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Protein-Phospholipid Interactions in Blood Clotting

James H. Morrissey, Rebecca L. Davis-Harrison, Narjes Tavoosi, Ke Ke, Vincent Pureza, John M. Boettcher, Mary C. Clay, Chad M. Rienstra, Y. Zenmei Ohkubo, Taras V. Pogorelov, and Emad Tajkhorshid

Depts. of Biochemistry, Chemistry and Pharmacology, College of Medicine, University of Illinois at Urbana-Champaign, Urbana, IL, USA

Abstract

Most steps of the blood clotting cascade require the assembly of a serine protease with its specific regulatory protein on a suitable phospholipid bilayer. Unfortunately, the molecular details of how blood clotting proteins bind to membrane surfaces remain poorly understood, owing to a dearth of techniques for studying protein-membrane interactions at high resolution. Our laboratories are tackling this question using a combination of approaches, including nanoscale membrane bilayers, solid-state NMR, and large-scale molecular dynamics simulations. These studies are now providing structural insights at atomic resolution into clotting protein-membrane interactions.

Keywords

MEMBRANES; PHOSPHOLIPIDS; TISSUE FACTOR; FACTOR VIIa; NMR

Introduction

Most steps of the clotting cascade require the assembly, on a suitable membrane surface, of serine proteases, protein cofactors and substrates. In particular, a suitable surface must contain exposed phosphatidylserine (PS), as might be found on activated platelets or on damaged or lysed cells. Membrane binding of blood clotting proteins is essential, since releasing them from the membrane reduces their catalytic activities many thousand-fold [1]. This requirement serves to limit blood clotting reactions to areas of trauma or inflammation.

A number of ideas have been proposed to explain how the membrane surface enhances the rate of activation of blood clotting proteins, including by increasing the local concentration of reactants; inducing conformational changes; and restricting the movement of proteins relative to each other. It is possible that all of these mechanisms are important. In spite of their critical importance, though, we still lack a detailed understanding of how blood clotting proteins interact with membrane surfaces. In this article, we describe ongoing studies in our laboratories in which we are applying new approaches to address this question.

*Corresponding author: James H. Morrissey, Biochemistry Department, College of Medicine, University of Illinois at Urbana-Champaign, 417 Med. Sci. Bldg., MC-714, 506 S. Mathews Ave., Urbana, IL 61801, USA, Tel.: +1 217 265-4036, Fax: +1 217 265-5290, jhmorris@illinois.edu.

Conflicts of interest statement

J.H.M. is a coinventor on patents covering some of the technologies mentioned in this article.

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Influence of the local membrane composition on blood clotting reactions

It is well known that plasma membranes contain membrane microdomains with distinct local lipid compositions, notably including sphingomyelin-rich lipid rafts [2]. Interestingly, even binary mixtures of phospholipids in simple liposomes can spontaneously segregate into membrane microdomains, such as those observed when mixtures of neutral phospholipids like phosphatidylcholine (PC) and anionic phospholipids like PS are incubated with plasma concentrations of calcium ions [3,4]. We therefore hypothesize that clotting proteins will tend to partition into locally PS-rich membrane microdomains, representing “hot spots” for blood clotting reactions. Thus, a complete understanding of how membrane surfaces contribute to blood clotting requires knowledge of the effects of membrane nanodomain composition on clotting factor activity, together with a picture, at atomic resolution, of how blood clotting proteins interact with phospholipids.

Nanodiscs as a platform for studying protein-membrane interactions

Stephen Sligar and colleagues have developed a stabilized, nanometer-scale phospholipid bilayer system, termed Nanodiscs, for studying protein-membrane interactions in a manner that allows strict control over the local composition of the membrane surface [5,6]. Nanodiscs consist of a discoidal bilayer ringed and stabilized by “Membrane Scaffold Protein” (MSP), which was engineered from apolipoprotein A-I and optimized for high levels of expression in *E. coli*. Nanodiscs are prepared in self-assembly reactions by first solubilizing phospholipids and MSP in a suitable detergent, and then slowly removing the detergent [6]. Efficient, reproducible, and simple to perform, Nanodisc self-assembly yields monodisperse preparations whose bilayer sizes and compositions are under strict experimental control. The Sligar laboratory has extensively characterized the physical properties of the supported bilayers in Nanodiscs, showing that they reflect the bilayer state in liposomes, including bilayer thickness, mean area per phospholipid, phase transition temperature, metal ion interactions, and ability to support a wide range of protein-membrane interactions [5,7–13]. More recently, longer versions of MSP have been engineered that allow the formation of Nanodiscs with a range of bilayer diameters (from about 8 nm to over 12 nm) [6].

It is also possible to embed integral membrane proteins into the nanobilayers contained in Nanodiscs. This is accomplished by including the desired detergent-solubilized membrane protein in Nanodisc self-assembly reactions, and has been successfully demonstrated for a wide variety of different membrane proteins [7–11,14–16]. Recently, we showed that tissue factor (TF) can be incorporated into Nanodiscs with bilayers of varying PS composition. This study allowed us to demonstrate that highly active complexes of TF with factor VIIa (FVIIa) can be assembled on 8 nm-diameter bilayers, with catalytic activities rivaling those of TF:FVIIa assembled on conventional liposomes [16]. Furthermore, we found that the binding affinity of factor X (FX) for nanoscale bilayers increased monotonically as the % PS increased, reaching maximal binding affinity at >80% PS. We also found that the total number of FX binding sites per bilayer also increased with increasing PS content. At saturation, the molar ratio of FX to PS was about 1 to 8, consistent with the idea that a FX binding site on the membrane surface consists of a cluster of about 8 PS molecules. This is similar to results from previous studies using liposomes, which estimated that each FX molecule interacts with about five PS molecules.

Maximal rates of FX activation by TF:FVIIa on nanobilayers required $\geq 70\%$ PS, reaching rates equivalent to those observed with TF-liposomes containing 20–30% PS [16]. We interpret these results to mean that activation of FX by the TF:FVIIa complex occurs preferentially on PS-rich “hot spots” on the membrane surface.

Currently, we are extending these studies using nanoscale bilayers to investigate the phospholipid dependence of other protease-cofactor pairs in blood clotting. For example, we have been able to assemble highly active prothrombinase complexes on Nanodiscs with 12 nm-diameter phospholipid bilayers, although the details of the phospholipid requirements of the prothrombinase and TF:FVIIa complexes are somewhat different (unpublished observations).

Computational approaches to studying clotting factor-membrane interactions

Recent advances in computing power and molecular dynamics (MD) methodology have now made it possible to conduct all-atom MD simulations over meaningful time scales of membrane proteins embedded in, or interacting with, phospholipid bilayers. This technology was recently used to conduct the first large-scale MD simulation of a γ -carboxylate-rich domain (GLA domain) interacting with a PS bilayer, in which the FVIIa GLA domain was shown to interact with multiple PS molecules [17]. This study demonstrated that some of the tightly bound Ca^{2+} ions stabilize GLA domain folding, while other tightly bound Ca^{2+} ions participate in interactions with PS headgroups. Interestingly, some of the interactions between the FVIIa GLA domain and the bilayer involved interactions with serine moiety of PS, while others predominantly involved the phosphate moiety. This study also demonstrated relatively deep penetration of the GLA domain into the membrane, with the tightly bound Ca^{2+} ions of the GLA domain being located around the level of the phosphates of the bilayer [17]. Currently underway in our laboratories are extensive additional MD simulations exploring the interaction of other membrane-binding domains in blood clotting with the membrane surface, as well as simulations of the interactions of TF with the membrane (Ohkubo & Tajkhorshid, submitted for publication).

Solid-state NMR studies of protein-membrane interactions

High resolution structures of a number of blood clotting proteins have been solved using x-ray crystallography, but always in the absence of the membrane. Solution NMR is capable of producing high-resolution protein structures, but this is problematical for large lipid-protein complexes owing to limitations imposed by their slow rate of molecular tumbling. On the other hand, we have recently been successful in employing magic-angle spinning solid-state NMR methods [18] to conduct high-resolution studies of membrane proteins in liposomes or Nanodiscs [19,20], and, for example, have successfully assigned chemical shifts of the helical membrane protein, Dsbb [21]. Thus, the combination of new solid-state NMR methods and the use of stabilized nanoscale bilayers now permits high-resolution studies of large, membrane-associated proteins in their native bilayer environments. We are applying these technologies to understand how blood clotting proteins bind to membrane surfaces. As a first step, we have focused our attention on determining the precise conformations of PC and PS headgroups in the presence of calcium ions and GLA domains, including internuclear distances and bond angle measurements among ^{13}C , ^{15}N , ^{31}P and ^1H nuclei. These measurements will enable experimental confirmation and elaboration of the MD-based GLA domain insertion models discussed above. We are also using solid-state NMR and nanoscale bilayers to probe conformational changes within GLA domains and interfacial residues in the TF:FVIIa complex. The ultimate goal is to obtain atomic-resolution structures of blood clotting proteins assembled on biologically relevant membrane surfaces.

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