteins can be efficiently incorporated into Nanodiscs during the self-assembly reactions (Fig. 1.14B). Tissue factor (TF) is one of the integral membrane proteins that has been successfully integrated into Nanodiscs\textsuperscript{47,48}.

Compared to liposomes, Nanodiscs provide improved signal-to-noise ratios in biophysical and spectroscopic studies of protein-membrane interactions. Furthermore, on Nanodiscs long-range recruitment of anionic phospholipids can be avoided. Using Nanodiscs we have complete control over the composition of the membrane surrounding the clotting reaction enzyme complexes and can study the effects of the phospholipid nanoenvironment on the GLA domain-membrane interactions\textsuperscript{71}. 
References


Chapter 2

Molecular determinants of phospholipid synergy in blood clotting

Introduction

Proteins that bind to phosphatidylserine (PS) are implicated in diverse processes such as intracellular signal transduction cascades; vesicle fusion and neurotransmitter release; plasma membrane-cytoskeleton interactions; phagocytosis of apoptotic cells; and blood clotting. Indeed, most of the steps in the coagulation cascade require the reversible, Ca-dependent association of clotting proteins with membrane bilayers containing exposed PS. Releasing these proteins from the membrane surface renders them thousands of times less active. An exemplar is the initiation of clotting, triggered when the plasma protease, factor VIIa (fVIIa), binds to the integral membrane protein, tissue factor (TF). The resulting TF:fVIIa complex activates two membrane-bound zymogens, factors IX (fIX) and X (fX), by limited proteolysis. fVIIa, fIX and fX are homologous proteins that interact with PS-containing membranes via their GLA domains, so-named because they are rich in gamma-carboxyglutamate (Gla). GLA domains are the most common membrane-binding motif in blood clotting, but in spite of their importance, we lack a detailed understanding of their interaction with the membrane at atomic resolution. This study provides a new view of how lipids collaborate to promote the binding of GLA domains to membrane surfaces and trigger blood clotting.

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A puzzling feature of TF:fVIIa is its requirement for unphysiologically high PS levels (~30% PS) for optimal procoagulant activity when TF is incorporated into liposomes with mixtures of PS and phosphatidylcholine (PC). The answer may come from phosphatidylethanolamine (PE). By itself, PE (in PE/PC liposomes) supports little to no clotting activity, but when PE is incorporated into PS/PC liposomes, it dramatically decreases the PS requirement for optimal activity of membrane-bound protease blood clotting reactions, including proteolytic activation of fX by the TF:fVIIa complex \(^5\), inactivation of fVa by activated protein C \(^6\), activation of prothrombin by the fVa:fXa (prothrombinase) complex \(^7\) and activation of fX by the fVIIIa:fIXa (intrinsic tenase) complex \(^8\). Of the four major plasma membrane phospholipids, PC and sphingomyelin are abundant on the outer surface while PS and PE are actively sequestered to the inner leaflet (Fig. 2.1A). Cell lysis, damage or activation causes externalization of PS and PE, which promotes clotting reactions \(^9\). PE is considerably more abundant than PS, so the ability of small amounts of PS to support blood clotting reactions in the presence of larger amounts of PE represents a more physiologic environment for assembling clotting reactions.

How PE “synergizes” with PS is not known, but Zwaal et al. \(^3\) and Gilbert & Arena \(^8\) have summarized the following hypotheses from the literature: PE-1: PE, being a non-lamellar phospholipid, induces the formation of PS-rich microdomains which are more efficient in supporting clotting reactions. PE-2: Clotting proteins contain binding site(s) for PE (or a combination of PE and PS). PE-3: PE has hydrogen bond donors but PC does not; when PE forms hydrogen bonds with adjacent PS molecules it induces PS headgroup conformations that are more favorable for binding to clotting proteins. PE-4: The PC headgroup is so bulky and highly hydrated \(^10,11\) that it sterically hinders access of GLA domains to adjacent PS molecules.

Our previous studies demonstrated approximately one fX membrane-binding site for every 6 to 8 PS molecules, which is about equal to a GLA domain’s membrane footprint \(^15\). Here we present a novel, general explanation for GLA domain interactions with membranes which we term the *Anything But Choline (ABC)* hypothesis, triggered by detailed molecular dynamics (MD) simulations of GLA domains associating with PS-containing bilayers (Fig. 2.1B) \(^13,16\). The ABC hypothesis proposes two key types of GLA domain-phospholipid interactions: A single “phospho-L-serine-specific” binding site per
GLA domain (Fig. 2.1C,D), and multiple “phosphate-specific” interactions, in which phospholipid headgroups bend to allow their phosphates to form coordination complexes with tightly bound Ca\(^{2+}\) in the GLA domain (Fig. 2.1E). The unique “phospho-L-serine-specific” binding site was independently identified in the crystal structure of lyso-PS bound to the prothrombin GLA domain (Fig. 2.1C) \(^{14}\) and in MD simulations of fVIIa GLA domain binding to PS-bilayers (Fig. 2.1D) \(^{13}\). The proposed “phosphate-specific” interactions have been repeatedly observed in our MD simulations of GLA domains associating with PS-containing bilayers \(^{13,16}\). We propose that PS can provide both interactions, while PC can provide neither (because its bulky choline headgroup sterically hinders access of proteins to its own phosphate moiety). PE can provide phosphate-specific but not phospho-L-serine-specific interactions, explaining why PE/PC bilayers poorly support clotting, while PE synergizes with small amounts of PS.

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**Fig. 2.1- GLA domain-PS interactions.** (A) Depicted are the four most abundant plasma membrane phospholipids. Sphingomyelin and PC are abundant in the outer leaflet, while PS and PE are largely restricted to the inner leaflet; this membrane asymmetry is lost following platelet activation or cellular trauma \(^{12}\). (B) Membrane-bound model of the human fVII GLA domain obtained from MD simulations on the surface of a PS bilayer \(^{13}\). GLA domain backbone is a green tube, Gla residues are white, tightly bound Ca\(^{2+}\) are yellow spheres, and interacting PS are in stick representation. (C,D) The proposed phospho-L-serine-specific binding site independently observed in (C) the crystal structure of bovine prothrombin fragment 1 (PDB 1NL2 \(^{14}\)) and (D) our MD simulations of the fVIIa GLA domain-membrane complex \(^{13}\). In C and D, two Arg contributing to the site are purple sticks and the uniquely bound lyso-PS and DOPS molecules are drawn using stick representations. (E) Examples of two phosphate-specific interactions from B.
A strong prediction of the ABC hypothesis is that there is nothing unique about the ethanolamine headgroup in PE other than that it is not as bulky as PC. Thus, any phospholipid whose headgroup can bend to allow its phosphate to contribute phosphate-specific interactions with GLA domains should synergize with PS, a prediction inconsistent with the PE-specific hypotheses outlined above. In this study, we use a combination of biochemical techniques, solid-state NMR (SSNMR) analyses and MD simulations to compare the predictions of the ABC hypothesis versus PE-specific hypotheses to explain how phospholipids synergize to support fX activation by TF:fVIIa.

**Materials and Methods**

*Materials*—Diacylglycerol (DAG) and phospholipids (except for D-PS and PS*) were from Avanti Polar Lipids (Alabaster, AL); Bio-Beads® SM-2 adsorbent was from Bio-Rad (Hercules, CA); D-serine was from Sigma-Aldrich (St. Louis, MO); U-^{13}C,^{15}N-L-serine was from Cambridge Isotope Laboratories (Andover, MA); human fVIIa was from American Diagnostica (Stamford, CT); bovine prothrombin was from Haematologic Technologies (Essex Junction, VT); human fX and α-thrombin were from Enzyme Research Laboratories (South Bend, IN); and NTA Biacore sensor chips were from GE Healthcare Bio-Sciences (Piscataway, NJ). Recombinant, membrane-anchored TF and the membrane scaffold protein for Nanodisc production were expressed in *E. coli* and purified as described. PS with D-serine (D-PS) and PS* were synthesized from natural isotopic abundance POPC via phospholipase D-catalyzed headgroup exchange, using either D-serine (for D-PS) or U-^{13}C,^{15}N-L-serine (for PS*), as previously described. Prothrombin fragment 1 was prepared by digestion of bovine prothrombin with human α-thrombin and purified by ion-exchange and size-exclusion chromatography essentially as described.

*TF-Liposome Preparation and Measurement of FX Activation*—TF was incorporated into liposomes of varying phospholipid composition as described previously using Bio-Beads® SM-2 and 20mM sodium deoxycholate. Initial rates of fX activation by TF:fVIIa assembled on TF-liposomes were quantified as described previously, typically using 100 nM fX, 5 pM fVIIa, and 500 pM TF.

*SPR Analyses of FX Binding to Nanoscale Bilayers*—TF-free Nanodiscs of varying phospholipid composition were prepared as described. FX binding affinities for
nanoscale bilayers were quantified using SPR analyses (on a Biacore 3000 instrument) as described, with Nanodiscs immobilized on Ni\(^{2+}\)-NTA sensor chips via the oligohistidine tag on the membrane scaffold protein \(^{15}\). As previously reported, binding isotherms were plotted from maximal, steady-state RU values versus the fX concentration flowed over the chip surface, from which \(K_d\) values were derived by fitting the single-site ligand binding equation to the data \(^{15}\).

**MAS-SSNMR Spectroscopy**—SSNMR studies were performed on Varian (Fort Collins, CO) InfinityPlus spectrometers. POPS*/POPC Nanodisc spectra were acquired at 600 MHz with a Varian T3 HXY 3.2 mm probe at an MAS rate of 10,000 ± 3 Hz or 13,333 ± 2 Hz. The variable temperature gas was maintained at 90 ± 10 scfh flow, and the reported sample temperatures take into account thermocouple calibration and frictional heating due to MAS, calibrated with ethylene glycol \(^{22}\). All experiments utilized tangent ramped cross-polarization \(^{23}\) with TPPM decoupling \(^{24}\) at ~80 kHz nutation frequency.

The SSNMR studies were carried out by Mary Clay (Dr. Rienstra lab) and Dr. Rebecca Davis-Harrison (Dr. Morrissey lab).

**MD Simulations**—Four bilayers each composed of 1,188 lipids and solvated by water at approximately a 43:1 water/lipid ratio were simulated. The lipids were all composed of palmitoyloleoyl (PO) phospholipids, but with different headgroups, namely PE, CH\(_3\)PE, (CH\(_3\))\(_2\)PE, and PC, respectively. All simulations were performed using NAMD2 \(^{25}\), utilizing the CHARMM22 force field with \(\phi/\psi\) cross term map (CMAP) corrections \(^{26}\) for proteins and CHARMM36 parameters for lipids \(^{27}\). The TIP3 model \(^{28}\) was used for water. All simulations were performed using an NPT ensemble at 1.0 atm and 298 K, and with a time step of 2 fs. Constant pressure was maintained using the Nosé-Hoover Langevin piston method \(^{29,30}\), while constant temperature was maintained by Langevin dynamics using a damping coefficient \(\gamma\) of 0.5 ps\(^{-1}\) applied to all heavy atoms. Non-bonded interactions were cut off after 12 Å with a smoothing function applied after 10 Å. The particle mesh Ewald (PME) method \(^{31}\) was used for long-range electrostatic calculations with a grid density greater than 1 Å\(^{-3}\). As a measure of accessibility, solvent accessible surface area (SASA) of the lipid phosphates was calculated in VMD \(^{32}\), where a probe of a specified radius representing molecular species of various sizes is rolled.
over the phosphate groups and the surface available for direct contact with the probe is integrated. Molecular images were generated using VMD \textsuperscript{32}.

The MD simulations were carried out by Taras Pogorelov (Dr. Tajkhorshid lab).

\textbf{Results}

\textit{Many Phospholipids Synergize with PS to Enhance FX Activation by TF:FVIIa}—The ABC hypothesis predicts that many phospholipids can synergize with PS to enhance fX activation by TF:FVIIa, while PE-centric hypotheses rely on specific properties of the ethanolamine headgroup. (Here we refer to PS with the usual L-serine in its headgroup as L-PS, and PS with D-serine as D-PS.) To test these hypotheses, we measured rates of fX activation by TF:FVIIa on TF-liposomes containing binary lipid mixtures (0-30% L-PS; balance = PC), or ternary lipid mixtures in which the sum of L-PS plus the other test lipid equaled 30% (balance = 70% PC). Lipids tested included PE, phosphatidylglycerol (PG), phosphatidylinositol (PI), phosphatidic acid (PA) and D-PS.

Consistent with our previous report \textsuperscript{5}, PE strongly synergized with L-PS to support fX activation, left-shifting the L-PS response relative to L-PS/PC liposomes (Fig. 2.2A). PG also strongly synergized with L-PS while supporting very low rates of fX activation in the absence of L-PS (Fig. 2.2A). On the other hand, PE did not synergize with PG, as the effects of these lipids were strictly additive (Fig. 2.2B). This is consistent with the ABC hypothesis, which predicts the same role for PE and PG — i.e., providing phosphate-specific interactions. Figs. 2A and B employed dioleoyl (DO-) phospholipids to ensure that they were always above the phase transition, since palmitoyloleoyl PE (POPE) has a relatively high transition temperature. For other phospholipid mixtures, we used palmitoyloleoyl (PO-) phospholipids; the results (cf. Figs. 2A and 2C) confirm that PG synergizes with L-PS equally well whether PO- or DO-phospholipids were employed. Phospholipid synergy is thus independent of any subtle bilayer packing differences due to DO- versus PO-phospholipids. PA and PI also strongly synergized with L-PS while supporting very low rates of fX activation in the absence of L-PS (Fig. 2.2C). Taken together, these results demonstrate that PE’s ethanolamine moiety is not required for phospholipid synergy, as lipids with very different headgroups synergized equally well with L-PS.
Fig. 2.2- All tested glycerophospholipids synergize with L-PS to enhance fX activation by TF:fVIIa on liposomes, but PE does not synergize with PG. (A) Normalized rates of fX activation by fVIIa on TF-liposomes prepared with binary lipid mixtures (0-30% L-DOPS; balance = DOPC), or ternary lipid mixtures in which the sum of L-DOPS plus DOPE or DOPG equaled 30% (balance = 70% DOPC). (B) Rates of fX activation by fVIIa on TF-liposomes prepared with ternary lipid mixtures in which the sum of DOPG + DOPE equaled 30% (balance = 70% DOPC). (C) Normalized rates of fX activation by fVIIa on TF-liposomes prepared with binary lipid mixtures (0-30% L-POPS; balance = POPC), or ternary lipid mixtures in which the sum of L-POPS plus the other test lipid equaled 30% (balance = 70% POPC). Lipids tested included phosphatidylglycerol (POPG), phosphatidylinositol (POPI), phosphatidic acid (POPA) and D-POPS. FX activation rates for each TF-liposome preparation in panels A and C were normalized to the rate with 30% L-PS/70% PC. Data are mean ± standard error; n = 3 to 10. Headgroup structures for lipids in this experiment are included on the right.

Components of the prothrombinase and fVIIa:fXa complexes bind much more weakly to membranes if L-PS is replaced by D-PS (reviewed by Zwaal et al. 3). To our knowledge, PS headgroup stereospecificity has not been examined for fX activation by TF:fVIIa. We prepared TF-liposomes with binary lipid mixtures (0-30% L-PS, balance = PC), or ternary lipid mixtures of a total of 30% L-PS + D-PS (balance = 70% PC). D-PS poorly supported fX activation on its own, but it strongly synergized with L-PS (Fig. 2.2C). This is fully consistent with the ABC hypothesis, since D-PS should not support phospho-L-serine specific interactions but should provide phosphate-specific interactions with GLA domains.

PG and D–PS Synergize with L–PS to Enhance FX Binding to Membranes—Multiple protein-phospholipid interactions are present when TF:fVIIa activates fX, but the
dominant membrane contribution to catalysis appears to be fX binding to membranes adjacent to TF:fvIIa. We therefore quantified fX binding to nanobilayers using surface plasmon resonance (SPR) as described. Consistent with our previous findings, the $K_d$ for fX binding to Nanodiscs decreased with increasing L-PS content. The L-PS dose-response was left-shifted in the presence of 40% PG (Fig. 2.3A), demonstrating that PG synergizes with L-PS to support fX binding to bilayers. We also examined fX binding to nanobilayers containing either 50% L-PS, 50% D-PS, or 25% L-PS + 25% D-PS (balance in all cases = 50% PC; Fig. 2.3B). FX bound less to bilayers with 50% D-PS compared to 50% L-PS, while bilayers with 25% L-PS + 25% D-PS performed as well as those with 50% L-PS. Thus, D-PS synergizes with L-PS to enhance membrane binding of fX.

**Fig. 2.3- PG and D-PS synergize with L-PS to enhance fX membrane binding.** (A) Binding affinities of fX to Nanodiscs with varying L-POPS content with or without 40% POPG (balance = POPC). Nanodiscs were immobilized on Biacore sensorchips, after which steady-state levels of fX binding were quantified; $K_d$ values were derived from the resulting binding isotherms. Data are mean ± standard error; $n = 3$. (B) Steady-state binding of fX to nanoscale bilayers, quantified by SPR, using Nanodiscs with 50% POPC and either 50% L-POPS, 50% D-POPS, or 25% L-POPS + 25% D-POPS.

**Methylation of PE Headgroups Diminishes Synergy**—PC differs from PE by three N-methyl groups (cf. Fig. 2.4). We therefore asked how the presence of one or two N-methyl groups on PE would affect synergy with L-PS, using TF-liposomes containing binary lipid mixtures (0-30% L-PS, balance = PC), or ternary lipid mixtures of 0-30% L-PS plus either 30% PE, 30% N-monomethyl PE or 30% N-dimethyl PE (balance = PC).
The results (Fig. 2.4A) show a progressive diminution in synergy when one or two N-methyl groups are added to PE.

**Importance of the Phosphate Groups of Phospholipids for Synergy**—The experiment in Fig. 2.2C showed that PA synergized well with L-PS to support fX activation. We next asked whether PA’s phosphate is required, by comparing the ability of PA and diacylglycerol (DAG) to synergize with L-PS to enhance fX activation by TF:fVIIa. TF-liposomes were prepared containing binary lipid mixtures (0-30% L-PS, balance = PC), or ternary lipid mixtures of either a total of 30% PA + L-PS, or a total of 30% DAG + L-PS (balance = PC). DAG, which lacks a phosphate group, weakly synergized with L-PS compared to robust synergy with PA (Fig. 2.4B).

![Fig. 2.4](image-url)

**Fig. 2.4- Influence of PC methyl groups and PA phosphate on fX activation by TF:fVIIa.** (A) Rates of fX activation by fVIIa on TF-liposomes prepared with varying L-DOPS and either no DOPE, 30% DOPE, 30% CH₃-DOPE or 30% (CH₃)₂-DOPE (balance = DOPC). (B) Rates of fX activation by fVIIa on TF-liposomes prepared with binary lipid mixtures (0-30% L-POPS; balance = POPC), or ternary lipid mixtures in which the sum of L-POPS plus either POPA or DAG equaled 30% (balance = 70% PC). (DAG had palmitoyloleoyl acyl chains.) In both panels, fX activation rates for each TF-liposome preparation were normalized to the rate with 30% L-PS/70% PC; data are mean ± standard error; n = 3 to 7. Headgroup structures for lipids in this experiment are included on the right.
MD Simulations of Phosphate Accessibility in Bilayers—To investigate the molecular basis of phospholipid synergy, especially in terms of the accessibility of lipid phosphate groups, four independent 40 ns MD simulations of bilayers were performed in which the lipid headgroups were progressively transformed from PE to N-monomethyl PE, N-dimethyl PE and PC, paralleling the experimental setting in Fig. 2.4. Each simulation included 1,188 lipids distributed equally in the two leaflets to provide statistically meaningful data. Analyses of solvent accessible surface areas (SASA; Fig. 2.5) clearly show that each N-methyl group diminished the accessibility of the phosphate group for all probes with radii >1.5 Å (approximately the size of water), with the difference in phosphate exposure also being evident from top-view snapshots shown for representative frames taken from the simulations (Fig. 2.5, bottom). Although protein binding to membranes may induce lipid rearrangement, these measurements clearly show that the choline headgroup of PC sterically hinders access to PC’s phosphate group.

Fig. 2.5- MD simulations show that PC has restricted phosphate accessibility. (Top) MD simulations showing that adding N-methyl groups to PE reduces the solvent-accessible surface areas (SASA) of the phosphate group: PE (red), CH₃-PE (magenta), (CH₃)₂-PE (brown), and PC (black). SASA are calculated using varying probe radii for the last 20 ns of 40 ns MD simulations of 1,188 lipids. For comparison, water (radius 1.4 Å), Arg (guanidinium group radius ~2.5 Å), and the FVIIa GLA domain ω-loop (radius ~8 Å) are shown, in order to compare the results to the approximate size of functional groups that could interact with the phosphate groups. (Bottom) Visual comparison of phosphate exposure in the bilayer simulations (phosphate is black; remainder of the lipid is white).
SSNMR Evidence for a New PS Chemical Environment Induced by GLA Domains—We recently showed, using magic angle spinning SSNMR, that Ca\textsuperscript{2+} induces two distinct, equally populated conformations of L-PS headgroups in close spatial proximity, consistent with Ca\textsuperscript{2+} promoting L-PS clusters \textsuperscript{18}. In the present study, we employed this approach to investigate PS-GLA domain binding interactions, using PS with uniformly \textsuperscript{13}C,\textsuperscript{15}N labeled L-serine (termed PS\textsuperscript{*}) incorporated into Nanodiscs. When L-PS engages a GLA domain’s proposed phospho-L-serine-specific binding site, its headgroup should take up a unique conformation observable by SSNMR. 2-D \textsuperscript{13}C-\textsuperscript{13}C spectra were acquired of 30\% PS\textsuperscript{*}/70\% PC Nanodiscs prepared with Ca\textsuperscript{2+} in the absence (Fig. 2.6, blue trace) or presence (red trace) of saturating levels of prothrombin fragment 1 (which contains the GLA domain).

![SSNMR spectroscopy](image)

**Fig. 2.6-** SSNMR spectroscopy demonstrates a novel PS headgroup environment induced upon GLA domain binding to bilayers. A \textsuperscript{13}C-\textsuperscript{13}C 2-D SSNMR spectrum in blue of 30\% POPS\textsuperscript{*}/70\%POPC Nanodiscs (25 ms DARR mixing; 2.6 hr at 13 °C; 13.333 kHz MAS rate). Overlaid in red is a spectrum of 30\% POPS\textsuperscript{*}/70\% POPC Nanodiscs in the presence of prothrombin fragment 1 (50 ms DARR mixing; 50 hr at 13 °C; 10.000 kHz MAS rate). Both spectra were acquired on a 600 MHz (\textsuperscript{1}H frequency) spectrometer. Insert is an expansion of serine C\textsubscript{α}-C\textsubscript{β} regions. PS1 and PS2 represent equally abundant chemical environments for PS headgroups in the presence of Ca\textsuperscript{2+}, while PS3 represents a novel PS headgroup environment induced by Ca\textsuperscript{2+} plus prothrombin fragment 1.

In the presence of fragment 1, we observed a new correlation pattern at 58, 67, and 175 ppm, corresponding to a new chemical environment for L-PS headgroups (termed PS3 in Fig. 2.6). This is in addition to the two nearly equally populated, Ca\textsuperscript{2+}-induced headgroup configurations termed PS1 and PS2 that we also observed in our previous study \textsuperscript{18}. The individual cross-peaks made up the following fractions of the total L-PS
intensities: PS1, 42±3%; PS2, 41±2%; and PS3, 17±2%. SPR studies and MD simulations suggest that 6-8 L-PS molecules constitute a GLA domain binding site. The observation that 17±2% of the L-PS headgroups are found in the PS3 chemical environment upon GLA domain binding is consistent with the idea that one L-PS molecule per GLA domain is involved in the phospho-L-serine-specific binding interaction, with the rest of the PS molecules participating in phosphate-specific interactions (as depicted in Fig. 2.1).

Discussion

The impetus for this study was to explain why PE, a relatively abundant plasma membrane phospholipid, poorly supports clotting reactions in bilayers composed of binary mixtures of PE and PC, but strongly promotes clotting reactions when small amounts of PS are also included. We reasoned that solving this conundrum would allow us to develop a general mechanism for how GLA domains interact with PS-containing membranes. To investigate this question, we studied fX activation by TF:fVIIa assembled on liposomes with a wide variety of lipid compositions. Existing hypotheses to explain PE/PS synergy (PE-1 to PE-4, above) focus on specific properties of PE’s ethanolamine headgroup. In contrast, the ABC hypothesis proposes that GLA domains bind to bilayers via a single phospho-L-serine-specific binding interaction together with multiple phosphate-specific interactions. In binary PS/PC mixtures, PS has to provide both types of interaction since the bulky choline moiety of PC sterically hinders GLA domains from interacting with its own phosphate group. (The same situation would also apply to sphingomyelin, whose phosphocholine headgroup is identical to that of PC. Since PC and sphingomyelin are the predominant phospholipids on the outer leaflet of the plasma membrane, and since PS is sequestered to the inner leaflet, this explains the generally anticoagulant surface of healthy, intact cells.) PE collaborates with PS to create membrane binding sites for GLA domains because PE provides the more numerous phosphate-specific interactions, freeing up limited amounts of PS to engage in the single phospho-L-serine-specific interaction per GLA domain. We tested these hypotheses by examining how various phospholipids synergize with PS to enhance fX binding to bilayers, and to enhance fX activation by TF:fVIIa in liposomes. Glycerophospholipids with a wide variety of different headgroups were fully as effective as PE in synergizing with PS, including those with myo-inositol (PI), glycerol (PG), D-
serine (D-PS), or even just a bare phosphate (PA). We also found that PE did not synergize with PG in the absence of L-PS; instead, the effects of PE and PG were strictly additive. This is consistent with the ABC hypothesis, which predicts that while PE and PG can both provide phosphate-specific interactions, they cannot provide the essential phospho-L-serine-specific interaction. The fact that TF-liposomes containing binary mixtures of D-PS and PC poorly supported fX activation by TF:fVIIa recapitulates similar findings with prothrombinase and fVIIIa:fIXa. On the other hand, D-PS synergized strongly with L-PS to support TF:fVIIa activity, which is fully consistent with the ABC hypothesis, since D-PS should retain the ability to enter into phosphate-specific interactions with GLA domains.

Hypotheses PE-1 to PE-4 are inconsistent with our findings: 1) PE-1 (i.e., non-lamellar PE promotes PS-rich microdomains) cannot explain our results since both lamellar and non-lamellar phospholipids synergized with PS. Furthermore, the nanoscale bilayers in Nanodiscs preclude long-range clustering of PS into microdomains, yet they strongly supported PG/PS and D-PS/L-PS synergy for fX binding. 2) PE-2 (i.e., PE-specific binding sites in GLA domains) likewise cannot explain our finding that phospholipids with headgroups structurally unrelated to ethanolamine all strongly synergized with PS. In addition, progressive diminution of PE/PS synergy as one or two N-methyl groups were added to PE (by progressively inhibiting access to its own phosphate) is consistent with the ABC hypothesis but inconsistent with PE-2, which predicts an abrupt loss of synergy when the PE ethanolamine is chemically altered. We also showed, using MD simulations, that accessibility of the phosphate groups to objects the size of amino acids or the ω-loop of GLA domains is progressively diminished as the ethanolamine moiety of PE contains one, two or three N-methyl groups. 3) PE-3 (i.e., hydrogen-bonding between PE and adjacent phospholipids alters PS headgroup conformations) is also inconsistent with our findings, since the various PS-synergizing headgroups tested cannot engage in the same kind of hydrogen bonding interactions that ethanolamine can. 4) PE-4 is the most similar to the ABC hypothesis, the chief difference being that PE-4 proposes that bulky PC sterically hinders access of GLA domains to adjacent PS molecules, while the ABC hypothesis proposes that the PC headgroup sterically hinders access of GLA domains to PC’s own phosphate. PE-4 is not completely ruled out by many of the experiments presented here, but DAG (which contains no phosphate group) synergized weakly with PS, compared with full synergy observed with PA. Weak PS
synergy with DAG is consistent with the notion that simply removing the bulky choline reduces some steric hindrance to the bilayer in general. However, this experiment argues that lipids need to retain their phosphate moieties in order to synergize fully with PS, in agreement with the ABC hypothesis.

Electrostatics per se cannot explain PE/PS synergy, since we observed identical PS synergy with phospholipids having very different net charges, including PE (neutral); D-PS, PI and PG (-1); and PA (-1 to -2). Nor is it necessary for the synergizing phospholipid to be zwitterionic or even to have an amino group, as PA, PI, and PG lack these features. The common feature required for a phospholipid to synergize with PS appears to be a phosphate unshielded by a bulky headgroup.

It is possible that when L-PS engages the phospho-L-serine-specific binding site in a GLA domain, it induces a conformational change in the GLA domain that promotes the phosphate-specific interactions (and in fact PS has been proposed to be an allosteric regulator of clotting protein function 33). This may help explain why GLA domains bind so weakly to membranes containing relatively high amounts of anionic phospholipids other than L-PS.

Our SSNMR results provide direct evidence for a unique PS headgroup environment induced when a GLA domain binds to PS-containing bilayers. The finding that approximately 1/6 of the total PS* signal intensity is in this chemical environment is fully consistent with the ABC hypothesis, which predicts that about this fraction of the PS molecules in a PS/PC bilayer will be engaged in the single phospho-L-serine-specific binding interaction per GLA domain, provided that a GLA domain binding site consists of about 6 or so PS molecules, a notion which is supported by both our MD simulations (Fig. 3.1B) 13,16 and SPR-based binding studies 15. Based on these stoichiometries from Nanodisc-based studies, one might expect maximal rates of fX activation on liposomes with approximately 5% L-PS + 25% synergistic phospholipid. When we examined fX activation by TF:vIIa on TF-liposomes, maximal fX activation generally required somewhat more than 5% L-PS in combination with non-PC phospholipids. One explanation could be that L-PS is more readily clustered into nanodomains in the presence of Ca²⁺ than are other non-PC phospholipids.
This study provides a new explanation for how plasma membrane phospholipids synergize to create binding sites for GLA domain-containing blood clotting proteins, using fX activation by TF:fVIIa as the exemplar. In future studies, it will be interesting to test the predictions of the ABC hypothesis versus PE-specific hypotheses on other membrane-dependent clotting reactions, e.g., activities of the prothrombinase, fVIIIa:fIXa and protein S-activated protein C complexes. The nature of the proposed phospho-L-serine-specific and phosphate-specific binding sites deserve considerably greater scrutiny and are the subject of our ongoing studies aimed at achieving an atomic-scale understanding of the binding of GLA domains to membrane bilayers.
References


Chapter 3- Part A

Factor VII and protein C are phosphatidic acid-binding proteins

This chapter is a part of a manuscript, under preparation.

Introduction

It has long been known that changes in the phospholipid composition of cell membranes profoundly regulate blood clotting. This is because many of the proteins of the plasma clotting cascade, both procoagulant and anticoagulant, bind reversibly to membrane bilayers containing exposed anionic phospholipids, with phosphatidylserine (PS) being reported in many studies to have the greatest ability to accelerate blood coagulation. In this paper we provide evidence that, surprisingly, phosphatidic acid (PA) enhances the activity of certain membrane-bound blood clotting reactions more effectively than does PS.

The most common membrane-binding domains of proteins in the mammalian blood clotting cascade are \(\gamma\)-carboxyglutamate-rich (GLA) domains, located at the N-terminus of four procoagulant proteins—prothrombin (PT), factors VII (fVII), IX (fIX) and X (fX)—and three anticoagulant proteins—protein C (PrC), protein S (PrS) and protein Z (PrZ). Although these seven GLA domains are very similar in sequence and structure, their membrane binding affinities vary by about three orders of magnitude. For example, fVIIa and activated PrC (APC) bind relatively weakly to PS-containing membranes, while fX binds with relatively high affinity. To our knowledge, however, no study has directly compared the membrane binding properties of all seven human GLA domain-containing blood clotting proteins side-by-side under the same conditions. It has previously been shown that several of the plasma clotting proteins, including components of the fVa:fXa complex, the fVIIa:flXa complex and the tissue factor (TF):fVIIa complex, bind much more weakly to membranes if PS has \(d\)-serine in its headgroup (\(d\)-PS) versus the naturally-occurring \(L\)-serine (\(L\)-PS), but to our knowledge the PS headgroup stereospecificity of GLA domain binding has not been evaluated for all seven of these proteins.
Accordingly, in this study we used a surface plasmon resonance (SPR)-based approach to quantify the binding of the seven human GLA domain-containing clotting proteins to supported nanoscale phospholipid bilayers (Nanodiscs) containing 50% L-PS, or 50% D-PS, or 50% PA (with the balance being 50% phosphatidylcholine, PC). We now report that, while all seven proteins bound more extensively to nanobilayers containing L-PS versus D-PS, two of them—fVIIa and APC—bound more extensively to nanobilayers containing PA rather than PS. Furthermore, the proteolytic activities of fVIIa and APC were more strongly enhanced when liposomes contained PA rather than PS, although mixtures of PS and PA gave the highest activities. And finally, we show that certain phosphatidylinositol (PI) phosphates also supported enhanced enzymatic activity of fVIIa and APC.

**Materials and Methods**

**Materials**—Materials were from the following suppliers: phospholipids (except for D-PS), Avanti Polar lipids (Alabaster, AL); phospholipase D (Streptomyces sp.), Biomol International (now Enzo Life Sciences, Ann Arbor, MI); Tyrode’s salts, TRAP (thrombin receptor activating peptide), A23187 and D-serine, Sigma-Aldrich (St. Louis, MO); recombinant human fVIIa, C339-GCT; human fVa, APC, PrZ, PrS and DAPA, Haematologic Technologies (Essex Junction, VT); bPT and human fX, fIX, and PT, Enzyme Research Laboratories (South Bend, IN); NTA Biacore sensor chips, GE Healthcare (Piscataway, NJ); RGDS, Bachem(Torrance, CA); and Pooled normal plasma, King Bio-Medical, Inc. (Overland Park, KS). Recombinant membTF 5, sTF 6,7 and membrane scaffold protein 8 were purified from E. coli expression as described. D-PS was synthesized as described9. Liposomes were prepared using Bio-Beads® SM-2 (Bio-Rad; Hercules, CA) and 20 mM sodium deoxycholate as described 10. Generally PO- forms of phospholipids were used for preparing liposomes and Nanodiscs, unless otherwise is noted. PE was always used in the form of DOPE.

**SPR Analyses of Protein Binding to Nanoscale Bilayers**—Nanodiscs of desired phospholipid composition were prepared and used to quantify protein binding via SPR using a Biacore 300 instrument (GE Healthcare) as previously described 4, except that 0.2% bovine serum albumin was included in the buffers in which fVIIa binding was quantified. Briefly, the maximal, steady state RU values were plotted vs. protein...
concentration, to which the single-site ligand binding equation was fitted to yield $K_d$ values and maximal binding stoichiometries.

*Rates of fX Activation and fVa Inactivation*—Initial rates of fX activation by fVIIa or sTF:fVIIa in the presence of liposomes were quantified at room temperature as described typically using 100 nM fX and either 1 nM fVIIa, or 100 pM fVIIa + 20 nM sTF. Initial rates of fVa inactivation by APC were quantified at room temperature in a two-stage assay as described with minor modifications. In the first stage, 50 pM APC, 3 nM fVa and 20 µM phospholipids were incubated for 0-30 min, and in the second stage the remaining fVa cofactor activity was quantified in the presence of 1 nM fXa, 1.5 µM PT and 10 µM DAPA, where the first stage was dilute 1 to 5 into the second stage. For liposomes lacking PS-phospholipids (100%PC, or 30% PA + 70%PC, or 30% PI4P + 70% PC), 6 µM of 5% PS + 95% PC liposomes was added to the second stage.

*PLD Treatment of Activated Human Platelets*—Citrated blood was collected via atraumatic venipuncture from healthy human volunteers and centrifuged for 10 min at room temperature at 200 x $g$. The platelet-rich plasma (PRP) supernatant was removed. Platelets were purified from platelet rich plasma by gel filtration on a Sepharose 2B column using calcium free Tyrode’s/BSA buffer as the running buffer. Platelet fractions were pooled together and centrifuged at room temperature at 1,000g for 10min. Platelets were resuspended at 2.05 x 10⁸ platelets/ml in Tyrode’s buffer containing 300 µg/mL RGDS peptide to prevent aggregation, then activated at 37 ºC with agitation for 10 min with 10 µM TRAP followed by an additional 10 min with 20 µM A23187. Activated platelets were then incubated with varying concentrations of PLD for 1 hr at 37 ºC, after which they were diluted to 1.02 x 10⁶ to quantify initial rates of fX activation by sTF:fVIIa as described above for liposomes, except that Tyrode’s buffer was used in place of HBSAC.

*Clotting assays*—Clotting assays were performed in a Start4 coagulometer (Diagnostica Stago). For sTF-clotting assays, 50µL mixtures of sTF and liposomes were added to the coagulometer’s cuvettes, and incubated at 37ºC for 60sec, then 50 µL prewarmed pooled normal human plasma was added to the mixtures of sTF and liposomes and incubated for 60 more seconds. To start the clotting reaction, 50µL prewarmed 25mM CaCl₂ was added to the cuvette and the times to clot formation (PT) were measured. The final concentration of 100nM sTF and 50 µM phospholipids were used for these
experiments. FXa-clotting assays were triggered by adding fXa to plasma, bypassing the fX activation step of clotting reactions. 50µL liposome samples were incubated at 37°C for 120sec, then 50µL prewarmed pooled normal plasma was added, followed by 50µL of mixture of 25mM CaCl₂ and 1nM fXa to start the clotting reaction. After that the times to clot formation (PT) were measured. The final concentration of phospholipids was 50µM.

**Results**

*Phospholipid Specificity in Binding of Clotting Proteins to Membranes*—To evaluate the phospholipid specificity of membrane binding for all seven of the known GLA domain-containing clotting proteins, we quantified their binding to nanoscale phospholipid bilayers immobilized on Biacore chip surfaces, using Nanodiscs containing 50% L-PS or D-PS (balance = 50% PC). As a general control for anionic phospholipids, we also used Nanodiscs containing 50% PA (balance = 50% PC). In addition to examining the seven human clotting proteins with GLA domains, we also examined bovine PT (bPT; abbreviations without a preceding “b” all refer to human clotting proteins), as its PS-binding properties have been extensively studied in the past.

Binding results presented in Fig. 3.1 show that all eight proteins bound preferentially to bilayers containing L-PS compared to D-PS; thus, their membrane binding is stereospecific for L-serine in the PS headgroup.

While most of the proteins exhibited saturable binding to 50% L-PS nanobilayers, fVIIa gave no evidence of saturation even at 1 µM fVIIa (Fig. 3.1H), consistent with previous estimates of its $K_d$ of about 15 µM for binding to PS/PC liposomes. Similarly, PrZ bound relatively weakly to 50% L-PS nanobilayers, and furthermore the binding isotherms for both PrZ and PrS were sigmoidal (possibly due to protein aggregation). Therefore, reliable binding parameters for fVIIa, PrS and PrZ could not be calculated using 50% L-PS nanobilayers, although qualitative differences in their phospholipid binding specificities could be readily discerned. And finally, some of the proteins did not bind extensively enough to nanobilayers containing 50% D-PS and/or 50% PA to allow reliable determination of their binding parameters over the protein concentration ranges tested.
Table 1 summarizes the binding parameters that could be determined for fIX, fX, bPT, PT and APC. $K_d$ values for binding of these proteins to 50% l-PS nanobilayers ranged from 0.4 µM (fX) to about 3 µM (APC and PT). Binding stoichiometries for four of these proteins (fIX, fX, PT and bPT), expressed as the number of protein molecules bound at saturation per leaflet on 50% l-PS nanobilayers, were similar to each other, ranging from 5.0 to 6.6 protein molecules/leaflet (Table 1). We previously demonstrated that the type of Nanodiscs used in this study contain 67 ± 1 total phospholipid molecules per leaflet $^4$. Therefore, for these four proteins, there was one protein binding site per 5 to 7 l-PS molecules, which is in reasonably good agreement with our previous calculation of one binding site for fX for every 7.9 l-PS molecules $^4$. On the other hand, the maximal binding stoichiometry for APC on 50% l-PS nanobilayers was 3.29 ± 0.11 protein molecules/leaflet, corresponding to about one protein binding site per 10 l-PS molecules.
Table 3.1. Binding parameters for clotting proteins on Nanoscale bilayers of varying anionic phospholipid composition

<table>
<thead>
<tr>
<th>Phospholipid composition*</th>
<th>fX</th>
<th>fIX</th>
<th>PT</th>
<th>bPT</th>
<th>APC</th>
<th>fVIIa</th>
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<tr>
<td></td>
<td>$K_d$ (µM)$^\dagger$</td>
<td></td>
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<tr>
<td>50% L-PS</td>
<td>0.38 ± 0.005</td>
<td>1.5 ± 0.1</td>
<td>3.1 ± 0.3</td>
<td>2.0 ± 0.2</td>
<td>3.0 ± 0.4</td>
<td>nd$^\ddagger$</td>
</tr>
<tr>
<td>50% d-PS</td>
<td>1.07 ± 0.06</td>
<td>1.2 ± 0.2</td>
<td>4.2 ± 0.6</td>
<td>3.2 ± 0.3</td>
<td>3.6 ± 0.3</td>
<td>nd</td>
</tr>
<tr>
<td>50% PA</td>
<td>0.85 ± 0.18</td>
<td>1.08 ± 0.1</td>
<td>3.3 ± 0.4</td>
<td>2.4 ± 0.1</td>
<td>2.1 ± 0.4</td>
<td>1.7 ± 0.1</td>
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<table>
<thead>
<tr>
<th>Binding stoichiometry (protein molecules bound/leaflet)$^\ddagger$</th>
<th></th>
<th></th>
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<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>50% L-PS</td>
<td>5.6 ±0.4</td>
<td>6.6 ± 0.2</td>
<td>5.0 ± 0.1</td>
<td>6.2 ± 0.2</td>
<td>3.3 ± 0.1</td>
</tr>
<tr>
<td>50% d-PS</td>
<td>2.9 ± 0.2</td>
<td>2.9 ± 0.4</td>
<td>3.0 ± 0.2</td>
<td>4.8 ± 0.1</td>
<td>2.5 ± 0.2</td>
</tr>
<tr>
<td>50% PA</td>
<td>2.8 ± 0.04</td>
<td>3.3 ± 0.5</td>
<td>3.3 ± 0.2</td>
<td>4.4 ± 0.3</td>
<td>6.8 ± 1.0</td>
</tr>
</tbody>
</table>

*Nanodiscs contained the indicated anionic phospholipid (balance = 50% PC).
†Values (mean ± standard error; n = 3) are derived from fitting the one-site ligand binding equation to binding isotherms such as those depicted in Fig. 3.1.
‡nd, not determined.

As an additional control for headgroup specificity, we quantified the binding of all eight proteins to nanobilayers containing 50% PA, the simplest phospholipid. As expected, six of these proteins (fIX, fX, PT, bPT, PrS and PrZ) showed much less binding to PA-containing bilayers at each input protein concentration than to L-PS-containing bilayers (Fig. 3.1A-F). Very unexpectedly, however, almost three to four times as much fVIIa protein bound to nanobilayers containing 50% PA compared to bilayers containing 50% L-PS (Fig. 3.1H). This was similar for APC, as about twice as much of this protein bound to nanobilayers containing 50% PA compared to 50% L-PS (Fig. 3.1G). The binding of both fVIIa and APC to 50% PA nanobilayers was saturable and yielded a $K_d$ of 1.7 ±1.0 µM and a binding stoichiometry of 4.7 ± 1.5 fVIIa molecules/leaflet for fVIIa, a $K_d$ of 2.1 ±0.4 and a binding stoichiometry of 6.8 ± 1.0 APC molecules/leaflet for APC (Table 1). This corresponds to about APC binding site per 5 PA molecules, which is about half of what was required for APC to bind to PS (i.e., we calculated one APC binding site per 10 L-PS molecules). Thus, APC and fVIIa recognize PA preferentially over L-PS or d-PS when binding to membranes, as revealed by their higher binding stoichiometries on PA-nanobilayers.

Enhancement of fVIIa Enzymatic Activity by PA—It is well known that reactions of the blood clotting cascade involving GLA domain-containing proteins are greatly accelerated in the presence of membranes containing anionic phospholipids, with PS exhibiting the
highest activity by far \(^1\). (For the remainder of this report, we refer to \(\text{L-PS}\) simply as “PS.”) The surprising ability of \(\text{fVIIa}\) to bind to PA-containing nanobilayers prompted us to examine how liposomes with PA would modulate the rate of \(\text{fX}\) activation by \(\text{fVIIa}\), with or without its protein cofactor, TF. In particular, we used either the isolated ectodomain of TF (sTF) or fully membrane-anchored TF (membTF).

We first measured rates of \(\text{fX}\) activation by sTF:fVIIa in the presence of 50 \(\mu\)M liposomes with varying proportions of PA and PS (balance = PC), and normalized the rates to that observed with 30% PS/70% PC. As expected, binary mixtures of PS and PC (open squares in Fig. 3.2A) showed increasing rates of \(\text{fX}\) activation as the PS content increased, being maximal at 30% PS/70% PC. In parallel experiments, we varied the proportions of PA and PS such that the total PA + PS was kept at 30% and the balance was 70% PC (open circles in Fig. 3.2A). Liposomes containing 30% PA/70% PC (y-intercept) supported a rate of \(\text{fX}\) activation 6.8-fold higher than liposomes with 30% PS/70% PC, and the maximal rate was observed when liposomes contained 5% PS/25% PA/70% PC (17.5-fold higher than with 30% PS/70% PC). We also measured rates of \(\text{fX}\) activation by fVIIa alone, which showed a very similar ability of PA to enhance \(\text{fX}\) activation (Fig. 3.2B), although higher fVIIa concentrations were required in the absence of its protein cofactor. As with sTF:fVIIa, the optimal liposome composition was 5% PS/25% PA/70% PC and yielded a similar increase in activity (23.1-fold) over that observed with 30% PS/70% PC. This optimal phospholipid composition likely results from a compromise between preferential binding of fVIIa to PA (with or without sTF) and preferential binding of fX to PS (Fig. 3.1 and ref. \(^3\)).

In contrast to experiments with sTF, when membTF is employed as the cofactor for fVIIa, almost all the binding energy holding the two proteins together on the membrane surface comes from protein-protein interactions, and in fact the GLA domain of fVIIa is not required for this protein to bind productively to membTF (which is an integral membrane protein \(^{13}\)). Consistent with this notion, liposomes containing combinations of PA and PS (totaling 30%) supported rates of \(\text{fX}\) activation by membTF:fVIIa that were never higher than the rate observed with 30% PS/70% PC liposomes (Fig. 3.2C). This argues that the contribution of PA to enhancing the rate of \(\text{fX}\) activation by fVIIa or sTF:fVIIa results directly from its superior ability over PS to recruit fVIIa to the membrane surface, rather than effects of fX or TF. **Synergy Between PA and**
Phosphatidylethanolamine (PE) to enhance sTF:fVIIa activity—We previously showed that PE synergizes strongly with PS to promote fX activation by the membTF:fVIIa complex, even though PE supports very little activity without PS. Furthermore, we demonstrated that most glycerophospholipids other than PC also synergize with PS to promote fX activation by membTF:fVIIa, in a mechanism we termed the ABC (Anything But Choline) hypothesis. In fact, Fig. 3.2C demonstrates typical synergy between either PA or PE with PS to enhance fX activation by membTF:fVIIa. We were curious, however, whether PE could synergize with PA (with or without PS) to enhance fX activation by sTF:fVIIa. Fig. 3.2D (solid symbols) shows that PE increased all the observed rates of fX activation by sTF:fVIIa, with or without PA, with maximal activity supported by liposomes containing 5% PS/25% PA/70% PC. This finding argues strongly that the ability of PA to enhance fX activation by sTF:fVIIa is not just due to the previously-documented “synergy” that can occur between PS and most other non-PC phospholipids to enhance fX activation by membTF:fVIIa (which appears largely to be due to enhanced recruitment of fX to the membrane surface).

In cell membranes, PA is a minor phospholipid, typically constituting some 1 to 4% of total cellular lipid. We therefore explored whether (patho)physiologically relevant levels of PA could enhance fVIIa enzyme activity. Accordingly, we examined rates of fX activation using liposomes containing 0-5% PA, along with 5% PS and 30% PE (balance = PC), which should better reflect the composition of plasma membranes. The results in Fig. 3.2E, normalized to the rate observed with 5% PS/30% PE/65% PC, show that even low levels of PA measurably enhanced fX activation by sTF:fVIIa. Thus, liposomes with 1.5% PA approximately doubled the rate of fX activation compared to liposomes without PA, while liposomes with 5% PA exhibited a 7.5-fold increase in activity.

Treating Platelets with Phospholipase D (PLD) to Enhance sTF:fVIIa Activity—PLD, a highly regulated enzyme in mammalian cells which is also expressed by certain pathogenic microorganisms, generates PA by removing phospholipid headgroups. We postulated that treating activated platelets with PA would expose additional PA on the platelet surface, which in turn should enhance the rate of fX activation by sTF:fVIIa. Indeed, PLD treatment of activated human platelets enhanced their ability to support sTF:fVIIa-mediated fX activation about six-fold (Fig. 3.2F). Boiling PLD, before the experiment, diminished its effect on enhancing fX activation rates.
Fig. 3.2. Enhancement of fVIIa enzymatic activity by PA. (A) Rates of fX activation by sTF:fVIIa in the presence of liposomes (balance = PC) containing either 0-30% PS (□), mixtures of PS and PG such that PS + PG = 30% (▼), or mixtures of PS and PA such that PS + PA = 30% (○). Rates were normalized to 30% PS/70% PC. (B) Rates of fX activation by fVIIa (no sTF) in the presence of liposomes (balance = PC) containing either 0-30% PS (□), or mixtures of PS and PA such that PS + PA = 30% (○). Rates were normalized to 30% PS/70% PC. (C) Rates of fX activation by fVIIa in the presence of membTF-liposomes (balance = PC) containing either 0-30% PS (□), mixtures of PS and PE such that PS + PE = 30% (■), or mixtures of PS and PA such that PS + PA = 30% (○). Rates were normalized to 30% PS/70% PC. (D) Rates of fX activation by sTF:fVIIa in the presence of liposomes (balance = PC) containing 30% PE plus either 0-30% PS (■), or mixtures of PS and PA such that PS + PA = 30% (●). Rates were normalized to 30% PS/70% PC. (E) Rates of fX activation by sTF:fVIIa in the presence of liposomes (balance = PC) containing 5% PS and 30% PE, plus 0-5% PA (○). The horizontal dotted line is the value for liposomes with 10% PS, 30% PE, 0% PA. Rates were normalized to 5% PS/30% PE/65% PC. (F) Rates of fX activation by sTF:fVIIa in the presence of activated platelets that had been pretreated with the indicated PLD concentrations. Rates were normalized to the rate with activated platelets not treated with PLD. Data in all panels are mean ± standard error (n = 3 to 7).

Enhancement of sTF:fVIIa Enzymatic Activity by Phosphatidylinositol (PI) Phosphates—
The PA headgroup contains a monoester phosphate, which can have a higher negative charge than the diester phosphates in most other phospholipids. We hypothesized, therefore, that the presence of a monoester phosphate in PA was responsible for its ability to enhance the binding of fVIIa and APC to membranes. Notably, phosphorylated derivatives of PI contain one or more monoester phosphates attached to the myo-inositol moiety, leading us to predict that PI phosphates may also support enhanced fVIIa enzymatic activity. Accordingly, we examined rates of fX activation by sTF:fVIIa using liposomes containing 0-5% PI phosphates along with 5% PS and 30% PE (balance =
The PI phosphates tested included PI that is singly phosphorylated at the 3, 4, or 5 position of myo-inositol (PI3P, PI4P, or PI5P), as well as doubly phosphorylated PI (PI(3,4)P2, PI(3,5)P2, or PI(4,5)P2) and triply phosphorylated PI (PI(3,4,5)P3). The results (Fig. 3.3A) showed that, PI containing one monoester phosphate at position 4 (PI-4P) markedly enhanced the rate of fX activation, showing the highest activity. Notably, when liposomes contained 5% PS and 30% PE, the rate of fX activation was approximately doubled when the liposomes had 1.5% PI4P (compared to no PI4P; Fig. 3.3A).

![Fig. 3.3. Enhancement of fVIIa enzymatic activity, and membrane binding, by PI4P.](image)

(A) Rates of fX activation by sTF:fVIIa in the presence of liposomes (balance = PC) containing 5% PS and 30% PE, plus 0-5% PI4P (○). The horizontal dotted line is the value for liposomes with 10% PS, 30% PE, 0% PA. Rates were normalized to 5% PS/30% PE/65% PC. (B) Rates of fX activation by sTF:fVIIa in the presence of liposomes (balance = PC) containing mixtures of PA and PI4P such that PA + PI4P = 30% (▼). Rates were normalized to 30% PI4P/70% PC. (C) Binding of fVIIa to Nanodiscs containing either 20% L-PS (■), 20% PA (▼), or 20% PI4P (●) (balance = 80% PC). Binding data were acquired and analyzed as in Fig. 3.1. (D) Rates of fX activation by sTF:fVIIa in the presence of liposomes (balance = PC) containing either 0-30% PS (□), or mixtures of PS and PI4P such that PS + PI4P = 30% (◊). Rates were normalized to 30% PS/70% PC. (E) Rates of fX activation by sTF:fVIIa in the presence of liposomes (balance = PC) containing 30% PE plus either 0-30% PS (■), or mixtures of PS and PI4P such that PS + PI4P = 30% (♦). Rates were normalized to 30% PS/70% PC. Data in all panels are mean ± standard error (n = 3 to 6).

We also examined rates of fX activation by sTF:fVIIa on liposomes made with mixtures of PA and PI4P, where PA + PI4P was kept constant at 30% and the balance was 70%PC. The relation between PA and PI4P was linear, suggesting that PA and PI4P support same binding interactions for fX activation by sTF:fVIIa on phospholipid
membranes (Fig. 3B). This was also tested by comparing the binding of fVIIa to Nanodiscs made with 20% either POPA, or PI4P, or POPS. fVIIa bound to PA and PI4P membranes similarly, preferring both phospholipids to PS (Fig. 3C).

We further examined the effect of PE on its ability to enhance fX activation by sTF:fVIIa, using liposomes containing 0 to 30% PI4P + PS, either without PE (Fig. 3.3D) or with 30% PE (Fig. 3.3E). Optimal enhancement of fX activation without PE occurred when liposomes contained 10% PI4P, 20% PS, 70% PC, while optimal enhancement with PE occurred when liposomes contained 10-20% PI4P, 10-20% PS, 70% PC. This effect of PE on the enhancement of fVIIa activity by PI4P is thus very similar to that observed with PA (Fig. 3.2D).

Enhancement of APC Enzymatic Activity by PA and PI4P—Since APC bound more extensively to PA-Nanodiscs than to PS-Nanodiscs, we tested the ability of PA-containing liposomes to accelerate the rate of proteolytic inactivation of factor Va (fVa) by APC. Liposomes were made with binary mixtures of PS and PC (0-30% PS; balance = PC) or mixtures of PS + PA or PS + PI4P, in which the total amount of anionic lipid equaled 30% and the balance was 70% PC. Initial rates of fVa inactivation by APC were normalized to the rate on 30% PS/70% PC liposomes (Fig. 3.4).

Enhancement of APC Enzymatic Activity by PA and PI4P—Since APC bound more extensively to PA-Nanodiscs than to PS-Nanodiscs, we tested the ability of PA-containing liposomes to accelerate the rate of proteolytic inactivation of factor Va (fVa) by APC. Liposomes were made with binary mixtures of PS and PC (0-30% PS; balance = PC) or mixtures of PS + PA or PS + PI4P, in which the total amount of anionic lipid equaled 30% and the balance was 70% PC. Initial rates of fVa inactivation by APC were normalized to the rate on 30% PS/70% PC liposomes (Fig. 3.4).

Fig. 3.4. Enhancement of APC enzymatic activity by PA and PI4P. Rates of fVa inactivation by APC were quantified in the presence of liposomes (balance = PC) containing either 0-30% PS (■), mixtures of PS and PA such that PS + PA = 30% (●), or mixtures of PS and PI4P such that PS + PI4P = 30% (◊). Rates were normalized to 30% PS/70% PC. Data are mean ± standard error (n = 3).
Both PA and PI4P enhanced the rate of fVa inactivation, with maximal rates observed with PA-liposomes containing 5% PS, 25% PA, 70% PC, or with PI4P-containing liposomes containing 10% PS, 20% PI4P, 70% PC. The rates were increased fivefold and twofold, respectively, compared to 30% PS liposomes. Thus, as we found for fVIIa, not only does APC bind more extensively to bilayers containing PA than PS, its enzymatic activity is also enhanced when either PA or PI4P are included in liposomes that also contain PS.

Shortening the TF-clotting times by PA—To further study the effect of PA on enhancing the rates of fX activation by sTF:fVIIa, clotting assays were performed. The sTF-clotting times on liposomes with varying proportions of PA and PS (balance = PC) were measured. As expected, binary mixtures of PS and PC (open squares in Fig. 3.5A) showed decreasing clotting times as the PS content increased, with the shortest clotting time being at 30% PS/70% PC (51.6 ± 0.6 sec). In parallel experiments, we varied the proportions of PA and PS such that the total PA + PS was kept at 30% and the balance was 70% PC (closed circles in Fig. 3.5A). Consistent with our previous results, liposomes made with 10% PS/20% PA/70% PC, or 20% PS/10% PA/70% PC supported 34.2 ± 2.8 sec and 34.4 ± 0.5 sec sTF-clotting times, which were significantly lower than the clotting times on 30%PS/70%PC liposomes.

Fig. 3.5. Shortening the TF-clotting times by PA. (A) sTF-clotting times, and (B) fXa-clotting times in the presence of liposomes (balance = PC) containing either 0-30% PS (□), or mixtures of PS and PA such that PS + PA = 30% (●). 50μM phospholipids was used, the final concentration of 100nM sTF was used in (A), and everything was diluted in HBA. Data are mean ± standard error (n=3).
fXa-clotting assays on the same liposomes (Fig. 5B) only showed the synergy between PS and PA phospholipids for supporting the prothrombinase complex, and PS/PA liposomes did not support shorter fXa-clotting times, compared to 30%PS/70%PC liposomes.

Discussion

In this paper we show the PS stereospecificity of GLA domains of clotting proteins, and also compare their binding affinities for different phospholipid membranes. We also show how, surprisingly, two of the GLA domains, GLA domain of fVIIa and APC, prefer PA and PI-4P to PS molecules.

The driving force for this project was to compare binding of GLA domains of clotting proteins side by side and under similar conditions, and to determine their PS stereospecificity by SPR technique. Based on the older studies, the binding affinities of these GLA domains for phospholipid membranes is in a wide range, varying from nanomolar binding affinities (fX) to micromolar (fVIIa) $K_d$ values. The binding affinities that could be determined by SPR technique in our experimental setup were all in the micromolar range for 50% L-PS / 50% PC membranes. This might be because of using 50% PS in Nanodiscs, which is higher than physiologic PS concentration in activated platelet membranes. High PS concentrations were used to differentiate between bindings to different types of phospholipid membranes and to be able to determine the $K_d$ values accurately. The $K_d$ values for human protein S and human protein Z could not be determined accurately since the binding events were not following a hyperbolic binding curve after injection of concentrations more than 500nM on phospholipid membranes, probably due to precipitation or slow dissociation from membranes. If precipitation is not the case, the binding curves for these two proteins, looks sigmoidal which suggest a cooperative binding of these proteins to membranes, presumably through dimerization, e.g. for protein S. All proteins tested preferred phosphatidylyserine with L-serine (L-PS) in its headgroup to D-serine (D-PS). The binding affinities for L-PS and D-PS membranes are in the same order of magnitude. Binding to D-PS is a result of phosphate specific interactions and in some cases serine specific interactions, and binding to L-PS is a result of the same set of interactions along with phospho-L-serine specific interactions.
The $K_d$ values are a result of these interactions and it is possible that with $K_d$ s for L-PS and D-PS we are studying different types of interactions.

PA was used as the negative control. Since PA only has a phosphate group in its headgroup, if binding to D-PS is a result of phosphate-specific interactions, PA should show the same binding. fX, fIX, PT and bPT bound to D-PS and PA more or less in the same way, consistent with the ABC hypothesis predictions. Slightly better binding to PA compared to D-PS in some cases could be a result of a higher negative charge in PA headgroup at physiologic pH. Human protein S (PrS) and human Protein Z (PrZ) preferred D-PS to PA, which could be a result of serine specific interactions. Surprisingly, human APC and fVIIa preferred PA to PS. This was surprising because the GLA domains of clotting proteins have always been considered to prefer PS to other types of phospholipids. This finding was tested by measuring the effect of PA presence in phospholipid membranes on fX activation by fVIIa and fVa inactivation by APC. The results showed that in the presence of PA, the rates of fX activation by fVIIa or sTF:fVIIa, and fVa inactivation by APC on phospholipid membranes compared to 30% PS were about 15 fold and 4 fold increased respectively, which supported the Biacore findings.

fVIIa and APC act as important key points in blood clotting cascade. The reactions that are catalyzed by these two proteins require both enzyme complex and substrate to be bound to phospholipid membranes for proper functioning of the enzymatic reaction. fVIIa and APC bind phospholipid membranes with low affinities. Enhancing the binding affinity of these proteins for membranes have been the subject of several previous studies. Here we showed that although fVIIa and APC bind very weakly to PS molecules, their binding affinities for membranes are significantly enhanced when PA is incorporated into membranes.

Phosphatidic acid (PA) is one of the most important phospholipids in cell membranes with roles ranging from being the intermediate for synthesis of several types of phospholipids in cells, to activating several signaling pathways through binding to its effector. Beside that, PLD which is a physiologically and pathologically regulated enzyme that catalyzes PA production. Due to these constant changes in PA production and consumption, PA concentration in cell membranes varies between 1 to 4%; these
changes usually are the signals for binding of effector proteins and initiation of signaling pathways.

PA headgroup is a phosphomonoester group with two ionizable groups: pKₐ₁= 3.0 and pKₐ₂=6.9-7.9. So at physiologic pH, the first group is completely deprotonated, but the second group can be protonated or deprotonated, depending on the pH and the local environment around the PA head group. Multivalent cations, or amine, or hydroxyl groups can drive deprotonation of the second ionizable group in PA headgroup. So at physiologic pH, PA charge varies between -1 and -2 depending on its local environment. PA can act as a pH sensor in cells; changes in its headgroup charge resulted from changes in local pH, would provide the signal for binding of PA effector proteins.

Our hypothesis is that the phosphomonoester group in PA headgroup is the reason for fVIIa and APC preference for PA over PS. To test this hypothesis, phosphorylated phosphatidylinositol (PI) derivatives, PIPs, were tested. The results showed that PI-4P behave very similarly to PA for supporting fX activation by sTF:fVIIa, and fVa inactivation by APC.

Like PA, PI-4P have phosphomonoester group in their headgroups, are present in membranes at low concentrations and their synthesis is regulated. Any changes in the concentration of these phospholipids results in triggering the signaling pathways. Our hypothesis was that the phosphomonoester group in PA headgroup is the binding site for GLA domains of fVIIa and APC. The results of our experiments showed that PI-4P enhances rates of enzymatic reactions similar to PA, which shows the importance of these phospholipids in blood clotting reactions.

A few studies have shown exposure of PA and PI-4P to the outer leaflet of human erythrocyte, or PA mediated binding of gram negative pathogens to host cells, requiring PA exposure to the outer leaflet. When platelets are activated, their inner leaflet phospholipids become exposed to the outer leaflet and the contents of their granules are released. PA and PI-Ps are considered inner leaflet phospholipids but when platelets are activated they can become exposed to the outer leaflet.
Enhancing fVIIa and APC binding affinities for phospholipid membranes enhances the rates of enzymatic reactions catalyzed by these enzymes. The rates of these enzymatic reactions depend on proper binding of both enzymes and their substrates to phospholipid membranes. The substrates for these reactions, fX and fVa respectively, require different amounts of PS for proper binding to membranes. The balance between PA and PS requirement for each reaction would differentiate between rates of pro- and anti- coagulant reactions catalyzed by fVIIa and APC, depending on local phospholipid mixture of those phospholipid membranes.
References


Chapter 3- Part B

Clotting assays

Results

To compare PS- and PA-liposomes’ ability to support clotting, TF- and fXa-clotting assays were performed on liposomes made with various mixtures of phospholipids and compared.

Fig. 3.6- (A) TF- and (B) fXa-clotting assays are triggered by addition of tissue factor or fXa to the clotting reaction. (A) sTF clotting assay with 100nM sTF and 50 µM phospholipids, all diluted in HBSA with 130mM NaCl. (B) fXa clotting assay with 33.3 µM phospholipids, everything diluted in HBSA with 130mM NaCl.

In TF-clotting assays, both fX activation by TF:fVIIa and prothrombin activation by fXa:fVa determine the speed of clotting reaction. FXa-clotting assays are focused on prothrombin activation by fXa:fVa, and are independent of liposomes’ ability to support fX activation by TF:fVIIa.

We first compared different types of liposomes by means of TF-clotting assays (Fig. 3.6A). The results showed that under the experimental conditions used, PA did not improve clotting times, compared to PS. This might be the result of fVa preference for PS, which affects the prothrombinase reaction downstream of fXa generation reaction.
To test that, liposomes were compared in their ability to support the fXa clotting assays (Fig. 3.6B). The similar trend between Fig.s 3.6A and 3.6B shows that the fVα preference for PS affects the TF-clotting assay results.

The fXa-clotting assays indicate that, under the conditioned used, we need at least 20%PS for efficient prothrombinase activity. As the next step, 20%PS/10%PA and 30%PS liposomes were compared. In Fig. 3.7, sTF clotting times at different phospholipid concentrations were measured.

Based on these results, we know that 20%PS/10%PA liposomes support shorter clotting times compared to 30%PS liposomes. Taking into account the results in Fig. 3.7, 50 µM phospholipid was chosen for the next step.

As the next step, the effect of addition of 30%PE to a phospholipid mixture of liposomes was tested. As is shown in Fig. 3.8, at a 50 µM phospholipid concentration, 30% PE shortened the clotting times on PS liposomes; while on PA liposomes, addition of 30%PE slightly shortened the clotting times on PS/PA liposomes. The clotting times at higher PS concentrations were very similar in PS/PE and PS/PA/PE liposomes, with PA and PA/PE addition to PS at lower PS concentrations having the greatest effect on shortening the clotting times (Fig. 3.8A).
Fig. 3.8. Shortening the TF-clotting times by PA. (A) sTF-clotting times, and (B) fXa-clotting times in the presence of liposomes (balance = PC) containing either 0-30% PS (□), or mixtures of PS and PA such that PS + PA = 30% (○), without (open symbols), or with (closed symbols) 30%PE. 50µM phospholipids was used, the final concentration of 100nM sTF was used in (A), and everything was diluted in HBA. Data are mean ± standard error (n=3).

The same liposomes were then used for fXa clotting assays, which showed higher effects of PE on PS liposomes than on PS/PA liposomes. The shortest clotting times were supported by 30%PS and 30%PS/30%PE liposomes. PE did not affect the PS/PA liposomes very much, while PS/PA liposomes supported a similar clotting time to the PS/PE liposomes (Fig. 3.8B).

To find out how sTF binding to phospholipid membranes affects the clotting assays, TF-liposomes were used instead of sTF and liposomes. Fig. 3.9 shows TF clotting assays on TF-liposomes made with PS or PS+PA=30%, with and without 30%PE.
The effect of 30%PE on TF-liposomes (Fig. 3.9) on lowering the TF-clotting times is far less than what was observed on liposomes (Fig. 3.8). Interestingly, clotting times on PS/PA and PS/PA/PE TF-liposomes were longer than on PS and PS/PE TF-liposomes.

To find out if PA incorporation in liposomes can lower the limit of detection for fVIIa in plasma, various concentrations of fVIIa were spiked in plasma, and TF clotting times on 30%PS or 20%PS/10%PA liposomes were compared\(^1\) (Fig. 3.10).

As is shown in Fig. 3.10, clotting times on 20%PS/10%PA liposomes were shorter than those on 30%PS liposomes. This difference is more significant at lower fVIIa concentrations. Moreover, based on the slope of the two lines, 30%PS is more sensitive to change in fVIIa concentrations, compared to 20%PS/10%PA. Although the clotting times are different, 20%PS/10%PA does not lower the limit of fVIIa detection significantly.

As the next step, fVII-immunodeleted plasma (from Haematologic Technologies, Essex Junction, VT) was used and TF-clotting times were measured on liposomes, with fVIIa spiked at low concentrations (Fig. 3.11A). In another experiment, no tissue factor was used in the clotting assay and high concentrations of fVIIa were spiked into plasma (Fig. 3.11B).
Fig. 3.11 - TF-clotting assay (A) and clotting assay without TF (B) on liposomes. (A) fVIIa was spiked into fVII-immunedeficient plasma, at low concentrations. 100nM sTF and 100 µM phospholipid was used and everything was diluted in HBA. (B) High concentrations of fVIIa were used to start the clotting assay. 100µM phospholipid was used and everything was diluted in HBA.

The TF-clotting assays in Fig. 3.11A again do not show a significant difference between PS and PS/PA liposomes. Interestingly, 30%PA/30%PE liposomes are least affected, compared to the other liposomes, with changes in fVIIa concentration.

The clotting assays on liposomes (Fig. 3.11B) show 30%PS/30%PE and 20%PS/10%PA (with and without PE) support the shortest clotting times compared to the other liposomes. 30%PA and 30%PA/30%PE liposomes are less sensitive to changes in fVIIa concentration and support longer clotting times.

**Discussion**

The goal of this study was to find out if PA liposomes shorten the clotting times compared to PS liposomes. Our initial experiments showed that clotting assay results were not exactly as we expected them to be. In fX activation experiments, we used purified proteins, and everything was at equilibrium. In clotting assays, a lot of proteins in plasma can bind to phospholipid membranes, there is a competition for binding to membranes, and at the time that clotting occurs, the system is not at equilibrium.

One of the factors that probably affected the clotting assays very much was the prothrombinase reaction downstream of the fX activation reaction. Since fVa binds best to PS liposomes, this step favors PS liposomes and affects the final clotting results (Fig. 3.6). Higher PS concentrations were required in PS/PA liposomes to see the PA effect in
clotting assays (Fig. 3.7). Also, HBA, buffer without NaCl, was used to prepare samples, since higher salt concentrations in HBSA would result in longer clotting times.

As is shown in Fig. 3.8A, on PS/PA liposomes, the clotting times under the new conditions used, on 10% PS / 20% PA or 20% PS / 10% PA liposomes were dramatically lower than the clotting times on 30% PS liposomes.

Addition of PE to the phospholipid mixture shortened the clotting times on PS and PS/PA liposomes, with the biggest effect being on PS liposomes (Fig. 3.8A).

If what was observed on PS/PA liposomes in Fig. 3.8A was mainly a result of synergy between PS and PA, the PS/PE and PS/PA clotting times should have been in the same range. But this was not the case, and PS/PA liposomes supported shorter clotting times compared to PS/PE liposomes, especially at lower PS concentrations.

The fXa clotting assays, shown in Fig. 3.8B, tested the same liposomes as in Fig. 3.8A for supporting the prothrombinase assay. PS/PA liposomes supported slightly shorter clotting times compared to PS/PE liposomes, while the clotting times on PS liposomes were significantly higher than for the other types of liposomes. PA and PE have very similar effects on fXa clotting times (Fig. 3.8B), which showed that these two phospholipids had the same role, synergizing with PS phospholipids to support the prothrombinase reaction.

The TF-clotting assays on TF-liposomes showed the importance of fVIIa binding to phospholipid membranes. In TF-clotting reactions with sTF, fVIIa binding to phospholipid membranes is important and determines the rates of the clotting reactions. On TF-liposomes, fVIIa binding to membranes is not significantly affected by fVIIa binding to membranes, and the results of TF-clotting assays on TF-liposomes (Fig. 3.9) were found to be similar to fXa-clotting assays (Fig. 3.8B). The result of this experiment was not conclusive, as the clotting times on PS/PA and PS/PA/PE liposomes were longer than those on the PS/PA liposomes. If PA affects TF incorporation in liposomes, it can lower TF concentration on TF-liposomes in PS/PA and PS/PA/PE membranes, and as a result we might have had less TF on PS/PA liposomes.

The TF-clotting assays on 30%PS, and 20%PS/10%PA liposomes (Fig. 3.10), showed that 20%PS/10%PA liposomes support shorter clotting times compared to 30%PS,
especially at lower fVIIa concentrations. The clotting times on PS/PA liposomes were less affected by the concentration of fVIIa, which showed that binding of fVIIa to these membranes is tighter than on 30%PS liposomes.

In clotting assays in which fVIIa was spiked in fVII-immunodepleted plasma (Fig. 3.11A) and in clotting assays without tissue factor, triggered by spiking high concentrations of fVIIa (Fig. 3.11B), if 30%PS is used as the reference, we can see that in both cases, liposomes without PS were less sensitive to changes in fVIIa concentrations. Fig. 3.11B shows that 20%PS/10%PA liposomes were also less affected by changes in fVIIa concentration, compared to 30%PS liposomes, indicating that PS provides sensitivity to the fVIIa concentration. Additionally, in the presence of sTF (Fig. 3.11A), clotting times on 30%PA/30%PE liposomes were shorter than or similar to the clotting times on 30%PS liposomes, but in the absence of sTF (Fig. 3.11B), the clotting times on 30%PA/30%PE liposomes were longer than on 30%PS liposomes. Also, incorporation of PA in PS/PA or PS/PA/PE liposomes did not shorten the clotting time compared to 30%PS/30%PE liposomes, in the absence of sTF.

These results show that sTF is required for shorter clotting times on PA membranes and this might again be due to the prothrombinase complex preference for PS phospholipids. In the absence of sTF, the fX activation by fVIIa cannot compete with prothrombin activation. In clotting assays without tissue factor (Fig. 3.11B), the intrinsic pathway and intrinsic tenase play an important role in driving clotting reactions. The C1 and C2 domain of fVIIIa, similar to that of fVa, requires PS phospholipids to bind to phospholipid membranes, and as a result, PS affects the clotting times the most (Fig. 3.11B).

The fact that on PS liposomes, the clotting times are more sensitive to changes in fVIIa concentration, could show that there is stronger binding on PA or PA/PE membranes.
References


Chapter 4

Effect of mixtures of calcium and magnesium on blood clotting

This chapter is a part of a manuscript, under preparation.

Introduction

In chapter one, the role of calcium and magnesium ions in folding of GLA domains of blood clotting proteins was discussed. Physiologically, there is about 1.25mM free Ca$^{2+}$ and 0.5mM free Mg$^{2+}$ in plasma. For maximum enzymatic activity in blood clotting reactions, calcium and magnesium ions should be present at, or more than, their physiologic concentrations.

Calcium ions are essential for the folding of, and membrane binding of, GLA domains of clotting factors. The physiologic concentration of calcium (1.25mM), without magnesium, is suboptimal and does not support occupancy of all calcium binding sites in the GLA domain, while saturating levels of calcium ions, ranging from 2.5 to 5mM, can support the maximum rates of enzymatic reactions in the absence of magnesium ions.

Magnesium ions cannot support clotting without calcium ions; this shows magnesium’s inability to support GLA domains binding to anionic phospholipid membranes on its own. In fact, the crystal structure of the GLA domain of fXa prepared in the presence of magnesium ions (without any calcium ions) demonstrated that the ω-loop of its GLA domain, that is responsible for its membrane binding, had a disordered structure. On the other hand, the crystal structures of GLA domains prepared in the presence of Ca$^{2+}$ or mixtures of Ca$^{2+}$ and Mg$^{2+}$, demonstrated a strong bivalent ions-GLA domain network being formed, building the keel part of GLA domain, and allowing it to bind to phospholipid membranes.

In the crystal structures of GLA domains prepared in mixtures of calcium and magnesium, 2-3 of 7 calcium binding sites in GLA domains were occupied by
magnesium ions (discussed in chapter 1). At suboptimal calcium concentrations, magnesium ions occupy specific binding sites in the GLA domain that can further support binding of GLA domain containing clotting factor to phospholipid membranes. It has experimentally been shown that presence of magnesium ions along with calcium ions enhances the affinity of enzymes for the membrane and cofactor. Phospholipid synergy studies, discussed in chapter 2, were carried out at 2.5 mM calcium concentration. Here we studied the effect of physiologic concentrations of calcium and magnesium ions on blood clotting reactions.

**Materials and Methods**

**Materials**—Phospholipids were from Avanti Polar Lipids (Alabaster, AL); Bio-Beads® SM-2 adsorbent was from Bio-Rad (Hercules, CA); human fVIIa was from American Diagnostica (Stamford, CT); human fX was from Enzyme Research Laboratories (South Bend, IN). Recombinant, membrane-anchored TF was expressed in *E. coli* and purified as described.

**TF-Liposome Preparation and Measurement of FX Activation**—TF was incorporated into liposomes of varying phospholipid composition as described previously using Bio-Beads® SM-2 and 20 mM sodium deoxycholate. Initial rates of fX activation by TF:fVIIa assembled on TF-liposomes were quantified as described previously, typically using 100 nM fX, 5 pM fVIIa, and 500 pM TF.

**Results**

Physiologic concentrations of Ca$^{2+}$ and Mg$^{2+}$ support synergy between PE and PS—To find out if incorporation of magnesium along with calcium ions in fX activation reactions affects synergy between PE and PS, rates of fX activation by TF:fVIIa on TF-liposomes were compared. Liposomes were prepared with mixtures of DOPS and DOPE, where DOPS+DOPE=30% (balance=70%PC).

As is shown in Fig. 4.1, at physiologic concentrations of Ca$^{2+}$ and Mg$^{2+}$ (1.25 mM Ca$^{2+}$ + 0.5 mM Mg$^{2+}$), PE strongly synergized with PS to support fX activation. Compared to 2.5 mM Ca$^{2+}$, physiologic mixtures of calcium and magnesium are as good or even better in supporting the PE/PS synergy for fX activation by TF:fVIIa on TF-liposomes. Suboptimal
concentrations of calcium (less than 2.5 mM), in the absence of magnesium ions, are insufficient to properly support fX activation by TF:fVIIa. PE/PS synergy still happens at 1.25 mM Ca\(^{2+}\), although to a lesser extent.

**Fig. 4.1- Physiologic mixtures of Ca\(^{2+}\) and Mg\(^{2+}\) support synergy between PE and PS.** TF-liposomes were prepared with DOPS (open symbols) or mixtures of DOPS and DOPE (closed symbols), where DOPS+DOPE=30%. Rates of fX activation by TF:fVIIa on these liposomes were measured at 1.25mM Ca\(^{2+}\) (shown in black), or at 2.5 mM Ca\(^{2+}\) (in blue), or at 1.25 mM Ca\(^{2+}\) and 0.5 mM Mg\(^{2+}\) (in red). Data are mean ± standard error; n = 3.

These results showed that PE can synergize with PS at various calcium concentrations. The highest rates are reached at or above the physiologic concentrations of calcium and magnesium; while in the absence of magnesium, calcium concentrations above 2.5 mM are required.

*Magnesium enhances rates of fX activation by TF:fVIIa at any calcium concentration*—To find out if increasing calcium concentrations would make the presence of magnesium ions redundant, rates of fX activation by TF:fVIIa on PS/PE liposomes, at increasing calcium concentrations, with and without 0.5 mM magnesium, were compared.

Rates of fX activation in mixtures of calcium and magnesium ions, normalized to those rates at the same calcium concentrations without magnesium (Fig. 4. 2A), showed that
at lower calcium concentrations, the effect of magnesium ions is more pronounced. Furthermore, liposomes with lower PS contents are more affected by the addition of magnesium, than those made with higher PS concentrations.

Fig. 4.2- Magnesium enhances rates of fX activation by TF:fVIIa at any calcium concentration. Rates of fX activation by TF:fVIIa on selected TF-liposomes in the presence of increasing calcium concentrations along with 0.5 mM magnesium were normalized to fX activation rates (A) in the presence of the corresponding calcium ion concentrations, without magnesium (e.g. (Rates at 1.25 mM Ca²⁺ and 0.5 mM Mg²⁺)/ (Rates at 1.25 mM Ca²⁺)) or; (B) in the presence of calcium concentrations with a similar ionic strength to those with mixtures of calcium and magnesium (e.g. (Rates at 1.25 mM Ca²⁺ and 0.5 mM Mg²⁺)/(Rates at 1.75 mM Ca²⁺)). Data are mean ± standard error; n = 3.

If fX activation rates in the presence of mixtures of calcium and magnesium ions are normalized to those at calcium concentrations with the same ionic strength, rate enhancements are less pronounced (Fig. 4. 2B), while rates of fX activation in the presence of calcium and magnesium ions were always higher than those with just calcium ions (Fig. 4. 2A and B).

Discussion

Although the presence of Ca²⁺ is required for blood clotting, it has been shown that the affinity of clotting factors for cellular membranes, tissue factor, or anticoagulant agents is strongly enhanced when Mg²⁺ occupies the two external calcium binding sites in the GLA domain⁹,₁⁰,₁₄⁻₁₆. This is consistent with our findings (Fig. 4. 2), suggesting that Mg²⁺ enhances fX GLA domain binding to phospholipid membranes. Increasing Ca²⁺ concentration up to 5mM reduced the effect of Mg²⁺, but could not completely mask it, indicating that some of the calcium/magnesium binding sites are better stabilized by Mg²⁺.¹⁷
The GLA domain of fVIIa is crystalized both in the presence of Ca\(^{2+}\) (1DAN)\(^8\), or in mixtures of Ca\(^{2+}\) and Mg\(^{2+}\) (2A2Q)\(^3\). In the 2A2Q structure, three of the seven Ca\(^{2+}\) ions bound to the GLA domain in 1DAN, (Ca\(^{2+}\) 7, Ca\(^{2+}\) 5, Ca\(^{2+}\) 9), are replaced with magnesium ions and the folding of the GLA domain \(\omega\)-loop is different in these two structures.

The folding of the \(\omega\)-loop in crystals obtained in 5mM Ca\(^{2+}\) and 2.5 mM Mg\(^{2+}\), is similar to the 2A2Q structure, with the same three positions occupied by magnesium ions. In crystal structures prepared at 45mM Ca\(^{2+}\) and 5mM Mg\(^{2+}\), the folding of the \(\omega\)-loop is similar to that of 1DAN, although the Ca\(^{2+}\)-7 and Ca\(^{2+}\)-9 positions are occupied by Mg\(^{2+}\)\(^18\). Based on these results, it has been suggested that four calcium and three magnesium ions are bound to the GLA domain of plasma fVIIa, and high concentrations of calcium are required to switch the middle Mg\(^{2+}\) with a Ca\(^{2+}\)\(^19\).

In the X-ray structure of fIX bound to its ligand (1J34), the middle metal binding site is occupied by Ca\(^{2+}\) and the two external binding sites are occupied by Mg\(^{2+}\). It is shown that in this GLA domain structure, calcium ions directly interacted with Gla residues and the external binding sites were stabilized by Mg\(^{2+}\) ions because of magnesium's strong electrostatic interaction with the environment\(^17\). On the other hand, in the X-ray structure of fVIIa (2A2Q), the middle and external metal binding sites are occupied with magnesium ions, and the interactions between magnesium ions and Gla residues are established through the presence of crystallized water molecules\(^17\). These two structures can be the interconverting forms of the same system. If the system is present in a hydrophilic environment, like plasma, the middle binding site is occupied by magnesium, which can interact with the Gla residues through water molecules. When the \(\omega\)-loop is interacting with the phospholipid membrane, calcium is present in the middle binding site; the environment is hydrophobic and so the calcium molecules interact directly with Gla residues and would not need water molecules to keep the folding of the \(\omega\)-loop\(^17\).

The middle metal position in ligand-bound fIX at 5mM Ca\(^{2+}\) and 2.5 mM Mg\(^{2+}\) is occupied by calcium and in the ligand-free position by magnesium\(^4\,\!^2\). This is another example that is consistent with the suggested mechanism for switching the calcium and magnesium ions in the GLA domain, which suggests that the conformation of the \(\omega\)-loop in plasma clotting factors might not be optimal for binding to phospholipid membranes, where four calcium and three magnesium ions are bound to their GLA domains. The optimal
conformation might possibly be achieved by switching the middle magnesium ion with a calcium ion\textsuperscript{17,19}.

The fact that the synergy between PE and PS is not affected by mixtures of calcium and magnesium ions shows that the phosphor-L-seine and the phosphate-specific interactions are not sensitive to switching calcium with mixtures of calcium and magnesium.

As mentioned above, in the presence of mixtures of calcium and magnesium, when the GLA domain binds to the phospholipid membrane, the middle magnesium ion is switched with a calcium ion, providing the membrane binding folding of the $\omega$-loop. Since the phosphor-L-seine and phosphate-specific interactions are mainly involved with middle calcium ions, the synergy is not different between the all-calcium or calcium/magnesium conditions (Fig. 4. 1).

On the other hand, in the calcium/magnesium condition, the two external metal binding sites are occupied with magnesium ions, which provide better stabilization of these compared to calcium ions. This is probably the main reason for higher fX activation rates in the presence of magnesium ions (Fig. 4. 1) and greater effects of magnesium ions at lower calcium or PS concentrations (Fig. 4. 2A). At higher calcium concentrations or when rates are normalized to those at similar ionic strength conditions, the effect of magnesium ions is less, but is not completely masked (Fig. 4. 2), which shows that calcium can substitute for magnesium when the GLA domain binds to phospholipid membranes; but cannot stabilize the GLA domain on phospholipid membranes as strongly as magnesium does.
References


Chapter 5

Phospholipid specificity of prothrombin’s GLA domain for binding to phospholipid membranes

Introduction

It was shown that when the GLA domain of prothrombin binds to a PS membrane, it promotes formation of a new population of PS molecules, involved in PS-specific binding interaction with prothrombin’s GLA domain \(^1\). It has also been shown that PE synergizes with PS to enhance the activity of the prothrombinase complex\(^2\).

Based on the binding studies carried out in chapter 3, we already knew that fX binds specifically to phosphatidyl-L-serine (L-PS) phospholipids and binds much more weakly to the other anionic phospholipids. The results of prothrombin binding to these membranes showed that although prothrombin binds preferentially to L-PS phospholipids, the difference between binding to L-PS and the non-PS phospholipids is not as pronounced as that of fX. Furthermore, in chapter 2, it was shown that the rates of enzymatic reactions on 30% non-PS phospholipids are very low and there is a linear relationship between PE and PG for supporting fX activation by TF:VIIa enzyme complex. This shows that PE and PG basically have similar roles in these reactions.

The ABC hypothesis, introduced in chapter 2, was mainly focused on fX activation by TF:VIIa. To further test the ABC hypothesis for the prothrombinase complex, the first step is to study the phospholipid specificity of the prothrombin’s GLA domain.

Materials and Methods

**Materials**—phospholipids were from Avanti Polar Lipids (Alabaster, AL); Bio-Beads® SM-2 adsorbent was from Bio-Rad (Hercules, CA); human fXa and human prothrombin PT were from Enzyme Research Laboratories (South Bend, IN); human fVa and DAPA were from Haematologic Technologies Inc. (Essex Junction, VT).

**Liposome Preparation and Measurement of Prothrombin Activation**—Liposomes were prepared using varying phospholipid composition as described previously \(^3\) using Bio-
Beads® SM-2 and 20 mM sodium deoxycholate. Initial rates of prothrombin activation by fXa:fVa on liposomes were quantified at 37 °C by DAPA assays having final concentrations of 20 mM HEPES-NaOH pH 7.4, 100 mM NaCl, 2.5 mM CaCl₂, 0.1% BSA, 1.5 μM prothrombin, 25 pM fXa, 10 nM fVa and 10 μM DAPA. DAPA is a specific inhibitor of thrombin and when bound to thrombin, its fluorescence intensity is enhanced. Using DAPA, we could study thrombin generation in real time.

**Results**

*Non-PS phospholipids support the prothrombinase complex activity and can synergize with PE to enhance prothrombin activation*— The prothrombinase activity of fVa:fXa on liposomes made with either 30% DOPS or 30% DOPG or 30% DOPA (balance: DOPC), showed that the non-PS anionic phospholipids tested can support the prothrombinase activity with rates comparable to, but always less than the rates on PS membranes (Fig. 5.1). Rates of prothrombin activation on liposomes prepared with 0-30% either DOPS or DOPG or DOPA, with and without 30% DOPE (balance: DOPC), showed that non-PS anionic phospholipids can synergize with DOPE to enhance prothrombin activation by fVa:fXa; although higher concentrations of DOPG or DOPA were required to reach the maximum enzymatic activity, which was always less than the maximum enzymatic activity of PS membranes (Fig. 5.1).

![Fig. 5.1 - Non-PS phospholipids support prothrombin activation by fVa:fXa, and synergize with PE to enhance prothrombin activation. Liposomes were prepared with 0 – 30% DOPS (shown in black), DOPG (in red); or DOPA (in blue); with (closed symbols) or without (open symbols) 30% DOPE. The x-axis shows the DOPS, or DOPG, or DOPA concentration in liposomes; and the y-axis shows the rate of prothrombin activation in RFU/min. Data are mean ± standard error; n = 3 (The DOPA results were repeated once).](image-url)
Discussion

Prothrombin activation by fVa:fXa on liposomes requires proper interaction of prothrombin, fVa and fXa with phospholipid membranes. fVa binds to phospholipid membranes in a Ca\(^{2+}\)-independent manner via its C2 domain. Prothrombin and fXa bind to phospholipid membranes through GLA domains in a Ca\(^{2+}\)-dependent manner\(^4\). In the prothrombinase complex, the catalytic efficiency (\(k_{\text{cat}}/K_m\)) reaches a maximum at around 10%PS, and slowly decreases with further increase of the PS content \(^5,6\). This effect is shown to be different from that of other anionic lipids, which usually reach a plateau of maximal activity at 25-50%\(^6\).

The preference of either members of the prothrombinase complex for different phospholipid membranes results in increasing or decreasing rates of prothrombin activation on liposomes. The fX GLA domain prefers PS phospholipids to other anionic phospholipids\(^1,7\) and fVa is a PS-binding protein \(^8\). Although prothrombin’s GLA domain also prefers PS to other anionic phospholipids, its preference for PS over non-PS phospholipids is not as strong as that of fX\(^6\).

Here we showed that non-PS anionic phospholipids can promote prothrombin activation by fVa:fXa on liposomes, but the maximum rates on these liposomes are less than those on 5% DOPS membranes. This is consistent with previous findings, which suggested that the interaction with non-PS phospholipids is mainly through non-specific, electrostatic interactions\(^5\), and cannot reach maximum activities on PS membranes.

The synergy between PE and non-PS as well as PS phospholipids shows that PE is not playing the same role as that of PG or PA, in supporting prothrombinase activity. These results suggest that, since PG and PA can support the prothrombinase activity, they can bind to PS-specific binding sites in the prothrombin GLA domain. These bindings are not specific and are probably mainly through electrostatic interactions. PE, PG and PA can also satisfy the phosphate-specific interactions, resulting in synergy between PE and PG, or PE and PA.
**Future direction**

The next step in this study will be testing the ABC hypothesis for the prothrombinase complex, and finding out if PG and PA are able to synergize with PS in a same manner as PE phospholipids.

These studies were carried out on liposomes prepared using the Bio-Bead technique, with deoxycholate as the detergent. Another control for these results would be repeating these experiments on liposomes of bigger sizes, to make sure that the small size of the liposomes in this study is not limiting for prothrombinase complex binding and activity.
References


Conclusions

Binding of GLA domains of clotting proteins to phospholipid membranes requires presence of bivalent metal ions and the right mixture of phospholipids in the membrane; bivalent metal ions (Ca$^{2+}$ and Mg$^{2+}$) are essential for proper folding of GLA domains, clustering of phospholipid molecules, and bridging GLA domains to phospholipid membranes. Also, adequate amounts of the right phospholipids are required to support optimum binding of GLA domains to phospholipid membranes.

In this study, we benefitted from combining the biochemical and biophysical techniques with Nanodisc technology (Sligar lab), molecular dynamics (MD) simulations (Tajkhorshid lab) and solid state-NMR (SSNMR) (Rienstra lab), as a part of a collaborative project at university of Illinois. This approach enabled us to look into the protein-phospholipid interactions in blood clotting at a molecular scale.

In our studies, we first determined the molecular determinants of phospholipid synergy in blood clotting reactions, where we mainly focused on the GLA domains of fX and PT (chapter two). Then we carried out a systematic study of membrane binding of all of the GLA domains of clotting proteins, where we compared their binding affinities for various phospholipid membranes, and determined their phospholipid specificities (chapter three). The effects of bivalent metal ions, calcium and magnesium, on clotting reactions were studied in chapter four. We also did some preliminary experiments on prothrombin activation, and further tested the phospholipid specificity of prothrombin's GLA domain (chapter 4).

Here is a short description of our findings:

- In chapter two a new explanation for the synergy between PE and PS phospholipids was presented: the “ABC hypothesis”. There, we showed that binding of GLA domains to phospholipid membranes involves one “phospho-L-serine” specific interaction, and 5-6 “phosphate” specific interactions. Anything But Choline, can support the phosphate specific interactions and can synergize with PS phospholipids

- In chapter three, the binding affinities of GLA containing clotting proteins for phospholipid membranes were compared. The surprising results showed that
fVIlα and APC prefer PA phospholipids to PS. These results were further confirmed by enzymatic assays, platelet activation, and clotting assays. PI-4P, which has a phosphomonoester group in its headgroup, supported similar binding interactions. We, therefore, concluded that the phosphomonoester group in PA headgroup is the binding site for the GLA domains of fVIlα and APC.

- Binding of GLA domains to phospholipid membranes is also dependent on calcium and magnesium ions, which together support GLA domains’ folding, and their binding to phospholipid membranes. In chapter four, we showed that at higher calcium concentrations (at or above 2.5 mM), the effect of magnesium ions on fX activation by TF:fVIlα is very small; while at lower calcium concentrations, or on phospholipid membranes with lower PS contents, the effect of magnesium ions is more significant. Interestingly, we showed that calcium ions cannot completely mask the effects of magnesium ions, and even at higher calcium concentrations, addition of magnesium ions to the solution enhanced the rates of fX activation; this was more than a result of enhancing the cations’ concentration. Our results suggested that GLA domains are better stabilized on the phospholipid membranes when magnesium ions are present along with calcium ions.

- In chapter 5 we showed that although prothrombin can bind to other, non-PS, anionic phospholipids, none of the phospholipids tested could support as high rates of thrombin activation as PS membranes did. Also, compared to PS, higher concentrations of non-PS anionic phospholipids were required to support the lower enzymatic activities.

Our efforts to better understand the interactions of GLA domains with phospholipid membranes resulted in unraveling the molecular mechanisms underlying the phospholipid synergies in blood clotting. Our results also showed that while GLA domains of clotting proteins have similar characteristics, their phospholipid specificities can vastly differ from each other; even GLA domains with similar phospholipid specificities, have different degrees of binding affinities for phospholipid membranes.

There are several therapeutics available for treating patients suffering from different types of hemophilia and thrombosis, but lack of specific and efficient medications
requires scientist to look for new and more specific activators or inhibitors of the blood clotting cascade. This will not be possible unless we have a detailed knowledge of the nature of the interactions between blood clotting proteins and phospholipid membranes. Our studies were another step forward to better understand these binding interactions, and hopefully pave the way for future discoveries.