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Protein-membrane interactions: Blood clotting on nanoscale bilayers

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Summary

The clotting cascade requires the assembly of protease-cofactor complexes on membranes with exposed anionic phospholipids. Despite their importance, protein-membrane interactions in clotting remain relatively poorly understood. Calcium ions are known to induce anionic phospholipids to cluster, and we propose that clotting proteins assemble preferentially on such anionic lipid-rich microdomains. Until recently, there was no way to control the partitioning of clotting proteins into or out of specific membrane microdomains, so experimenters only knew the average contributions of phospholipids to blood clotting. The development of nanoscale membrane bilayers (Nanodiscs) has now allowed us to probe, with nanometer resolution, how local variations in phospholipid composition regulate the activity of key protease-cofactor complexes in blood clotting. Furthermore, exciting new progress in solid-state NMR and large-scale molecular dynamics simulations are allowing structural insights into interactions between proteins and membrane surfaces with atomic resolution.

Keywords

membranes; phospholipids; microdomains; GLA-domains; cofactors

Introduction

Most steps of the clotting cascade require a collection of serine proteases, protein cofactors, and protein substrates to assemble on a membrane surface. A suitable surface typically contains a mixture of neutral and anionic phospholipids, such as might be encountered following platelet activation or cell damage. While many anionic lipids can stimulate clotting reactions, phosphatidylserine (PS) is by far the most active. Membrane binding of blood clotting proteins is essential, since releasing these proteins from the membrane surface reduces their catalytic activities many thousand-fold [1]. How anionic membrane surfaces accelerate clotting reactions is not known with certainty, but a variety of explanations have been offered, including by increasing the local concentration of reactants, inducing specific conformational changes, and restricting the movement of proteins to two dimensions. In spite of the critical importance of membrane binding to blood clotting, we lack a detailed understanding of how any blood

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clotting protein interacts with phospholipid surfaces. This review focuses on recent advances that are permitting new insights into the role of the phospholipid bilayer in blood clotting.

Membrane microdomains and nanodomains

Plasma membranes contain membrane microdomains in which the local lipid and protein composition can vary from the bulk membrane. Notable examples include cholesterol- and sphingolipid-rich lipid rafts and caveolae [2,3]. Blood clotting reactions are typically studied on liposomes composed of mixtures of PS and neutral phospholipids like phosphatidylcholine (PC), but even such liposomes can spontaneously form membrane subdomains. For example, studies of giant unilamellar vesicles made from mixtures of anionic and neutral phospholipids demonstrated that Ca^{2+} could induce the anionic phospholipids to move over relatively long distances, coalescing into microdomains that were visible by light microscopy. Clustering became more pronounced in the presence of both Ca^{2+} and membrane-binding proteins [4,5]. These findings suggest that clotting proteins are likely to partition into or out of specific microdomains, seeking the membrane composition for which they have the highest affinity. We propose that blood clotting reactions take place preferentially on membrane “hot spots” consisting of Ca^{2+} -induced clusters of anionic phospholipids. Moreover, we further suggest that there are local differences in anionic lipid content at the nanometer scale. Unfortunately, we typically know only the average composition of the lipid bilayers that are used in experiments. A complete explanation of how membrane surfaces contribute to blood clotting requires an understanding of the composition of membrane nanodomains together with a picture, at atomic resolution, of how the membrane binding domains of blood clotting engage the bilayer.

Stabilized nanoscale bilayers (Nanodiscs) as a unique platform for studying protein-membrane interactions

Stabilized, nanometer-scale phospholipid bilayers, termed Nanodiscs, have been developed for studying protein-membrane interactions, allowing strict control over the local composition of the membrane surface. Nanodiscs consist of a phospholipid bilayer ringed and stabilized by two copies of “Membrane Scaffold Protein” (MSP). Engineered from apolipoprotein A-I, MSP is an amphipathic α -helical protein wrapped around a discoidal bilayer domain in a “belt” fashion [6,7]. MSPs have been optimized for high expression in *E. coli* and they allow spontaneous self-assembly of nanometer-scale discoidal bilayers [7]. Nanodisc self-assembly is efficient, reproducible and simple to perform, yielding monodisperse preparations whose bilayer domain sizes are under strict experimental control. The physical properties of the supported bilayers in Nano-discs have been extensively characterized, and they faithfully reflect the bilayer state in liposomes, including bilayer thickness, mean area per phospholipid, phase transition temperature (albeit slightly broadened), metal ion interactions, and ability to support a wide range of protein-membrane interactions [6,8–14]. Recently, next-generation Nanodiscs have been developed that encompass larger diameter bilayers. This was accomplished by lengthening the amphipathic helical “belt” of MSP with 1, 2 or 3 additional 22-mer helices, yielding Nanodiscs ranging in diameter from about 8 nm to over 12 nm [7].

Blood clotting reactions studied on nanoscale bilayers

When integral membrane proteins are included in Nanodisc self-assembly reactions, they embed into the bilayer just as they do during liposome formation [8–12,15–17], faithfully replicating their *in vivo* topology. We recently demonstrated that tissue factor (TF)-containing Nanodiscs prepared with suitable mixtures of PS and PC allow the assembly of highly active TF:FVIIa complexes on nanoscale bilayers, with catalytic activities rivaling those in liposomes [17,18]. This approach precludes long-distance (up to μm scale) recruitment of PS molecules

into membrane subdomains. Unlike with liposomes, the input PS content is precisely what blood clotting proteins “see” when they encounter the nanoscale bilayer surface. We found that the dissociation constant for FX binding to nanoscale bilayers decreased monotonically as the % PS increased, reaching maximal binding affinity at >80% PS. Interestingly, the number of FX binding sites also increased with increasing PS content. At saturation, 8.4 FX molecules bound per leaflet (or about 8 PS molecules per bound FX), consistent with the idea that a FX binding site consists of a small cluster of PS molecules. Previous studies using liposomes estimated that each FX interacts with about five PS molecules [19].

Activation of FX by TF:FVIIa on nanobilayers exhibited maximal catalytic efficiencies at 70% PS or higher, and were comparable to rates observed with TF-liposomes containing 20–30% PS [17]. These results argue that extremely high local PS content is required for optimal assembly and function of the TF:FVIIa complex, as might be found on membrane “hot spots” containing locally high PS concentrations.

This study also allowed us to address a long-standing question regarding substrate delivery to membrane-bound proteases such as TF:FVIIa. One can imagine at least two different mechanisms of substrate presentation to TF:FVIIa: Solution-phase FX might bind directly to the membrane-bound TF:FVIIa complex; or membrane-bound FX molecules might randomly move on the membrane surface via lateral diffusion or hopping to encounter TF:FVIIa [1,20–22]. Unlike the situation on liposomes, TF:FVIIa on Nanodiscs cannot access a large pool of membrane-bound FX. Instead, the nanobilayers can bind at most five or six FX molecules, which will be converted to FXa within two or three seconds. However, we observed sustained, linear rates of FX activation over 20 minute time courses, during which at least 2400 FX molecules were activated per TF:FVIIa [17]. The fact that turnover rates obtained with TF-Nanodiscs rival those of TF-liposomes demonstrates that the TF:FVIIa complex is not dependent on a large, preexisting pool of membrane-bound FX to serve as substrate.

We are now extending our studies using nanoscale bilayers to investigating the phospholipid dependence of the assembly and activity of other protease-cofactor pairs in blood clotting. We recently found that highly active prothrombinase complexes (FVa:FXa complexes) can be assembled on Nanodiscs engineered to encompass 12 nm-diameter phospholipid bilayers. The larger size of FVa (about four times the size of TF) necessitated the use of larger nanobilayer surfaces. Interestingly, the detailed phospholipid requirements of the prothrombinase differ from those of the TF:FVIIa complex (unpublished observations).

Computational approaches to studying clotting factor-membrane interactions on nanoscale bilayers

Although a number of x-ray crystal structures of clotting proteins have been solved, none of the structures include a membrane surface. For this reason, we lack a detailed, atomic-resolution understanding of how blood clotting proteins interact with phospholipid bilayers. On the other hand, recent advances in both computing power and in molecular dynamics (MD) methods now make it possible to conduct large-scale, all-atom MD simulations over meaningful time scales of membrane proteins interacting with phospholipid bilayers. The first large-scale MD simulation of a GLA domain interacting with a PS bilayer showed that the FVIIa GLA domain interacts simultaneously with multiple PS molecules [23]. This MD simulation also revealed quite different interaction modes between the GLA domain and various PS molecules, involving interactions between PS and the tightly-bound calcium ions of the GLA domain, as well as direct protein-lipid interactions. Of particular interest, some of the PS-GLA domain interactions involved the serine headgroup of PS, others were predominantly with the phosphate moiety, and some involved both the serine and the phosphate of PS. In contrast to previous ideas about the interaction of GLA domains with membrane

surfaces (which propose rather shallow penetration of the GLA domain into the bilayer), this model suggests that the structurally bound Ca^{2+} ions of the GLA domain are deeply immersed in the PS headgroup layer, at about the level of the phosphates. The so-called ω -loop of the GLA domain (containing three exposed hydrophobic side chains) is also fully inserted into the hydrophobic core of the bilayer [23]. Interestingly, some of the structurally bound Ca^{2+} ions of the GLA domain do not directly participate in membrane binding, but instead stabilize GLA domain folding, while other Ca^{2+} ions directly participate in anchoring the GLA domain to the membrane. In addition, membrane binding of the GLA domain is also stabilized by direct interactions between PS and certain amino acid side chains that are positioned optimally to reach the membrane head groups [23]. Additional MD simulations are underway that are revealing the membrane binding modes of other blood clotting proteins.

Solid-state NMR studies of protein-membrane interactions employing nanoscale bilayers

Solution-based NMR studies of proteins interacting with detergent micelles or bicelles [24] are feasible with modern high-field methods, and high-resolution structures of membrane proteins have been solved, including prominent recent examples [25–27]. However, the detailed lipid structure is not accurately reproduced in these sample preparations, and solution NMR investigations are ultimately limited by the rate of molecular tumbling of large lipid-protein complexes. On the other hand, magic-angle spinning solid-state NMR methods enable high-resolution studies in liposomes or Nanodiscs [28,29]. We have recently developed techniques for atomic-resolution structure determinations by solid-state NMR [30], and have demonstrated that most of the steps for structure determination can be applied directly to membrane-embedded systems; for example, we recently assigned chemical shifts of the helical membrane protein, DsbB [31]. The combination of new solid-state NMR methods and the ability to assemble clotting proteins on Nanodiscs offers tremendous possibilities for studying complexes of large, membrane-associated proteins in their native lipid environments. We are currently applying these technologies to solving the structures of key protein-membrane complexes in the blood clotting system and anticipate that these studies will reveal fundamentally new types of structural information at atomistic detail. For example, the precise conformations of PS and PC headgroups will derive from internuclear distance measurements among ^{13}C , ^{15}N , ^{31}P and ^1H nuclei. Site-resolved correlations between lipid and protein molecules will enable an experimental confirmation and elaboration of the MD-based GLA domain insertion models discussed above. And finally, the conformational changes within GLA domains and interfacial residues in the TF:FVIIa complex will be probed directly to shed light on this important region of the complex, which has so far been invisible to high-resolution structural methods.

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