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Plenary Paper

THROMBOSIS AND HEMOSTASIS

Cryo-EM structure of the prothrombin-prothrombinase complex

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KEY POINTS

- The prothrombin-prothrombinase complex reveals the spatial arrangement of its macromolecular components for the first time.
- The structure offers a molecular view of prothrombin activation along the meizothrombin pathway.

The intrinsic and extrinsic pathways of the coagulation cascade converge to a common step where the prothrombinase complex, comprising the enzyme factor Xa (fXa), the cofactor fVa, Ca^{2+} and phospholipids, activates the zymogen prothrombin to the protease thrombin. The reaction entails cleavage at 2 sites, R271 and R320, generating the intermediates prethrombin 2 and meizothrombin, respectively. The molecular basis of these interactions that are central to hemostasis remains elusive. We solved 2 cryogenic electron microscopy (cryo-EM) structures of the fVa-fXa complex, 1 free on nanodiscs at 5.3-Å resolution and the other bound to prothrombin at near atomic 4.1-Å resolution. In the prothrombin-fVa–fXa complex, the Gla domains of fXa and prothrombin align on a plane with the C1 and C2 domains of fVa for interaction with membranes. Prothrombin and fXa emerge from this plane in curved conformations that bring their protease domains in contact with each other against the A2 domain of fVa. The ^{672}ESTVMATRKMHDRLEPEDEE^{691} segment of the A2 domain closes on the protease domain of fXa like a lid to fix orientation of the active site. The ^{696}YDYQNRL^{702} segment binds to prothrombin and establishes the pathway of activation by sequestering R271 against D697 and directing R320 toward the active site of fXa. The cryo-EM structure provides a molecular view of prothrombin activation along the meizothrombin pathway and suggests a mechanism for cleavage at the alternative R271 site. The findings advance our basic knowledge of a key step of coagulation and bear broad relevance to other interactions in the blood.

Introduction

The hemostatic response to vascular injury entails a sequence of proteolytic events where inactive zymogens are converted to active proteases.[1] The cascade starts with exposure of tissue factor from the damaged endothelium and culminates with conversion of prothrombin to thrombin in a reaction catalyzed by the prothrombinase complex composed of the enzyme factor Xa (fXa), cofactor fVa, Ca^{2+}, and phospholipids.[1-3] The presence of fVa in the prothrombinase complex drastically increases the rate of prothrombin conversion to thrombin[4,5] in a cofactor-dependent activation that is paradigmatic of analogous reactions of the blood coagulation and complement cascades.[1,6] Elucidation of the molecular basis of the prothrombin-prothrombinase interaction therefore bears broad significance to blood physiology and to the large class of trypsin-like zymogens to which prothrombin belongs.[7]

Activation of prothrombin requires cleavage at 2 residues, R271 and R320, and proceeds along 2 possible pathways that generate the inactive intermediate prethrombin 2 (following initial cleavage at R271) or the active intermediate meizothrombin (following initial cleavage at R320).[2,4,8-10] The pathway of prothrombin activation by prothrombinase is context dependent in vivo. On the surface of platelets, activation proceeds along the prethrombin 2 pathway.[11,12] On nonplatelet surfaces such as red blood cells[13] or the endothelium,[8] activation proceeds along the meizothrombin pathway.[14] Importantly, this pathway produces immediate catalytic activity even before thrombin is generated by subsequent cleavage at R271[15] and is also preferred in vitro in the presence of synthetic liposomes[9,14,16,17] or microparticles.[18] The mechanism of prothrombin activation and the factors responsible for preferential cleavage at R271 or R320 have been thoroughly investigated.[9,14,16,19-23] Simple kinetic schemes have been devised,[20] along with more elaborate models based on conformational transitions of prothrombinase[14,19] or prothrombin.[21,24] Computational models of the prothrombin-prothrombinase complex have been proposed.[22,23,25] These previous developments have broadened our understanding of a key interaction of the blood coagulation cascade[2,8,26,27] and await validation from structural biology.

Significant progress has been made in the elucidation of the structural architecture of the three macromolecular components of the prothrombin-prothrombinase complex. Numerous X-ray crystal structures of fXa have been solved,[28,29] detailing the assembly of

the protease and epidermal growth factor (EGF) domains. Structures of prothrombin have revealed the organization of its entire multidomain architecture and the existence of two forms, open and closed, in allosteric equilibrium.[30-34] The open form is preferentially cleaved at R271 and initiates the prethrombin 2 pathway, whereas the closed form is preferentially cleaved at R320 and initiates the meizothrombin pathway.[31,33] More recently, cryogenic electron microscopy (cryo-EM) structures of fV and fVa[35] have unraveled the molecular organization of the cofactor, including the elusive A2 domain housing epitopes for prothrombin and fXa binding. These studies have laid the groundwork for solving a cryo-EM structure of the prothrombin-prothrombinase complex at near-atomic (4.1-Å) resolution. The structure shows the spatial arrangement of the prothrombin-fXa-fVa complex for the first time and reveals how the A2 domain of fVa brokers activation along the meizothrombin pathway.

Methods

Materials

Human fVa was purchased from Sigma (F0931). Prothrombin and fXa were made recombinantly as reported elsewhere[10,36-38] with the catalytic residues S525 (prothrombin) and S379 (fXa) replaced by Ala to prevent (auto)proteolysis. Nanodiscs containing phosphatidylserine only lipids provide optimal scaffolds for clotting factors[39] and were prepared by modifying established protocols.[40] Specifically, 412 μL phosphatidylserine (Avanti Polar Lipids; 840032C) was dispensed into a glass tube and air dried over N_2 before being dissolved in 0.5 mL of a buffer containing 20 mM Tris, 100 mM NaCl, and 50 mM sodium deoxycholate, pH 7.4. The lipid mixture was mixed in a 60:1 molar ratio with membrane scaffold protein MSP1E3D1 (Sigma; MSP08) resuspended in 20 mM Tris and 100 mM NaCl, pH 7.4, and incubated at 4°C for 40 minutes. The MSP1E3D1:lipid mix was then extensively dialyzed overnight in 20 mM Tris and 10 mM NaCl using a Slide-A-Lyzer cassette with a 10 000 molecular weight cut-off. After dialysis, the nanodiscs were further purified by gel filtration over a Superdex 200 Increase column with 20 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid, 150 mM NaCl, and 5 mM $CaCl_2$ as running buffer. Several prothrombin:fVa:fXa molar ratios were tested to generate a complex,[41] and eventually the 2:1:2 ratio and 0.01 mg/mL total protein optimized filtering for imaging. Other established methods like Grafix[42] and GraDeR[43] were used but failed to yield stable complexes. For the sample on nanodiscs, 0.01 mg/mL protein was added to 0.016 mg/mL nanodiscs based on the extinction coefficient of MSP1E3D1. Cryo-EM grids were Quantifoil 2/2 holey carbon grids, glow discharged prior to vitrification that was carried out on an FEI Vitrobot Mark IV (FEI). Varying blot forces between 2 seconds at −1 blot force and 3.5 seconds at −15 blot force were used for grid screening. A 2-second at −1 blot force yielded grids for collection. Data for the fVa-fXa complex on nanodiscs were collected on a 200-kV Glacios equipped with a Falcon IV direct electron detector, using a pixel size of 0.94 Å, a dose of 51.28 $e^-/Å^2$, and a defocus value of between −0.8 and −2.4 μm. Data for the prothrombin-fVa-fXa complex were collected on a 300-kV Titan Krios G3 cryo-TEM equipped with a GIF BioQuantum 968 energy filter and a Gatan K3 direct electron detector, using a pixel size of 0.413 Å, a dose of 50 $e^-/Å^2$, and a defocus value of between −1 and −3 μm.

Image processing, map calculation, and model building

Image stacks were aligned, binned, and dose weighted using MotionCor2.[44] Contrast transfer function (CTF) determination was performed using CTFFind4.[45] Motion-corrected and ctf-estimated images were curated according to CTF estimations of a maximum resolution of 4 Å. For the fVa-fXa complex on nanodiscs (supplemental Figure 1A, available on the *Blood* Web site), a total number of 572 677 particles were initially picked by cryoSPARC[46] and subject to multiple rounds of 2-dimensional classification, ab initio map generation, homogenous refinement, nonuniform refinement, and particle subtracted local refinement. Only 1 good class was obtained. Model building started from the fVa-fXa portion of the ternary prothrombin-fVa-fXa complex, using manual model in Chimera and COOT[47] with only 1 round of real-space refinement in Phenix.[48] For the prothrombin-fVa-fXa complex (supplemental Figure 1B), a total number of 12 260 048 particles were initially picked using cryoSPARC. Two-dimensional classification immediately gave classes that could be distinguished from fVa alone[35] by extra densities at the A1/A2 domain. Iterative ab initio and heterogenous classification yielded several different classes that was put through further rounds of homogenous refinement, nonuniform refinement, and particle subtracted local refinement before finally choosing the highest resolution class for model building. The structure 7KVE of fV[35] was used as starting model for fVa. The structures 6BJR[31] and 5EDM[30] of prothrombin in the closed and open forms were used as starting models for prothrombin. The structure 1XKB[29] of fXa was used as starting model for fXa. Full-length homology models for 6BJR and 5EDM were built using ITASSER[49] that was also used to build homology models for the entire Gla domain (residues 1-47) of fXa missing in 1XKB. Resolution of all maps was sufficient to locate overall protein arrangements, interactions, and major contacts. 7KVE could easily be placed into densities of all the different classes. Homology models of 5EDM (open form of prothrombin) could not be placed into any class. Conversely, the homology model of 6BJR (closed form of prothrombin) could be placed into all classes. The protease domains of both prothrombin and fXa were immediately apparent during refinement. Different homology models of residues 1 to 47 of fXa were fitted into the density of the Gla domain, and the model providing the best fit in Chimera was used. Further model building was carried out in COOT followed by only 1 round of real-space refinement in Phenix. Relevant parameters of the cryo-EM structures of fVa-fXa on nanodiscs and prothrombin-fVa-fXa are summarized in Table 1. Representative 2-dimensional class averages and gold standard Fourier shell correlation of masked refinement of maps and models are reported in supplemental Figures 2A-B and 3A-B for the fVa-fXa complex on nanodiscs and the prothrombin-fVa-fVa complex, respectively.

Results

Prothrombinase

The prothrombin-fVa-fXa complex was initially assembled on nanodiscs to reproduce conditions closest to physiologic, but only the fVa-fXa complex could be detected by cryo-EM at a resolution of 5.3 Å (Figure 1A; supplemental Figure 1A). In this structure of prothrombinase, fVa adopts a conformation similar to that of the free form solved recently[35] (root-mean-square-

Table 1. Structural parameters for the cryo-EM structures of fV and fVa

	Prothrombin-fVa-fXa complex (PDB ID:7TPP emd ID:26060)	fXa-fVa complex on nanodiscs (PDB ID:7TPQ emd ID:26061)
Map		
Voltage (kV)	300	200
Electron exposure (e-/Å^2)	50	51.28
Pixel size (Å)	0.413	0.94
Initial particle number	12260048	572677
Final particle number	330317	16853
Symmetry imposed	C1	C1
Resolution (unmasked, FSC threshold 0.143)	5.2	12.1
Resolution (masked, FSC threshold 0.143)	4.1	5.3
Model		
Refinement program	Phenix (real space)	Phenix (real space)
Number of protein atoms (non-H)	18513	14020
RMSD bonds (Å)	0.003	0.003
RMSD angles (°)	0.894	0.921
Ramachandran favored (%)	75.36	76.37
Ramachandran allowed (%)	21.81	20.52
Ramachandran disallowed (%)	2.83	3.11
All-atom clash score	9.9	12.3
MolProbity score	2.66	2.78

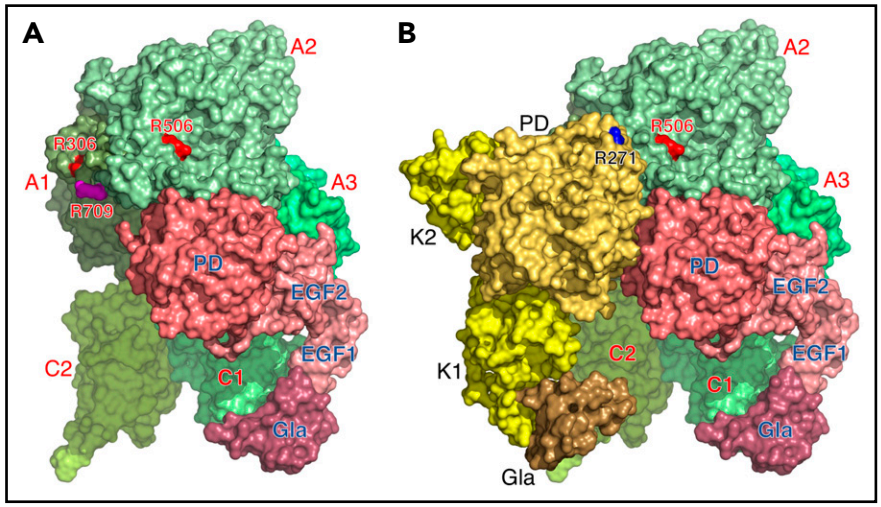

Figure 1. Cryo-EM structures of prothrombinase free and bound to prothrombin. (A) The structure was solved at 5.3-Å resolution and shows the 2 proteins of the prothrombinase complex, fVa and fXa, in surface representation. Images including the nanodiscs are given in supplemental Figures 1 and 2. The architecture of the A1 (smudge), A2 (pale green), A3 (lime green), C1 (lime), and C2 (lemon) domains is solved in its entirety, except for the N-terminal ^{1546}SNNGNRRNYY^{1555} sequence of the A3 domain immediately downstream of the site of thrombin activation at R1545.[5] The overall arrangement of fVa is very similar to that of the free form solved recently.[35] The architecture of fXa is fully resolved and provides a picture of the complete arrangement of the constitutive Gla (raspberry), EGF1 (dark salmon), EGF2 (salmon), and protease (deep salmon). The bound fXa features an overall conformation with the domains not vertically aligned but bent over the EGF1-EGF2 junction, as documented in several X-ray structures,[28,29] and additionally 90° at the EGF1-Gla domain junction (supplemental Figure 4C). Also shown are the sites of fVa inactivation by activated protein C at R306 and R506 (red) and the site of thrombin activation at R709 (magenta). The enzyme is positioned in the complex along the A2, A3, and C1 domains of fVa, with the Gla domain aligned with C1 domain on the plane of the nanodisc (see supplemental Figure 1A). A segment of the A2 domain lowers on the fXa to fix the protease domain for optimal interaction with an incoming substrate (see Figure 2 for details). (B) The structure was solved at 4.1-Å resolution and shows fVa and fXa in the same arrangement found on nanodiscs (see panel A), which supports the ternary complex as a genuine representation of the prothrombin-prothrombinase complex. The constitutive domains of prothrombin are colored in sand (Gla domain), pale yellow (EGF1), yellow (EGF2), and orange yellow (protease domain, PD), with the Gla domain aligned with the homologous domain of fXa and the C1 and C2 domains of fVa for interaction with membranes. Prothrombin faces fXa in the complex and is positioned along the A2, A1, and C2 domains of fVa. Visible in this orientation is the site of activation at R271 (blue), located above the protease domain of fXa, but not R320 that inserts into the active site of fXa (see Figure 2 for details). The overall architecture of prothrombin is similar to that of the closed form[31,33] (supplemental Figure 4A).

deviation (rmsd) = 3.29 Å over 1014 Cα atoms; supplemental Figure 4B) with the C1 and C2 domains standing perpendicular to the membrane and supporting the A1 and A3 domains. Regions disordered in the free form of fVa[35] become visible in the bound form. The A1 domain is resolved in its entirety and therefore is the A2 domain on top of the A1 and A3 domains. Missing is only the N-terminal ^{1546}SNNGNRRNYY^{1555} sequence of the A3 domain immediately downstream of the site of thrombin activation at R1545.[5] The architecture of fXa is fully resolved and reveals the complete arrangement of the Gla, EGF1, EGF2, and protease domains that aligns well (rmsd = 3.04 Å over 284 atoms; supplemental Figure 4C) with available X-ray structures of the Gla-domainless enzyme.[28,29] The Gla domain stands perpendicular to the membrane, but the EGF1 domain bends almost 90° and accentuates the overall curvature in the enzyme that positions the active site about 70 Å above the plane of the membrane and in optimal orientation for engagement of prothrombin in the closed form.

Prothrombin-fVa-fXa complex

Removal of nanodiscs yielded a structure of prothrombin bound to fXa and fVa at a significantly higher resolution of 4.1 Å (Figure 1B). Both fVa and fXa features conformations practically identical (rmsd = 0.17 Å over 1605 Cα atoms) to those detected on nanodiscs, supporting the 2 cryo-EM structures of the fXa-fVa complex obtained independently, with and without nanodiscs, as a genuine representation of prothrombinase. Prothrombin in the ternary complex is missing only the segment 157 to 170 in the flexible linker 2 region[30] and assumes a conformation similar to the closed form solved by X-ray studies (rmsd = 2.51 Å over 537 Cα atoms; supplemental Figure 4A)[31] that predominates in solution and changes little on interaction with prothrombinase.[31,50] The closed conformation enables prothrombin to align the Gla domain with the homologous domain of fXa and the C1 and C2 domains of fVa and then curve over the plane of the membrane to present R320 in the protease domain to the active site of fXa.

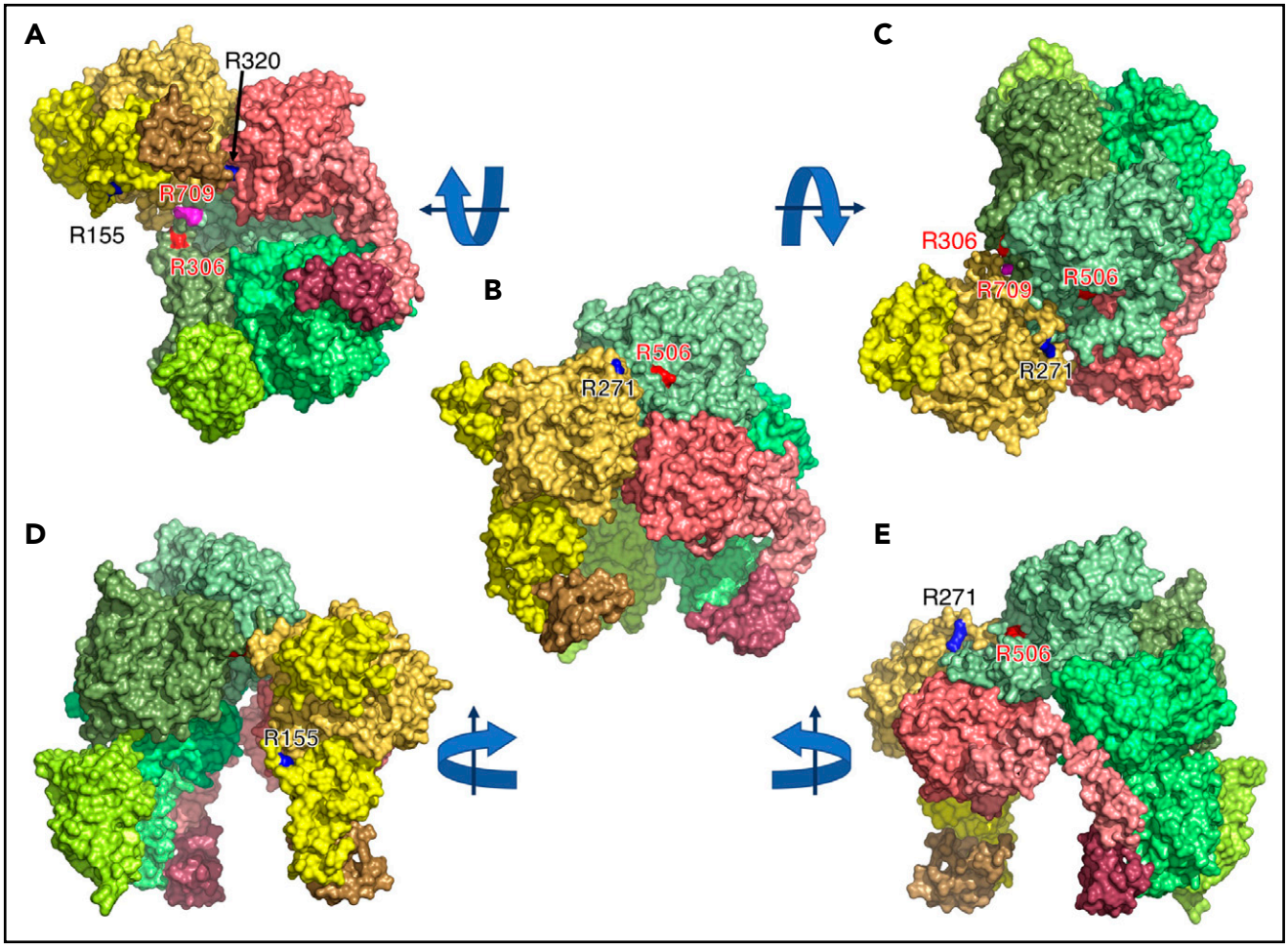

Figure 2. Cryo-EM structure of prothrombin bound to fVa and fXa. Molecular surface representation of the ternary complex of prothrombin fVa and fXa. The complex in Figure 1B is shown in different orientations obtained by 90° rotation of the reference view (B) as indicated by arrows. The constitutive domains of prothrombin, fVa and fXa are rendered in the same colors as in Figure 1B. Prothrombin engages prothrombinase through the protease domain (B,D-E) that binds to the A2 domain of fVa (B-D) and the protease domain of fXa (A-C). The protease domain of fXa is in close contact with the A2 domain of fVa (B,E). Labeled are the sites of prothrombin activation by prothrombinase at R271 (B-C,E) and R320, which is visible only looking up from the plane of the membrane (A) as it penetrates the active site of fXa. The preferred site of prothrombin activation in the absence of fVa, R155, is located on the side of prothrombin opposite to that facing fXa (D). The 672 to 691 region of the A2 domain of fVa changes conformation in the complex relative to the free form[35] (C,E) and closes like a lid on the protease domain of fXa while making direct contacts with R271 of prothrombin (C,E; see also Figure 5 for details).

Table 2. Main molecular contacts <5 Å among the components of the prothrombin-fVa-fXa complex

fVa-fXa	Prothrombin-fXa	Prothrombin-fVa
A511-L352(c170)	K307-Q240(c61)	L260-T305*
A511-I357(c175)	D318-R405(c222)	D261-K309*
F576-R347(c165)	D318-K408(c224)	D265-K309*
D577-R347(c165)	R320-E372(c188)	R266-A694*
T579-K351(c169)	E323-K330(c148)	E269-R505
T626-N348(c166)	D326-K242(c62)	R271-D697*
D628-R347(c165)		R310-Y698
E662-R306(c125)*		L312-Y698
E662-K420(c236)*		L313-L702*
E669-R306(c125)		P534-Y696*
E672-R306(c125)*		F535-Y696*
E672-Q360(c178)*		
E672-K414(c230)*		
V675-R424(c240)*		
E686-K276(c96)		
S1598-E82		
T1679-F84		
H1683-L91		
Y2021-E39*		

Residues are numbered sequentially and for fXa also according to chymotrypsin by parentheses.

*Residues of fVa not previously listed as potential epitopes of recognition of prothrombin or fXa by a recent review of biochemical data and computational models.[66] The review lists a total of 56 residues of the A2 domain and 35 residues of the A3 domain of fVa involved in the interaction with fXa. The cryo-EM structure documents only 11 residues in the A2 domain (A511, F576, D577, T579, T626, D628, E662, E669, E672, V675, E686), 3 of which not reported previously (E662, E672, V675), 3 in the A3 domain (S1598, T1679, H1683) and 1 in the C1 domain (Y2021) also not reported previously. The main interactions are shown in Figure 4B-C. The review also lists 23 residues in the A2 domain, 18 in the A3 domain and 10 in the C1 domain as interacting with prothrombin.[66] The cryo-EM structure documents 2 residues in the A1 domain (T305, K309) not reported previously, 6 residues in the A2 domain (R505, A694, Y696, D697, Y698, L702), 4 of which (A694, Y696, D697, L702) not reported previously, and no contacts involving the A3 and C1 domains.

The prothrombin-fVa-fXa complex is shaped like a dome (Figure 2), with the A2 domain of fVa at the top and a base accommodating the C1 and C2 domains of fVa and the Gla domains of prothrombin and fXa (Figure 2A) in optimal alignment for membrane binding. Prothrombin and fXa face each other against the fVa scaffold (Figure 2B), with fXa along the A2, A3, and C1 domains and prothrombin along the A2, A1, and C2 domains. Relevant contacts are listed in Table 2. Importantly, fXa and prothrombin interact mainly through their protease domains (Figure 2A-C) and with the A2 domain of fVa (Figure 2B-E) that is resolved in its entirety unlike its free form.[35] The EGF and Gla domains of fXa are close to the A3 and C1 domains of fVa (Figure 2A-B,E) but make only minor interactions (Table 2). The kringle and Gla domains of prothrombin are significantly separated from the A1 and C2 domains of fVa (Figure 2A-B,D). Indeed, formation of the ternary complex buries a surface area of 6318 Å^2, which is only 5.5% of the total accessible surface area of fVa (115 692 Å^2). The limited size of the interaction surface is consistent with the cofactor role of fVa to promote prothrombin activation mainly through a drastic increase in k_{cat}.[2,4,8]

The site of cleavage of prothrombin at R271 is tucked against D697 of the A2 domain of fVa and sits at a Cα-Cα distance of 34 Å from R320 that inserts into the active site of fXa (Figure 2A-D). A third site of cleavage at R155 (Figure 2D) is positioned >50 Å away from the primary specificity pocket of fXa. Finally, the sites of fVa inactivation by activated protein C at R306 and R506 (Figure 2A-D) remain exposed to solvent and 35 Å apart. Overall, the structure reveals a complex where fVa directs R320 into the active site of fXa and prevents alternative cleavages at R271 and R155, consistent with biochemical evidence that fVa drives activation mainly through the meizothrombin pathway[4] and in a position-dependent manner.[51]

Membrane binding module

The Gla domains of prothrombin and fXa along with the C1 and C2 domains of fVa define a membrane binding module (Figure 3) shaped like an isosceles triangle with longer sides >100 Å, connecting the Gla domain of prothrombin with the Gla domain of fXa and the C2 domain of fVa, and a base spanning 70 Å across these 2 domains. The size of the module explains the difficulty of assembling the prothrombin-fVa-fXa complex on nanodiscs only 100 Å across (supplemental Figure 1). Residues Y1903, W1904, Y1917, Y1956, and potentially Y2021 in the C1 domain are oriented for optimal interaction with the membrane, consistent with previous studies[52-54] and the recent cryo-EM structure of the free form.[35] The analogous docking platform of the C2 domain comprises residues W2063, W2064, and W2068, again in agreement with biochemical studies[55,56] and the recent cryo-EM structure.[35] Adjacent to the C1 domain, the Gla domain of fXa exposes F4, Y24, and Y44 for interaction with membranes. The Gla domain of prothrombin, widely separated from fXa and fVa, features F4 and potentially Y24 and F28 for optimal binding.

fVa-fXa interaction

The interaction of fVa and fXa leading to formation of the prothrombinase complex takes place with an affinity in the nanomolar range.[57,58] Contacts involve predominantly the A2 domain of fVa and the protease domain of fXa (Table 2) and recapitulate at higher resolution those revealed by the cryo-EM structure on nanodiscs (Figure 1A). The entire C-terminal segment 654 to 709 of the A2 domain was missing in the free form solved recently[35] because of intrinsic disorder but becomes structured on prothrombin binding. The segment contains important epitopes of recognition, especially the ^{672}ESTVMATRKMHDRLEPEDEE^{691} sequence that moves >7 Å from its position in fV to cover the protease domain of fXa like a lid (Figures 2A,C,E and 4A). An important electrostatic interaction involves R347(c165) (c = chymotrypsin numbering) of fXa with D628 and D577 of fVa, along with a possible cation-π interaction with F576 (Figure 4B-C). Residue R347(c165) was identified previously as being critical for fVa binding,[59,60] and this role is confirmed by the cryo-EM structure. Residue R306(c125) engages another hot spot of fVa nearby by contacting E669 and especially E662 and E672 that also interact with K420(c236) and K414(c230), respectively (Figure 4B-C). A strong electrostatic interaction involves K276(c96) with E686, and a hydrophobic interaction couples L352(c170) and I357(c175) with

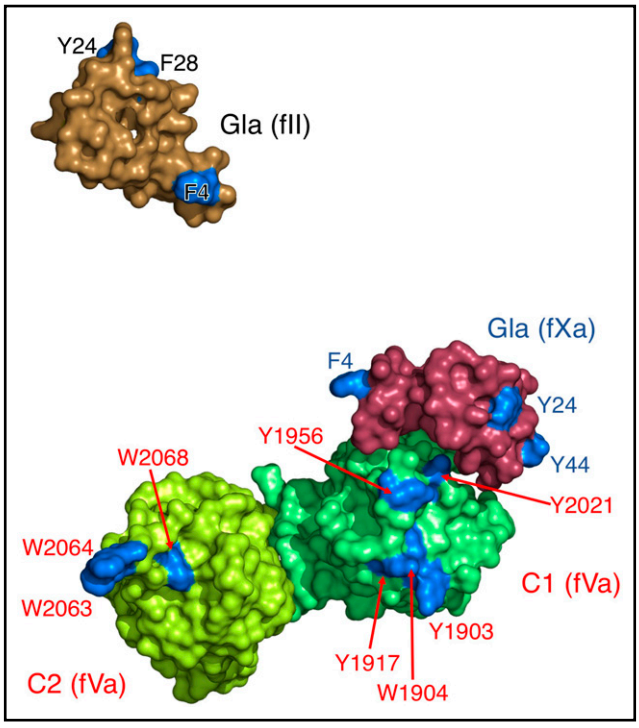

Figure 3. Membrane binding module of the prothrombin-prothrombinase complex. The Gla domains of prothrombin and fXa, along with the C1 and C2 domains of fVa, are colored as in Figure 1 and are shown in the same orientation as Figure 2A, that is, as seen from the plane of the membrane. The module features the Gla domain of fXa close, but not in contact, with the C1 domain of fVa, which is positioned relative to the C2 domain as seen in free fVa.[35] The Gla domain of prothrombin, conversely, is widely separated from the Gla domain of fXa and the C2 domain of fVa. The module has an overall triangular arrangement with sides of up to 90 Å (Gla domain of prothrombin from Gla domain of fXa), 70 Å (Gla domain of fXa from C2 domain of fVa), and 110 Å (Gla domain of prothrombin from C2 domain of fVa). Residues likely involved in direct interaction with the membrane are highlighted (blue).

A511 of fVa (Figure 4B-C). All these interactions orient the active site of fXa for optimal engagement of prothrombin (Figures 4C and 5). The ^{672}ESTVMATRKMHDRLEPEDEE^{691} segment of the A2 domain of fVa is flanked by the shorter segments ^{659}DDDED^{663} influencing the rate of cleavage of prothrombin by prothrombinase[61] and ^{695}DYDYQ^{699} affecting both the rate and pathway of prothrombin activation[62-64] (Figure 4A-B). These segments, not resolved in the structure of free fVa,[35] change little compared with the structure of fV and make no contacts with fXa, except for E662 that interacts with R306(c125) and K420(c236) (Figure 4B-C). Other residues in the A2 domain deemed important for fXa binding,[65,66] like E467, R652, and the entire segment ^{323}EYFIAAEEV^{331},[65,67] make no contacts with the enzyme. Outside of the A2 domain, 3 minor contacts are observed between A3 and EGF2 and a weak interaction between C1 and the Gla domain (Table 2). Overall, the fVa-fXa interaction involves a total of 15 residues of fVa (Table 2), 4 of which (E662, E672, V675, Y2021) add to a recent summary of biochemical and modeling studies.[66]

Prothrombin-prothrombinase interaction

The segment ^{311}ITREQRRHMKRWEYF^{325} at the boundary between the A1 and A2 domains of fVa is a potential epitope for APC recognition,[68] but only T305 and K309 in the A1 domain make minor contacts with prothrombin (Figure 4A; Table 2). The long segment ^{680}KMHDRLEPEDEESDADYDYQNRLAAALGIR^{709} in the A2 domain has been implicated in prothrombin binding and prothrombinase function.[62-64,66,69,70] The cryo-EM structure shows that only a small portion of this segment is involved in direct prothrombin binding (Figure 4A-B) and plays a crucial role in organizing the architecture of the 2 sites of cleavage at R271 and R320 (Figure 5). The segment ^{696}YDYQNRL^{702} separates the 2 sites of cleavage and provides directionality to the interaction of prothrombin with prothrombinase by sequestering R271 on one side and preparing R320 for interaction with the active site of fXa on the other side. Residue R271 is in strong H-bonding coupling with D697, which is reinforced by the nearby interaction between E269 and R505 of fVa and stabilized further by a hydrophobic interaction between Y696 and F535 of prothrombin. Docking of the R320 site on prothrombinase involves an extended recognition surface of P-P′ sites,[71] from P14 to P6′ (Figure 5). The proximal segment ^{307}KTERELLE^{314} encompasses a large insertion relative to the sequence of chymotrypsin[72] and has been shown to influence thrombin function and its generation from prothrombin.[73] Naturally occurring mutations in this region, like prothrombin Denver (E309K),[74] compromise activation and are associated with severe bleeding.[75] Residue K307 at P14 is in electrostatic interaction with Q240(c61) in the 60-loop above the entrance to the active site of fXa. Residue Y698 of fVa is in hydrophobic contact with L312 at P9 and in cation-π interaction with R310 at P11, whereas L313 at P8 contacts L702. The segment then makes a sharp turn that directs R320 to the active site of fXa, where it contacts E372(c188) next to D373(c189) in the primary specificity pocket that remains 7.1 Å away. The docking is stabilized by strong electrostatic interactions between D318 at P3 with both R405(c222) and K408(c224) in the Na^+ binding loop, which plays a role in prothrombin activation,[76] E323 at P3′ with K330(c148) in the autolysis loop, and D326 at P6′ with K242(c62) in the 60-loop (Figures 4C and 5). These observations confirm the importance of the sequence ^{695}DYDYQ^{699} as a competitive inhibitor of prothrombin activation by prothrombinase also capable of shifting preferential cleavage from R320 to R271.[62-64,69] Conversely, some previous assignments are not confirmed by the cryo-EM structure. The segment ^{473}GKGQPSVLQVVNLPI^{487} of prothrombin proposed as a fVa binding epitope[77] is mostly buried, as already reported in the structure of prothrombin,[10] and makes no contacts with fVa. There are no contacts between exosite I (residues 382-396) or the autolysis loop (residues 466-477) of prothrombin with either fXa or fVa (Figures 2B-D and 6), contrary to the suggestions of molecular models[22,23,25] and biochemical studies[27,77-79] reviewed recently.[66] Deletion of the kringles of prothrombin has suggested direct involvement of these domains in the interaction with fVa,[80,81] but there is no evidence of such contacts in the cryo-EM structure (Figure 2A,D). Overall, the fVa-prothrombin interaction involves only 8 residues of fVa (Table 2) and does not support involvement of the A3 and C1 domains proposed recently.[66] Likewise, interactions of fXa with prothrombin residues 205 to 220 of kringle 2,[82] the C terminus of the protease domain,[83] and the Gla domain[84] assigned from competition experiments are not confirmed by the cryo-EM structure. These effects are likely the result of conformational changes rather than perturbation of direct contacts.

Discussion

The cryo-EM structure presented in this study offers a 3-dimensional view of the initial encounter between prothrombin and prothrombinase that triggers activation along the meizothrombin pathway.[21,24,31,33] It has been suggested that the

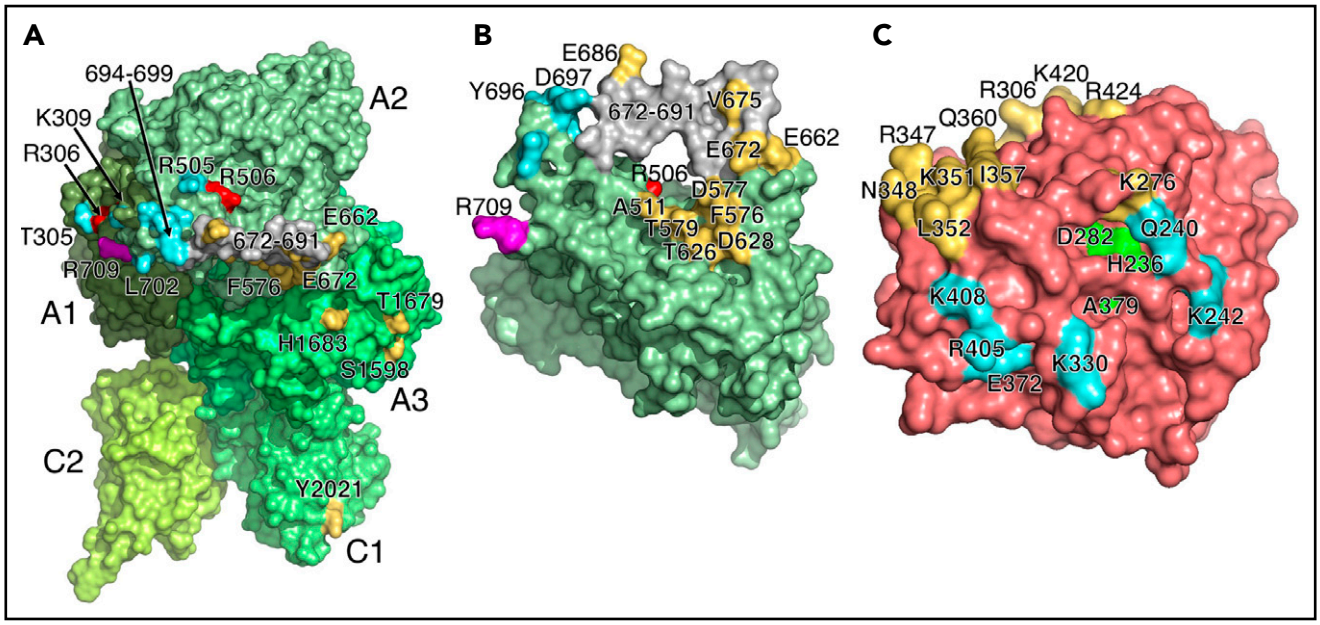

Figure 4. Interaction of fVa with fXa in prothrombinase. (A) Overall structure of fVa in surface representation, oriented as in Figures 1B and 2B, showing the residues involved in recognition of fXa (yellow) and prothrombin (cyan). The interaction with fXa involves mainly the A2 domain and few contacts in the A3 and C1 domains (Table 2). Residues in the 694 to 699 region (Table 2) are labeled as a group. The entire 672 to 691 segment (gray) moves >7 Å relative to the position in free fV[35] to close like a lid over the protease domain of fXa. Also shown are the sites of inactivation by activated protein C at R306 and R506 (red) and the site of thrombin activation of fV at R709 (purple). (B) Surface representation of the A2 domain of fVa oriented as in Figure 2A, with all other domains, fXa and prothrombin removed for clarity. The view reveals the individual residues of fVa important for fXa binding (yellow) and their position relative to the 672 to 691 segment (gray) and the epitopes for prothrombin binding (cyan). (C) Surface representation of the protease domain of fXa oriented as in Figure 2D and rotated 30° along the x-axis, with all other domains, fVa and prothrombin removed for clarity. Residues of the catalytic triad (green), with S379 replaced by Ala, are in the center. The view reveals the residues involved in fVa binding (yellow) as being located toward the C-terminal helix (K420, R434) and the 340 to 350 (c165-175) segment of the protease domain. Also shown are residues responsible for binding of prothrombin (cyan) around the active site entrance (Q240, K242, K33) and the Na^+ site region (E372, R405, K408). Particularly important is the strong electrostatic coupling of R347 of fXa with E572 and E662 of fVa, as identified by biochemical studies.[59,60]

function of fVa is to enhance prothrombin activation by providing a scaffold for assembly of the prothrombin-fXa complex rather than changing allosterically the conformation of fXa.[85] This conclusion is analogous to that reached for the cofactor-assisted activation of protein C by the thrombin-thrombomodulin complex[86,87] and is supported by the cryo-EM structure reported in this study. Changes in the conformation of fVa are limited to the ^{672}ESTVMATRKMHDRLEPEDEE^{691} segment in the A2 domain, which is disordered in the free form[35] but becomes structured and closes like a lid on the protease domain of fXa (Figures 1, 2, and 4). The change is an induced-fit step in the binding of fXa leading to formation of prothrombinase. Factor Xa does not fully extend over the plane of the membrane but curves at the junction between the EGF1 and Gla domains (Figures 1 and 2). Information on the position of the Gla domain in the free form of fXa, which is missing in current structures,[28,29] will establish if this curved conformation is induced by binding to fVa or is selected from an ensemble of preexisting conformers. Conversely, the curved conformation of prothrombin in the ternary complex resembles the closed form that predominates for the free zymogen in solution[31,33] and favors activation along the meizothrombin pathway.[31,32,50] In addition, binding of prothrombin has little effect on the conformation of prothrombinase (Figure 1), consistent with functional studies.[31,50] We conclude that the prothrombin-prothrombinase interaction initiating the meizothrombin pathway involves both induced-fit and conformational selection steps.[88] Assembly of the prothrombinase complex induces changes of the A2 domain of fVa and possibly of the EGF1-Gla domain junction of fXa. Binding of prothrombin to the complex requires selection of the closed form from the preexisting open-closed equilibrium in solution.

The relative arrangement of fVa and fXa revealed by the cryo-EM structure (Figure 2) is unlikely to be retained for processing the alternative site of cleavage of prothrombin at R271, which initiates the prethrombin 2 pathway on the surface of platelets.[11] Biochemical studies indicate that cleavage at R271 takes place with prothrombin in the open form[30,31,33,34] and the X-ray structure of prothrombin with residues 154 to 167 in the flexible linker 2 deleted[30] may be a good approximation of this conformation.[33] Swapping the closed with the open form in the cryo-EM structure points to changes needed to broker cleavage at R271 (Figure 6). The open form features a nearly vertical alignment of the Gla domain and kringles that rotates and pushes the protease domain up against fVa. This moves R320 up 18 Å from its position in the closed form (Figure 6B-C) to clash against E686 of fVa and K276 of fXa (Figure 6A). The rotation moves exosite I closer to fVa and the site of activation around R271 almost 20 Å closer to the active site of fXa. Therefore, transition from the closed to the open form prepares prothrombin to present R271 to the active site of fXa. The interaction likely requires a movement of the A2 domain of fVa back to the position seen in fV[35] and elongation of the fXa structure along its vertical axis. In this scenario, switching prothrombin activation from the meizothrombin (R320) to the prethrombin 2 (R271) pathway would cause conformational changes of all components of the prothrombin-prothrombinase complex. A critical test of this proposal will come from a cryo-EM

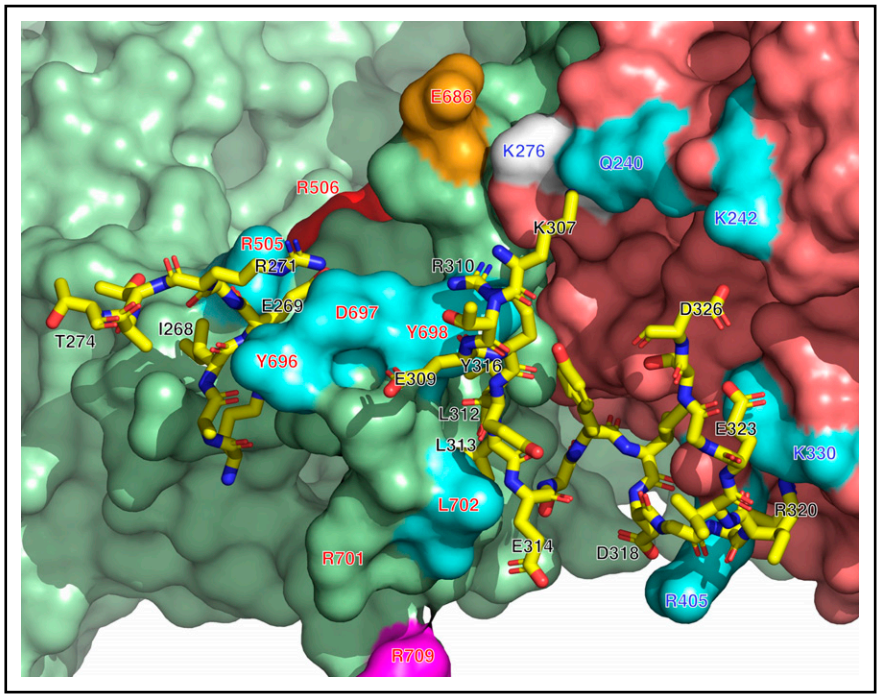

Figure 5. Interaction of prothrombin with prothrombinase. Molecular surface of interaction between the A2 domain of fVa (pale green surface), fXa (deep salmon surface), and prothrombin (yellow sticks) that details how fVa orchestrates preferential binding of R320 to the active site of fXa. Shown is the segment ^{269}EGRTAT^{274} comprising the site of cleavage at R271 and the longer segment ^{307}KTERELLESYIDGRIVEGSD^{326} comprising the site of cleavage at R320. Relevant epitopes of fVa and fXa for interaction with prothrombin are colored in cyan. The interaction between E686 of fVa (orange) with K276 of fXa (white) is also labeled, along with R506 and R709 of fVa. The segment ^{696}YDYQNRL^{702} separates the 2 prothrombin segments and sequesters R271 with a strong ionic interaction with D697 fixed by a nearby interaction between E269 and R505 of fVa. The segment then organizes the proximal portion of the R320 site for interaction with the active site of fXa. Flanking D697 are Y696 in hydrophobic interaction with F535 and Y698 coupled to R310 through a cation-π interaction and in hydrophobic contact with L312. The fragment then turns toward fXa where strong electrostatic interactions involve D318 at P3, E323 at P3′ and D326 at P6′ with residues R405, K370, and K242 of fXa, respectively. An additional ionic interaction is established between K307 of prothrombin and Q240 of fXa. As a result of these interactions, R320 penetrates the active site of fXa to initiate activation along the meizothrombin pathway.

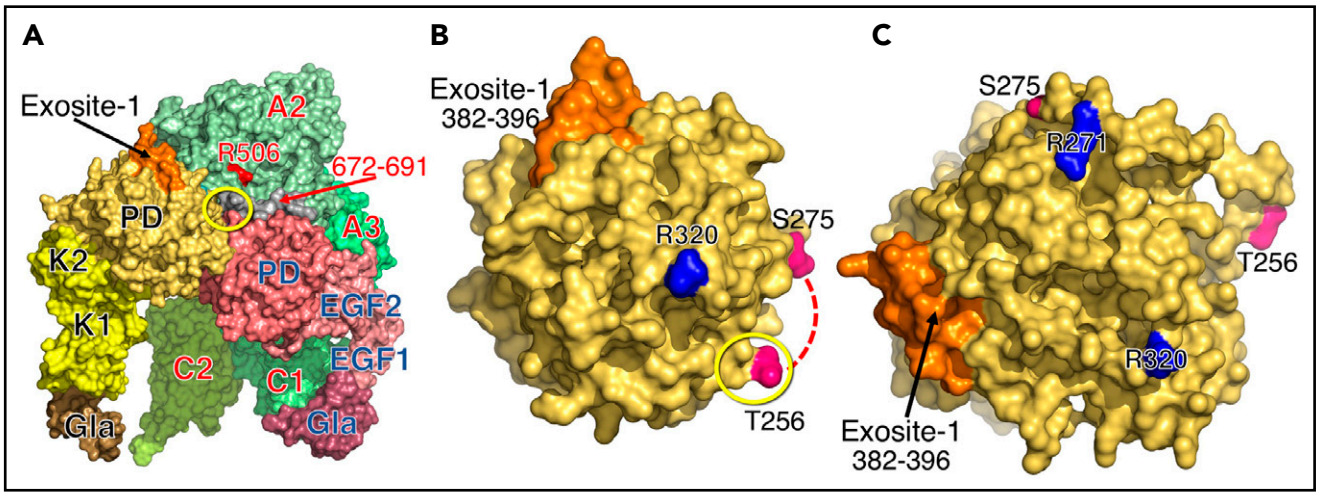

Figure 6. Putative interaction of prothrombin in the open form with prothrombinase. (A) The complex was obtained by overlaying the structure of prothrombin in the open form (PDB ID 5EDM)[30] to the closed form in the cryo-EM structure (Figures 1B and 2). The open form aligns the Gla domain with the homologous domain of fXa and the C1 and C2 domains of fVa. Unfortunately, the entire segment ^{257}GDGLDEDSDRAIEGRTAT^{274} containing the site of cleavage at R271 was not resolved in the 5EDM structure (B, dotted lines). Residue R320 moves 18 Å upward and clashes with the area of contact between E686 of fVa and K376 of fXa (yellow circle; see also Figure 5). A movement of the entire 672 to 691 region (gray) of the A2 domain would be necessary to accommodate the protease domain of prothrombin in the open form. Exosite-1 of prothrombin (residues ^{382}RIGKHSRTRYERNIE^{396}, orange) moves closer to but not in contact with fVa. (B-C) Protease domain of prothrombin in the open (B) and closed (C) forms obtained after rotation of panel A 90° clockwise along the y-axis (B) or directly from Figure 2E (C), with fVa, fXa, and the auxiliary domains (EGF1, EGF2, Gla) removed for clarity. Transition from the closed to the open form moves exosite 1 closer to fVa and relocates R320 18 Å upward from the position in the closed form (indicated in panel B by a yellow circle, for reference). The transition also causes a significant clockwise rotation of the entire segment 257 to 274 containing the R271 site (C), not visible in the open form (B, dotted lines), with the Cα-Cα distance between T256 and S275 shrinking from 37 to 16 Å. The rotation would bring R271 closer to the active site of fXa in the open form.

structure of prothrombinase bound to meizothrombin that is stabilized in the open form and can only be cleaved at R271.[31,50]

Knowledge of the precise arrangement of the 3 components of the prothrombin-fVa-fXa complex points to new targets for future mutagenesis studies (Table 2) and offers a much needed validation of existing paradigms, biochemical data, and structural models.[5,8,19,21-23,25,27,66] Although this complex may look different on nanodiscs or equivalent membrane surfaces, it is highly consistent with main features previously observed for its individual components by X-ray, cryo-EM, and single molecule spectroscopy studies in solution.[28,31,33,35,50,82] Hence, the conformations of prothrombin, fVa and fXa can be captured reproducibly with techniques that differ widely in the constraints imposed on each macromolecule. This gives biological relevance to the structural information derived by cryo-EM and motivates extension of similar approaches to the study of other interactions in the blood coagulation cascade.

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Authorship

Contribution: E.A.R. performed protein purification, biochemical preparations, structure determination, and analysis; B.S., M.J.R. and J.A.J.F. performed cryo-EM data acquisition; and E.A.R., B.S., M.J.R., J.A.J.F., and E.D.C. analyzed the results and prepared the manuscript.

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Footnotes

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The structures were deposited in the Protein Data Bank with accession code 7TPQ for the fVa-fXa complex on nanodiscs (Figure 1A) and 7TPP for the prothrombin-fVa-fXa complex (Figures 1B and 2).

The online version of this article contains a data supplement.

There is a *Blood* Commentary on this article in this issue.

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