Chapter 2
Factor VIII and Factor V Membrane Bound Complexes

Svetla Stoilova-McPhie

Abstract The formation of membrane-bound complexes between specific coagulation factors at different cell surfaces is required for effective blood clotting. The most important of these complexes, the intrinsic Tenase and Prothrombinase complexes, are formed on the activated platelet surface during the propagation phase of coagulation. These two complexes are highly specific in their assembly mechanism and function modulated by anionic membranes, thus offering desirable targets for pharmaceutical interventions. Factor V (FV) and factor VIII (FVIII) are highly homologous non-enzymatic proteins. In their active state, FVa and FVIIIa serve as cofactors for the respective serine proteases factor Xa (FXa) and factor IXa (FIXa), significantly increasing their catalytic activity. This is achieved by forming well organized membrane-bound complexes at the phosphatidylserine rich activated platelet membrane in the presence of Ca^{2+} ions. The tenase (FVIIIa/FIXa) complex, catalyzes the proteolytic conversion of FX to FXa. Subsequently the prothrombinase (FVa/FXa) complex catalyzes the conversion of prothrombin to thrombin, required for efficient blood clotting. Although significant knowledge of FV and FVIII biochemistry and regulation has been achieved, the molecular mechanisms of their function are yet to be defined. Understanding the geometric assembly of the tenase and prothrombinase complexes is paramount in defining the structural basis of bleeding and thrombotic disorders. Such knowledge will enable the design of efficient pro- and anticoagulant therapies critical for regulating abnormal hemostasis. In this chapter, we will summarize the findings to date, showing our achievement in the field and outlining the future findings required to grasp the complexity of these proteins.

Keywords Blood coagulation · Factor VIII · Factor V · Activated platelets · Membrane-Associated proteins · 3D structure · Cryo-Electron microscopy · Macromolecular complexes

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Introduction

Haemostasis is defined as arrest of bleeding, derived from the Greek words 'haeme' meaning blood and 'stasis' meaning to stop. It is the complex physiological process responsible for stopping blood loss that depends on a delicate equilibrium between pro- and anticoagulant forces. Hemostasis' main components are cells (platelets, and fibroblasts), soluble proteins (coagulation factors) and insoluble proteins (extracellular matrix proteins). The hemostatic system is activated upon vascular injury. Primary hemostasis leads to the formation of a platelet plug at the site of the injury. Secondary hemostasis leads to the generation of a fibrin clot that allows time for the injured endothelium to heal. The final stage—fibrinolysis activates the anticoagulant mechanisms necessary to dissolve the clot and restore blood flow. Secondary hemostasis is generally referred as blood coagulation, defined as the formation of insoluble, cross-linked fibrin by a sequential activation of coagulation factors and thrombin. Fibrin also stabilizes the primary platelet plug in larger blood vessels to stop hemorrhage (Gale 2011; Palta et al. 2014).

The blood coagulation process requires the following elements:

- Cells
- Enzymatic coagulation factors (zymogenes)
- Non-enzymatic coagulation factors (pro-cofactors)
- Calcium ions (Ca^{2+})
- Phosphatidylserine (PS) rich phospholipid (PL) membrane surface.

The coagulation process was initially described as a series of sequential proteolytic reactions, in which the activation of one clotting factor leads to the activation of the next, culminating in a thrombin 'burst' and fibrin generation. This model, known as a clotting cascade or 'waterfall model' explains well the clinical tests designed to characterize the main bleeding and thrombotic disorders at the time (Macfarlane 1964; Davie and Ratnoff 1964).

Traditionally the clotting cascade has been divided into intrinsic, extrinsic and common pathways (Fig. 2.1). This division has been helpful for interpreting laboratory results. The two clinical clotting tests: Activated Partial Thromboplastin Time (APTT) (Langdellet al. 1953; Quick 1950) for the intrinsic/common pathways and the Prothrombin Time (PT) (Quick 1950) for the extrinsic/common pathways represent well the property of blood to follow different mechanism of clotting in vivo and in vitro.

The intrinsic pathway follows the coagulation of blood in vitro that occurs when blood is in contact with a foreign body like glass, silica, kaolin. The extrinsic or FVII pathway follows the in vivo coagulation, where the 'intrinsic' tenase (FVIIa/TF) complex is formed during vascular injury producing small amount of FXa and thrombin that activates the cofactors VIII and V required for the assembly of the 'extrinsic' tenase and prothrombinase complexes on the activated platelet surface.

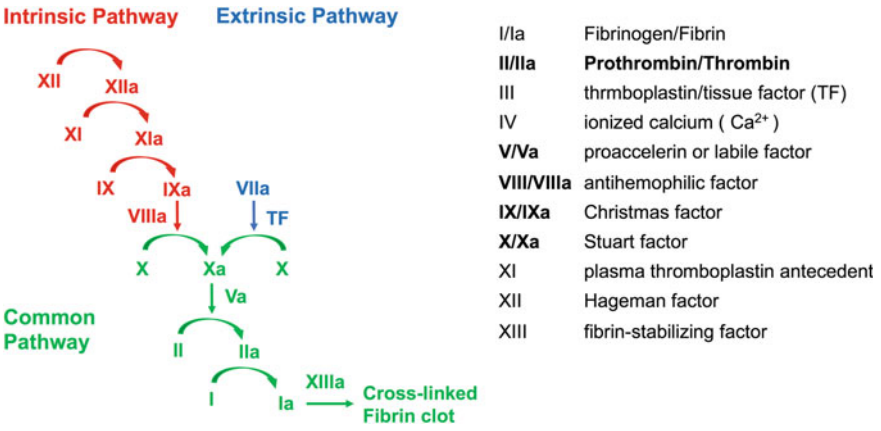


Fig. 2.1 Cascade model of blood coagulation. The coagulation factors are presented with roman numerals. The active form is denoted with a suffix 'a'. The factors in bold are the components of the tenase and prothrombinase complexes. Only the pro-coagulant pathways are indicated for clarity

These two complexes work in synchronization to generate a thrombin 'burst'—several orders of magnitude acceleration of thrombin activation, necessary for efficient hemostasis. The nomenclature of the coagulation factors participating in the clotting is shown in Fig. 2.1. In bold are the ones that participate in the 'extrinsic' tenase (FVIIIa/FIXa) and prothrombinase (FVa/FXa) complexes securing timely clotting. The significance of Ca ions (Ca^{2+}) and phospholipid (PL) surfaces for blood clotting are illustrated in Table 2.1.

Table 2.1 Clotting times of blood in vitro, in the presence and absence of different components. APPT and PT refer to the clotting tests: activated partial thromboplastin time and prothrombin time, and their components

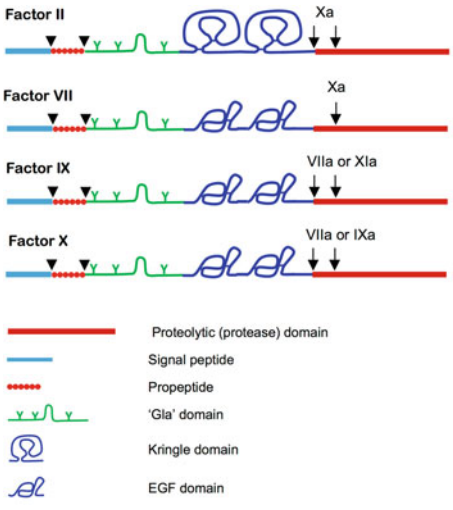
Clotting system	Clotting time
Whole blood	240–460 s (4–8 min)
Whole blood + EDTA or citrate	Infinite
Citrated platelet-poor plasma (PPP) + Ca^{2+}	120–240 s (2–4 min)
Citrated platelet-poor plasma + PL + Ca^{2+}	60–85 s
APTT: Citrated platelet-poor plasma + kaolin + PL + Ca^{2+}	21–32 s
PT: Citrated platelet-poor plasma + thromboplastin + Ca^{2+}	11–12 s

Thromboplastin contains FVII and PL

Clotting Factors

The coagulation factors are traditionally identified as roman numerals, based on the order in which they were isolated and identified historically. They circulate in plasma as inactivated and activated (denoted with 'a') proteins. The majority of clotting factors are precursors of proteolytic enzymes known as zymogens that circulate in blood as an inactive form. The activation of each zymogen is depicted by suffixing letter "a" to the Roman numeral identifying that particular zymogen (Fig. 2.1) (Palta et al. 2014). These proenzyme factors undergo a post translational modification (vitamin K dependent gamma (γ) carboxylation of glutamic acid residues) which enables them to bind calcium ions and adopt the suitable conformation required for binding to negatively charged membrane surfaces, such as the activated platelet surface. Deficiency of vitamin K or administration of vitamin K antagonists (*e.g.* warfarin) abolish this carboxylation and it is to date the most powerful anticoagulant drug (Fig. 2.2) (Hoffman and Monroe 2014).

Vitamin K-dependent proteins play a central role in the regulation of blood coagulation. The N-terminal γ-carboxyglutamic acid (Gla) domain anchors the proteins to cell membranes exposing phosphatidylserine in a Ca^{2+} dependent interaction. A



Coagulation Factors	(kDa)	(nM)	(%)
Factor II (Prothrombin)	72	1207 - 1727	40
Factor VII	50	7 - 13	5-10
Factor IX	**56**	**40 - 142**	**10-40**
Factor X	**59**	**101 - 162**	**10-15**
Factor V	**300**	**12 - 42**	**10-15**
Factor VIII	**280**	**0.36 - 1**	**10-40**

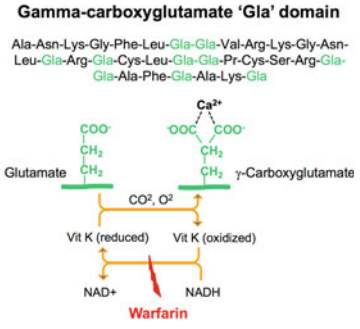


Fig. 2.2 Domains organization and similarities between the Vitamin K dependent coagulation factors in the propagation phase of coagulation. The proenzymes Factors II, VII, IX and X are highly homologous single peptides that comprise of a catalytic/protease domain at the C terminus, followed by a sequence of epidermal growth factor (EGF) or kringle domains, a γ-carboxyglutamic acid (Gla) domain, an activation region (propeptide) and a signal peptide at the N terminus. Upon synthesis, the signal and propeptide domains are cleaved (arrow heads) from the polypeptide chain, exposing the Gla domain to the negatively charged platelet membranes. Further proteolytic activation (arrows) exposes the serine protease site of the enzymes. The table on the right shows the size and concentration of the zymogenes and pro-cofactors in blood (Furie and Furie, 1992)

linear area of Ca^{2+} ions bind internally to the carboxyl groups of the Gla domain inducing a significant structural transition from a largely unfolded to a tightly folded domain capable to bind anionic membranes. The Gla domain-membrane interaction has a critical role in the assembly of the membrane-bound coagulation complexes leading to enhanced catalytic efficiency of the respective proteases. This phenomenon explains the fact that removing Ca^{2+} ions by citrate or EDTA prevents blood from clotting indefinitely (Table 2.1) (Huang et al. 2003; Furie and Furie 1988; Kalafatis and Mann 1997; Kalafatis et al. 1997; Zwaal et al.1998).

Cell Model of Coagulation

Since 2010 a new model of the coagulation process has been proposed—the cell based model that explains some aspects of hemostasis, which a protein-centric (cascade) model does not. The cascade theory is particularly useful for understanding the in vitro coagulation tests, but does not consider the central role of cell-based surfaces in the in vivo coagulation process (Hoffman and Monroe 2001). In the cell-based model, the coagulation process of thrombin generation and fibrin formation is represented on cell surfaces, as a sequence of three distinctive phases (Fig. 2.3):

1. *Initiation phase*: plasma factor (FVII) binds to tissue factor (TF) on subendothelial fibroblasts, auto activates to FVIIa and forms the TF-FVIIa "extrinsic tenase" complex that activates FX to FXa and produces a small amount of thrombin (FIIa).
2. *Propagation and Amplification phase*: The small amount of thrombin (FIIa) generated in the initiation phase activates the platelets. Cleaves FVIII from its carrier-the von Willebrand Factor (vWF). Activates FVIII and FV to FVIIIa and FVa, respectively. Then, it amplifies its own production through the assembly of the FVIIIa-FIXa "intrinsic tenase" complex and the FVa-FXa "prothrombinase" complex on the phosphatidylserine (PS)-rich surfaces of activated platelets. The two complexes work in synergy to amplify and propagate thrombin generation, producing a "thrombin burst".
3. *Fibrin formation*: The thrombin burst is essential for forming cross-linked fibrin (FIIa) from fibrinogen Factor I (FI), by cleaving fibrinogen to soluble fibrin, activating factor XIII (FXII to FXIIIa) is required to cross-link fibrin while concurrently inhibiting fibrinolysis. Calcium (Ca^{2+}) is critical at all stages of thrombin generation and fibrin formation.

It is important to note that from a spatial point of view, everything happens on the injured blood vessel (endothelial) surface. The small amount of thrombin generated by the 'intrinsic' tenase complex also activates the platelets, which includes translocating PS to the platelet surface. These activated platelets together with the vWF, form a platelet plug on the site of injury, which occurs almost simultaneously with the assembly of the tenase and prothrombinase complex.

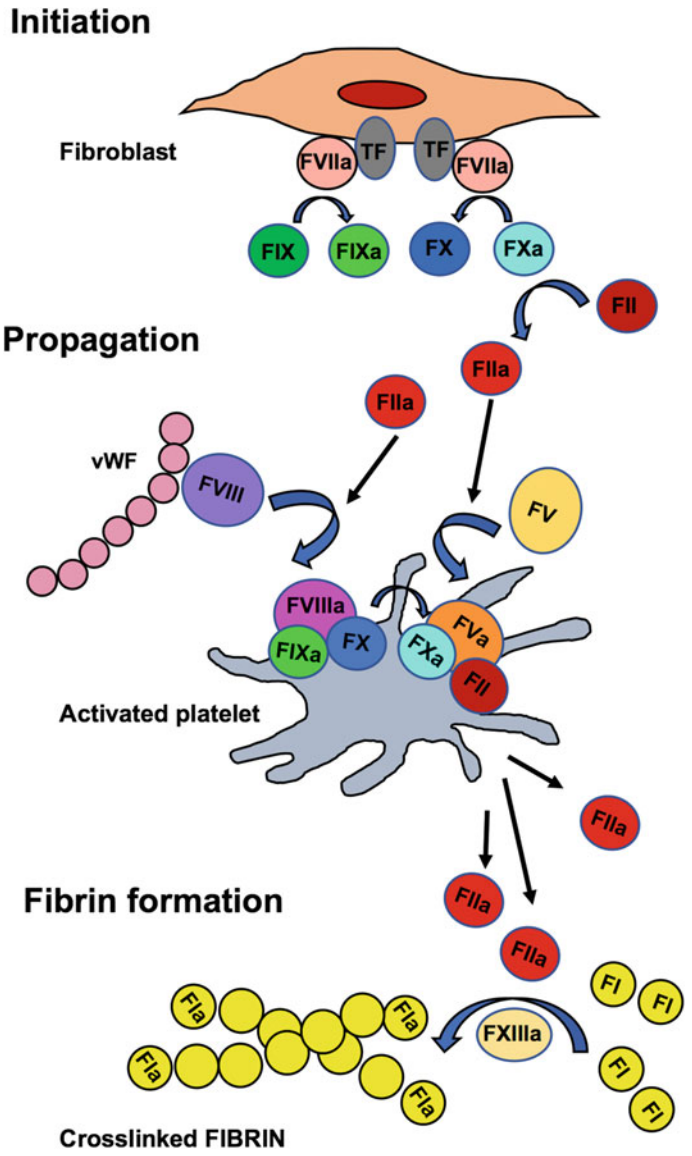


Fig. 2.3 Cell-based model of blood coagulation. Only the proc-coagulant pathways are included in this model, characterized by three phases: Initiation, Propagation and Firbrin formation. The first two phases of the cell model are directly linked to thrombin (FIIa) generation. In the first-initiation phase, -only small (initial) amount of FII are generated on endothelial cells (fibroblast) by the 'intrinsic' tenase complex assmbled on the surface of endothelial cells (fibroblast). In the second-propagation phase, FII generation is increased more than fivefold due to the synergy of the tenase and prothrombinase complex assembled on the activated platelet surface. The thrombin burst generated in the second phase secure accelerated generation of crosslinked fibrin (FIa) in the third-fibrinolysis phase, required to stop fast the blood loss

The models for the coagulation process presented in this introduction include only the pro-coagulant pathways. FVa and FVIIIa are also key players in the anticoagulant pathways, forming membrane-bound complexes with active protein C (APC) and protein S, leading to their inactivation. APC is a vitamin K-dependent serine protease, highly homologous to FVII, FIX an FX. Protein S is a modified vitamin K-dependent serine protease that has a gla-domain facilitating binding to anionic membrane surfaces but does not have a protease domain and proteolytic activity. The FV and FVIII membrane-bound complexes with APC are critical for fine tuning of the coagulation process, more pronounced for FVa, as FVIIIa is intrinsically unstable. In this chapter our focus will be on the pro-coagulant membrane-bound FV and FVIII complexes.

The Tenase and Prothrombinase Complexes

The tenase and prothrombinase complexes are tertiary complexes formed on the PS-rich activated platelet surface in the propagation phase of coagulation. The product from the tenase complex, FXa is the enzyme in the prothrombinase complex that catalyzes the conversion of prothrombin to thrombin. The presence of FVa and FVIIIa within the macromolecular enzyme complexes enhances the respective proteolytic reactions rate by several orders of magnitude, accelerating sufficiently thrombin generation to secure normal hemostasis. The two membrane-bound complexes work in synchronization and are pivotal steps in the coagulation cascade. (Figure 2.4) (Mann et al. 1990).

The serine proteases FIXa and FXa by themselves are capable of activating the respective zymogenes—FX and prothrombin (FII) in solution. The presence of a co-factor and the assembly of the membrane-bound complexes increases the catalytic activity of the enzymes ~ 300,000 times, as illustrated for the prothrombinase complex in Table 2.2.

The significance of the FVIII co-factor function and the tenase complex assembly is further illustrated by the fact that defects or deficiency of FVIII lead to a sever hemorrhagic disorder known as Hemophilia A. Hence FVIII is also known as the anti-hemophilia A factor (AHF) (Cawthern et al. 1998).

Factor V and Factor VIII

Blood coagulation factors V and VIII are two large multidomain glycoproteins that share high structural and functional homology and do not have enzymatic (proteolytic) activity (Mann et al. 1988; Kane and Davie 1988). Factors V and VIII are composed of three structural domains organized in the same order from the N- to the C-terminal: A1-A2-B-A3-C1-C2, with a high degree of amino acid identity (~40%) except for the B domains. The B domain consists of about 900 amino acids and

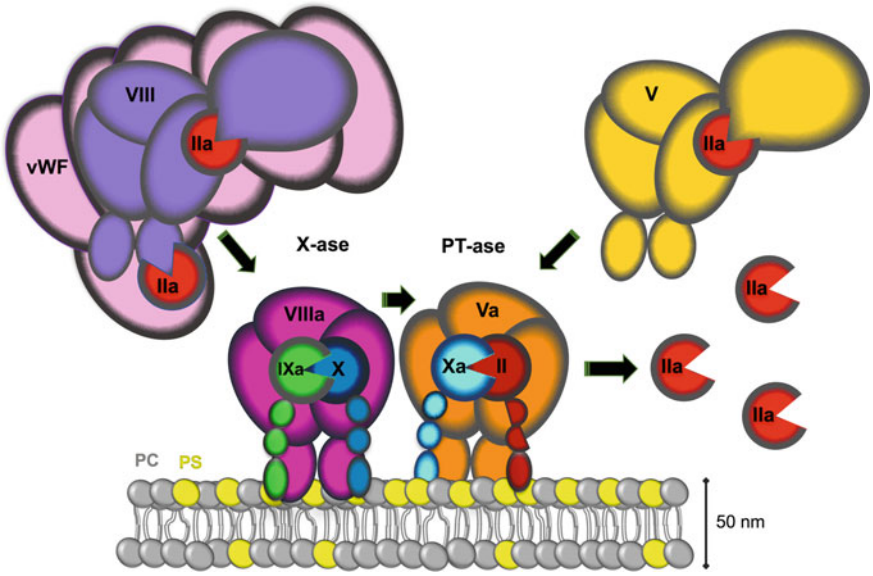


Fig. 2.4 The tenase and prothrombinase complexes. These drawing illustrates the proposed mechanism of the Tenase (X-ase) complex assembly between Factors VIIIa and IXa and the Prothrombinase (PT-ase) complex assembly between Factors Va and Xa on the activated platelet surface rich in PS. Xa is the product of the X-ase and the enzyme of the PT-ase complex. The PT-ase complex activates Prothrombin (II) to Thrombin (IIa). The separate domains of the proteins are sown as ovals/sphere. The membrane and protein complexes are drawn to scale

Table 2.2 Clotting times for FII (thrombin) activation by FXa. PL indicates phospholipids

Clotting factors	Rate of FII generation	Clotting times
II, Xa	1	3,024,000 s (35 days)
II, Xa, PL, Ca^{2+}	350	8640 s (2.4 h)
II, Xa, PL, Va, Ca^{2+}	21,000	150 s (2.5 min)
I, Xa, Platelets, Va, Ca^{2+}	302,400	10 s

The components of the prothrombinase complex In vitro are: FII, FXa, FVa, PL and Ca2 + . In vivo platelets serve the role of PL

is rich in *N*-and *O*-glycosylation sites, 25 for the Factor V and 18 for the FVIII, heavily glycosylated with N-linked oligosaccharides and are highly flexible (Fig. 2.5) (Nesheim et al. 1979; Rosing et al. 1980). The significance of the B domain uniqueness has not been fully understood. The A domains are about 330 amino acid and have 30% identity between each other and the three A domains of Curuloplasmin, a Cu-binding protein that circulates freely in blood. The C domains are about 150 amino acids and are highly homologous to the Discoidin 1 protein (Pemberton et al. 1997; Saenko et al. 2002).

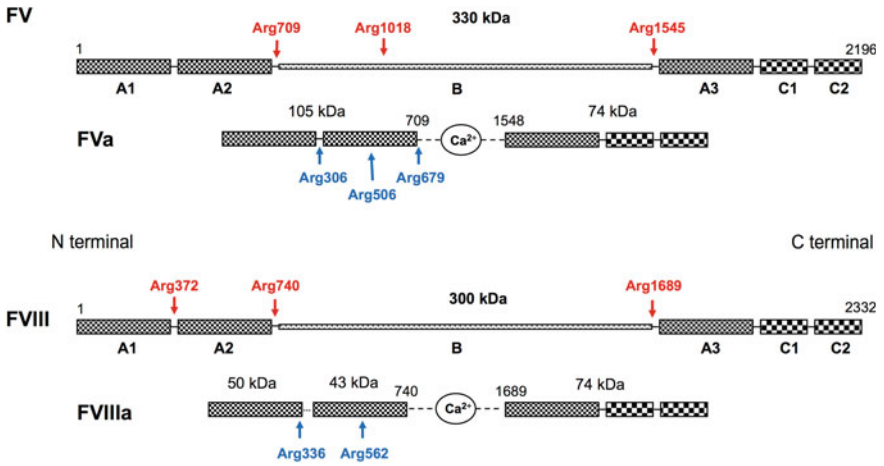


Fig. 2.5 Domains organization of Factor V and Factor VIII. FV and FVIII are expressed as single chain six domains proteins of ~ 300 kDa. The three A domains are homologous (~ 35% sequence identity) and contain the FX and FXa binding sites, respectively. The A2 and A3 domains are separated by a unique B domain that is not required for function. The C domains are also homologous (~ 40% sequence identity) and contain the membrane-binding sites. Upon proteolytic activation by Thrombin (red arrows) the B domain is excised and the FVa and FVIIIa active forms present as non-covalently bound heterodimers of a heavy chain (A1-A2 domains) and a light chain (A3-C1-C2) domains stabilize electrostatically by Ca^{2+} ions. The A1 and A2 domains in FVIII, further separate by proteolysis at Arg372, leading to the final active form presenting a heterotrimer of the A1 and A2 domains holding together solely by hydrophobic interactions and the light chain stabilized by both hydrophobic and electrostatic interactions. The blue arrows present the APC cleavage sites, leading to inactivation of FV and FVIII

The activated forms, FVIIIa and FVa act as co-factors to the serine proteases FIXa and FXa respectively, when assembled in a membrane-bound complex with a zymogen substrate FX and prothrombin, respectively (Fig. 2.4). These complexes are functional only when formed in the presence of calcium ions and anionic phospholipid surfaces, such as the PS rich activated platelet membrane. Calcium is needed to stabilize the Gla-domains of the vitamin K-dependent serine proteases so they can efficiently bind to the negatively charge activated platelet membrane (Huang et al. 2004). Calcium is also needed for the single calcium binding sites in FVIII and FV that are required for stabilizing the heavy and light chain of the proteins after activation (Fig. 2.5) but have not shown to be required for membrane-binding (Stoilova-McPhie et al. 2013). The assembly of the FVIIIa/FIXa and FVa/FXa complexes increases the Vmax of the FX and prothrombin activation ~5 orders of magnitude (Mann et al. 1988; Kane and Davie 1988; Nesheim et al. 1984a).

Although the knowledge of factor VIII and factor V and their regulation has progressed significantly over the last two decades, the molecular mechanisms by which their co-factor function leads to accelerated substrate cleavage remains to be fully defined. The difficulties in having homogeneous preparations of these factors have been overcome by the advent of recombinant DNA technologies and protein

purification procedures. The dynamics and complex interactions of these factors with their multiple partners (ions, membranes, activators and inhibitors) renders them a change for structural and functional studies to date. There has not yet been a consensus on the molecular models of FVIIIa and FVa within the membrane-bound tenase and prothrombinase complexes, despite existing crystal structures for FVIII and FV, the interacting proteases—FIXa and FXa and zymogen substrates—FX and prothrombin, respectively. Understanding the mechanism by which the structure of these factors morphs to fulfill their function is the endeavor of modern hematology (Bos and Camire 2010).

Factor V

Factor V (FV) is a large (Mr ~ 330 kDa), multidomain (A1-A2-B-A3-C1-C2), single chain glycoprotein that circulates in blood at a concentration of ~ 20 nM (~ 10 μg/ml). At physiological plasma concentrations, the pro-cofactor FV cannot assemble or function in the prothrombinase complex. Proteolytic processing of the B domain is an absolute requirement for the expression of cofactor function. Thrombin is the main physiological activator of FV and cleaves three peptide bonds (Arg709, Arg1018, and Arg1545) within the B domain that leads to the B domain removal and FV activation (Fig. 2.5). The active from—FVa, presents a heterodimer composed of a heavy chain (A1-A2; Mw = 105 kDa) and a light chain (A3-C1-C2; Mw = 71–74 kDa), which are associated through electrostatic forces supported by Ca^{2+} ions (Fig. 2.5) (Jenny et al. 1987; Mann and Kalafatis 2003; Suzuki et al. 1982; Esmon et al. 1973; Esmon 1979; Nesheim et al. 1984b; Stoilova-McPhie et al. 2008).

Deficiency of FV is associated with a rare bleeding disease (1:1,000,000) parahemophilia. Most mutations of FV are associated with venous thrombosis due to resistance to cleavage by APC, leading to the FVa remaining active and accelerated thrombin generation through the prothrombinase complex. The most common of these mutations is known as the FV Leiden (Asselta et al. 2006; Sun et al. 1994; Bos and Camire 2010).

Factor VIII

Factor VIII (FVIII) is expressed as a large (Mr ~ 330 kDa), heavily glycosylated multidomain (A1-A2-B-A3-C1-C2) proteins that circulates in vivo, as a complex with the von Willebrand factor (vWF)—a large (Mr ~ 2MDa) multidomain protein (Figs. 2.3 and 2.4). Once activated by thrombin it exists as a heterodimer resulting from limited proteolysis at the B-A3 junction and at additional sites in the B domain. This heterodimer consists of a variably sized heavy chain (A1-A2-B; 200-290 kDa) and a light chain (A3-C1-C2; ~ 80 kDa) that are non-covalently associated and correspond to the FVIII form purified from blood (plasma derived, pd) or expressed

in vitro (recombinant FVIII) (Fig. 2.5). The heavy chain interaction with the light chain serves various important roles in FVIII physiology, as it has been reported to stabilize the heterodimeric structure of FVIII, as well as to prevent proteolysis by FXa and APC. The FVIII heterodimer is an inactive pro-cofactor and it is subjected to further proteolysis to gain full cofactor activity (Fay 2004; Fay 2006). Factor VIII can be proteolytically activated by both thrombin and FXa. Thrombin cleaves three peptide bonds at Arg372, Arg740, and Arg1689, thereby generating FVIIIa, which is a heterotrimer composed of the A1 (Mr ~ 50 kDa) and A2 (Mr ~ 43 kDa) domains held together by hydrophobic forces, and the light chain (A3-C1-C2; Mr ~ 73 kDa) (Fig. 2.5). Once activated, FVIIIa biological activity decays over a short period of time (~ 6 min in vitro) due to the A2 domain dissociation from A1/A3-C1-C2 domains, a mechanism that contributes to the regulation of FVIIIa cofactor activity (Vehar et al. 1984; Fay et al. 1991; Fay and Smudzin 1992; Lollar and Parker 1991; Fay 2004; Fay 2006; Lenting et al. 1998).

Defect or deficiency of FVIII result in Hemophilia A, an X chromosome genetic disorder affecting 1:5000 males (Graw et al. 2005). Recombinant FVIII was launched as drug for Hemophilia A in 1992 and it was one of the first recombinant proteins approved for treatments in humans. Multiple commercial recombinant FVIII forms exist to date, that facilitate significant structural and functional studies (Antonarakis 1995; Oldenburg et al. 2004).

Despite the similarities in the structure and function of FV and FVIII, the molecular mechanisms of activation have been found to differ substantially. The inactive pro-cofactor is stable when purified from blood or expressed. The FV does not have a transient heterodimer form before the final activation step to FVa. Activated FV (FVa) forms a stable heterodimer that can be characterized structurally and functionally (Fig. 2.5 (Stoylova et al.1994; Stoilova-McPhie et al. 2008). The FVa inactivation is achieved by forming a membrane-bound complex with APC/ProteinS and FVIII. The inactivated FVai lacks its A2 domain that contains the main interactions sites with the serine protease FXa. The structure of the FVai (Adams et al. 2004) and consequent structures of FVa (not publicly available, because of ownership by pharmaceutical companies) are highly homologous between themselves and to the resolved FVIII structures (RMSD between the Cα atoms is ~ 1.5 Å). The mechanism of activation of FVIII is quite different. The stable form is a heterodimer of variable size which is an intermediate product between the stable complex of the pro-cofactor, FVIII with the vWF and the stable complex between the activated FVIII (FVIIIa) and the FIXa within the membrane-bound tenase complex (Fay 2006). To further stabilize the inactive FVIII heterodimer, B domain deleted (BDD-FVIII) constructs were generated producing FVIII homodimers that are more stable and homogenous in solution and can be studies structurally and functionally. The deletion of the B-domain increases the yield but does not affect the FVIII in vitro and in vivo activity (Parameswaran et al. 2005; Kessler et al. 2005). Cleavage between the A1 and A2 domains at position Arg372 is critical for the pro-coagulant activity of FVIII. The precise mechanism by which cleavage at Arg372 facilitates the transition to the active cofactor state remains to be determined (Nicolaes and Dahlback 2002; Bos and Camire 2010).

The Factor VIII Structures

The macromolecular organization of the FVIII domains in their membrane-bound form and their synchronization are the driving force behind FVIII activity and the assembly of the FVIIIa-FIXa complex on the activated platelet surface. The presence of multiple membrane-binding sites at the FVIII light chain interface is due to the flexibility of the molecule and specifically the flexible link between the C1 and C2 domains lacking a stable hydrophobic macromolecular interface (Stoilova-McPhie et al. 2013). While several structures of FVIII constructs organized in 3D crystals were resolved at ~ 3.6 to 4Å resolution by X-ray crystallography (Ngo et al. 2008; Shen et al. 2008; Svensson et al. 2008; Leksa et al. 2017; Smith et al. 2020), a different membrane-bound domain organization has been proposed from our cryo-EM studies (Fig. 2.6) (Stoilova-McPhie et al. 2002; Stoilova-McPhie et al. 2013; Miller et al. 2014; Dalm et al. 2015).

The structural basis of FVIII activation and the FVIIIa-FIXa complex assembly strongly depend on the FVIII domain organization and geometry. Until we have direct structural evidence for the FVIIIa-FIXa complex assembly on a membrane mimicking the activated platelets surface, the functional implications of FVIII structure will not be completely understood. The macromolecular forces that drive the

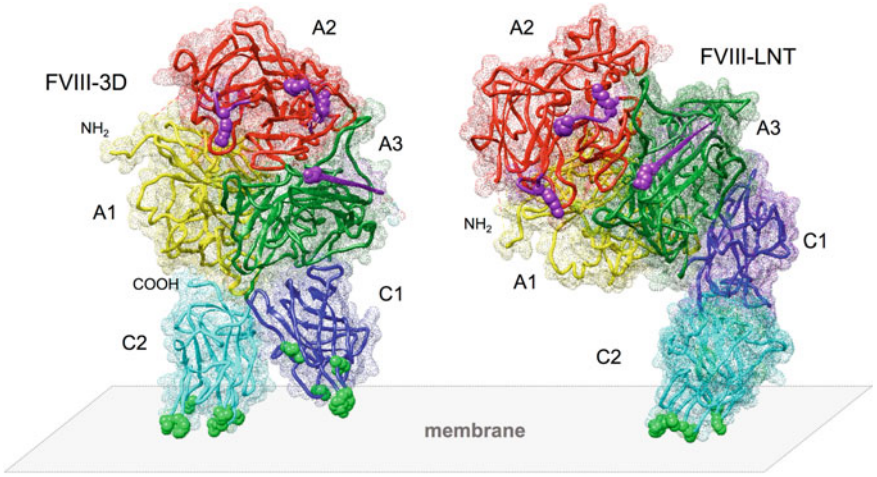


Fig. 2.6 FVIII structure organized in 3D (FVIII-3D) and helical crystals (FVIII-LNT). FVIII-3D is the BDD-FVIII structure as organized in 3D crystals (PDB: 3CDZ) resolved at ~ 4 Å resolution by X-ray crystallography (Ngo et al. 2008). The five FVIII domains are shown as ribbons and colored as follows: A1-yellow, A2-red, A3-green, C1-blue, C2-cyan. The FVIII membrane-binding residues identified on the C1 and C2 domains are shown in green. The FVIII residues identified at the FVIII-FIXa interface on the A2 and A3 domains are shown in purple. The N terminal (Ala1) is indicated with NH_2. FVIII-LNT is the FVIII structure based on the helical organization of the FVIII light chain bound to lipid nanotubes (3J2S) resolved by cryo-EM at 15 Å resolution (Stoilova-McPhie et al. 2013; Dalm et al. 2015). The lipid membrane surface is denoted with a grey rectangle

FVIII activation and the FVIIIa-FIXa complex assembly on a PS-rich membrane surface are fundamental for the successful design of membrane-mimetics modulating blood hemostasis at the FVIII level.

FVIII Structure as Helically Organized on Lipid Nanotubes

Self-organized lipid nanotubes (LNT) can be formed from Galactosylceramide (GC) lipids under specific conditions. These nanotubes are functionalized with specific ligands incorporated into the lipid bilayer. Any protein that can bind to these ligands can be subjected to helical organization and further structure determination by cryo-EM (Wilson-Kubalek et al. 2010; Egelman 2010). Single bilayer GC-LNT rich in PS, have been optimized in our group for the helical organization of FVIII. The geometry and lipid composition of these LNTs mimics the tubular protrusions (pseudopodia) formed by blood platelets upon their activation (Parmenter and Stoilova-McPhie 2008; Parmenter et al. 2008; Wassermann et al. 2010). Three recombinant FVIII proteins, two human and one porcine, were helically organized and the structure resolved by cryo-EM at 15Å and 14.5Å, respectively. All helical reconstructions showed that the membrane-bound FVIII macromolecules formed tightly organized and stable membrane-bound dimers (Fig. 2.7) (Stoilova-McPhie et al. 2013; Stoilova-McPhie et al. 2014; Miller et al. 2014; Dalm et al. 2015; Stoilova-McPhie 2017). The FVIII membrane-bound dimer organization was further confirmed by electron tomography of the helically organized FVIII on LNT (Miller et al. 2014; Dalm et al. 2015) and for FVIII bound to nanodiscs with the same lipid composition as the LNT (Stoilova-McPhie et al. 2014; Grushin et al. 2015; Grushin and Stoilova-McPhie 2017). The FVIII dimer interface was defined by rigid body docking of two FVIII-LNT FVIII structures (Fig. 2.6) within the cryo-EM map of the segmented dimer as shown on Fig. 2.7C. Our studies show that the main interaction interface between the two FVIII molecules is at the FVIII-heavy chain and predominantly at the level of the A2 domains, as only 8 of the 33 residues identified at the FVIII dimer interface within a cutoff of 8.7Å belong to the A1 domain (Fig. 2.8). Fourteen of these residues differ between the human and porcine sequence and eight are known Hemophilia A mutations (Stoilova-McPhie et al. 2014; Stoilova-McPhie 2017). The dimeric FVIII membrane-bound model proposed from our cryo-EM studies is supported by data reported for dimeric membrane-bound assembly of other coagulation factors, such as human FVIII, FIXa, bovine FVa and FXa (Stoylova, Mann and Brisson 1994; Stoilova-McPhie et al. 2014; Chattopadhyay et al. 2009; Miller et al. 2014).

The Supertenase Complex

The FVIIIa-FIXa complex assembly is regulated through high-affinity protein-lipid and protein-protein interactions. The dissociation constants (Kd) for the FVIIIa and FIXa membrane interactions are ~ 1 nM and for the FVIIIa-FIXa interaction ~ 15 nM

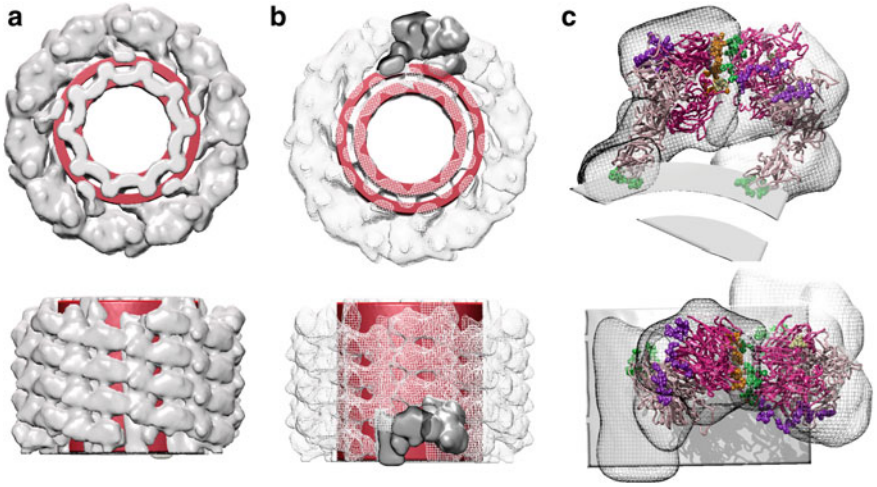


Fig. 2.7 Helical reconstruction of pFVIII-LNT. a. Helical reconstruction of porcine FVIII-LNT (light grey) from 10,433 segments at 14.5Å resolution (0.5 FSC) superimposed with the 3D reconstruction of a lipid nanotube (LNT) from 2000 segments (red). The helical parameters: twist – $\Delta\varphi = 35.5^{o}$ and rise - $\Delta z = 36.0$ Å converged after 200 IHRSR cycles. **b**. Segmentation showing the asymmetric unit cell (dark grey). **c**. Rigid body fitting of the FVIII-LNT structure within the EM map of the FVIII dimer. The FVIII-Light chain is shown in light pink and the heavy chain in hot pink ribbons. The FVIII-FVIII dimer interface is shown with green and orange spheres respectively. The FVIII-membrane interface is shown with green spheres and the FVIII-FIXa interface with magenta spheres. The top row shows views along the helical axis and the bottom row, views perpendicular to the helical axis (LNT membrane). The inner diameter of the LNT is 2.0 nm

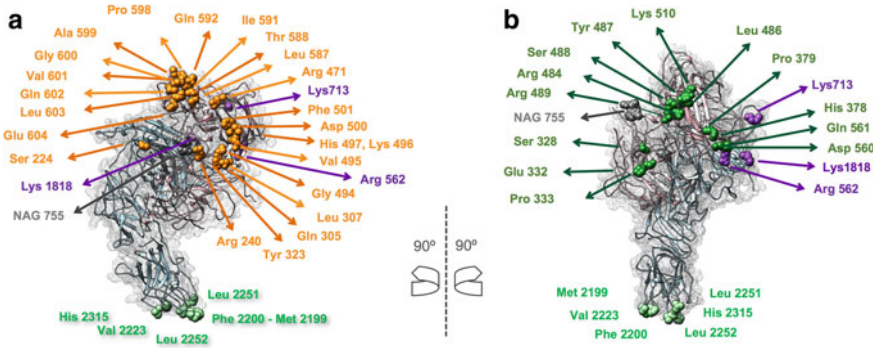


Fig. 2.8 The FVIII dimer interface. The FVIII dimer interface is shown parallel to the membrane surface. **a**. Interface of the monomer on the right in Fig. 2.7c facing the FVIII dimer interface. **b**. Interface facing the monomer on the left in Fig. 2.7c facing the FVIII dimer interface. There are 33 interactions between 22 amino acid residues from the 'left' monomer interface, shown with orange spheres with 13 residues from the 'right' monomer interface, shown as green spheres within a radius less than 7.8 Å. 14 of these residues differ between the human and porcine FVIII sequence, and 8 residues have known Hemophilia A (HA) mutations

(Ahmad, London and Walsh 2003). The extended FVIIIa-FIXa interface consists of four distinctive interaction sites, which have been identified through a number of functional studies assessing the inhibition by peptides, antibodies, direct mutagenesis, specific point mutations and cross-linking studies: (i) FVIIIa-A2 domain (558-565)—FIX-protease domain (helix 330-338) (ii) FVIIIa-A2 domain (707-713)—FIXa-protease domain (helix 301-303), (iii) FVIIIa-A3 domain (1811-1818) with FIXa-EGF1 domain and (iv) FVIIIa-C2 domain (2228-2240) with FIXa-Gla domain (Fig. 2.9) (Fay et al. 1994; Bajaj et al. 2001; Griffiths et al. 2013; Kolkman et al. 1999; Lenting et al. 1996a; Lenting et al. 1996b; Mathur and Bajaj 1999; Blostein et al. 2003; Soeda et al. 2009). Based on this knowledge combined with the resolved FVIII-2D (PDB: 3J2Q), FVIII-3D (3CDZ) and the FIXa (1PFX) structures, two very different models of the Tenase complex have been proposed (Brandstetter et al. 1995; Ngo et al. 2008; Autin et al. 2005; Stoilova-McPhie et al. 2002). In the first model FVIII (3CDZ) binds to FIXa in solution then undergoes a significant domain reorganization, such that all of the FVIII-LC domains interact with the membrane (Ngo et al. 2008) In the second model, FVIII (3J2Q) first undergoes significant domain reorganization upon binding to the membrane in a conformation suitable to bind FIXa

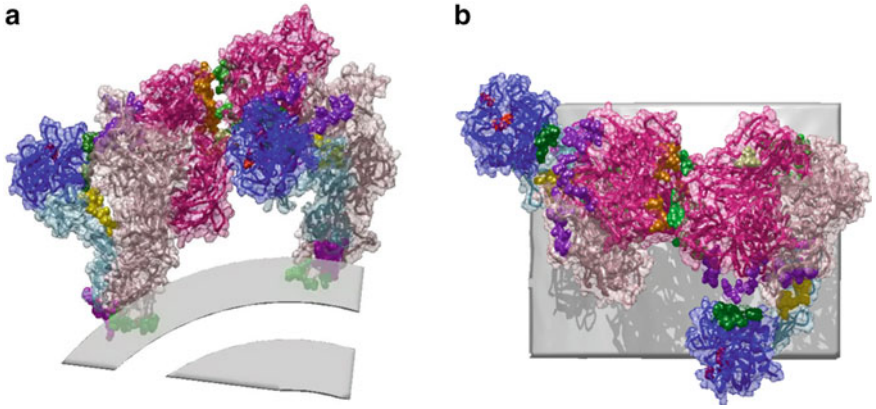


Fig. 2.9 The FVIIIa-FIXa complex. A, B are views along and perpendicular to the LNT surface. The FVIII-light chain is colored in light pink and the heavy chain in hot pink. The residues at the FVIII monomer-monomer interface are shown as green and orange spheres, respectively. The C2 domain membrane-binding residues are shown as light green spheres. The FVIII residues on the A2 and A3 domains identified to bind to the FIXa are shown was purple spheres. The FIXa (1PFX) heavy chain (protease domain) is colored in dark blue and the light chain holding the two EGF like domains in light blue. The FIXa membrane-binding Gla domain is shown as magenta spheres. The Asp39, His57 and Cys195 residues forming the FIXa serine protease active site are shown as red spheres. The identified primary FIXa binding sites for FVIIIa on the Protease domain are shown with green spheres: Lys132-Phe133-Gly134-Ser135 and Pro161-Leu162-Val163-Asp164-Arg165-Ala166-Thr167-Cys168-Leu169-Arg170. The secondary FIXa-FVIIIa interaction sites on the FIXa-FGF domains are shown with yellow spheres: Lys80-Asn81–Glu83-Leu84-Asp85-Ala86-Thr87-Cys88-Asp89-Ile90. The residues from the membrane-binding Gla domain are shown as cyan spheres. The LNT's bilayer structure as defined by cryo-EM is shown as a light grey surface (Stoilova-McPhie, 2017).

in the FVIIIa-FIXa complex (Stoilova-McPhie et al. 2002). Resolving the FVIII and FVIIIa membrane-bound structures as assembled on LNT by cryo-EM will be decisive in building a functional model of the FVIIIa-FIXa complex and understanding the critical role played by FVIII in hemostasis.

The dimeric FVIIIa-FIXa (supertenase) complex was modelled with the membrane-bound porcine FVIII dimer as a template, the porcine FIXa crystal structure (1PFX) and the identified FVIIIa-FIXa interaction sites imposing similar constraints as the ones used for the previous Tenase models. First, the FVIIIa and FIXa molecules were oriented such as to face each other at the appropriate height above the membrane (Mutucumarana et al. 1992). Second, the two molecules were rotated and translated in the membrane plane such as to accommodate the four identifed FVIIIa-FIXa interactions sites and secure that the membrane-binding FIXa-Gla and FVIIIa-C2 domains interact correctly with the lipid bilayer. Third, the FIXa active site (Asp39, His57, Ser195 catalytic triad) needs to be exposed to the substrate—FX.

Further, flexible fitting will be required to refine the model based on subnanometer resolution cryo-EM structures. None of the existing models, however, can accommodate all four FVIIIa-FXa interaction sites. The proposed dimeric model has the advantage that the substrate, FX can be positioned correctly on the membrane surface, such as it is stabilized by interaction with the FVIII-FX binding site identified on the A1 domain and accessible to the FIXa proteolytic/active site. The dimeric/multimeric organization of the tenase and prothrombinase complexes will also justify a structural synchronization of the protein function needed for more than five order of magnitude increase in the proteolytic activity for the respective enzymes (FIXa and FXa).

Structural Basis of FVIII Activation-Future Perspectives

Despite the considerable efforts have been made over the last two decades to define the mechanism of FVIII and FV activation, the structural basis of their co-factor functions is poorly understood. (Mann and Kalafatis 2003; Fay 2004). The active form of FV, FVa, is stable and we have previously obtained 2D crystals from membrane-bound bovine FVa (Stoylova et al. 1994). The active form of FVIII, FVIIIa is much more volatile and unstable for direct structural studies. The porcine FVIII (pFVIII) expressed and activated in our lab had sufficient stability and quantity for functional and structural studies by cryo-EM. The purity and integrity of the pFVIII and pFVIIIa were assessed as described by Lollar et al. (1993). The FVIII and FVIIIa were both helically organized on LNT at the same lipid composition and lipid to protein ratio, as previously described (Miller et al. 2014; Dalm et al. 2015). The current 3D reconstruction of pFVIII-LNT and pFVIIIa-LNT reached 12Å resolution at Fourier Shell Correlation coefficient, FSC = 0.5 (Fig. 2.10). Further segmentation of the helical volumes confirmed the dimeric organization of both porcine FVIII and porcine FVIIIa when bound to the LNT surface. This first membrane-bound FVIIIa structural data confirmed our previous observation from membrane-bound 2D

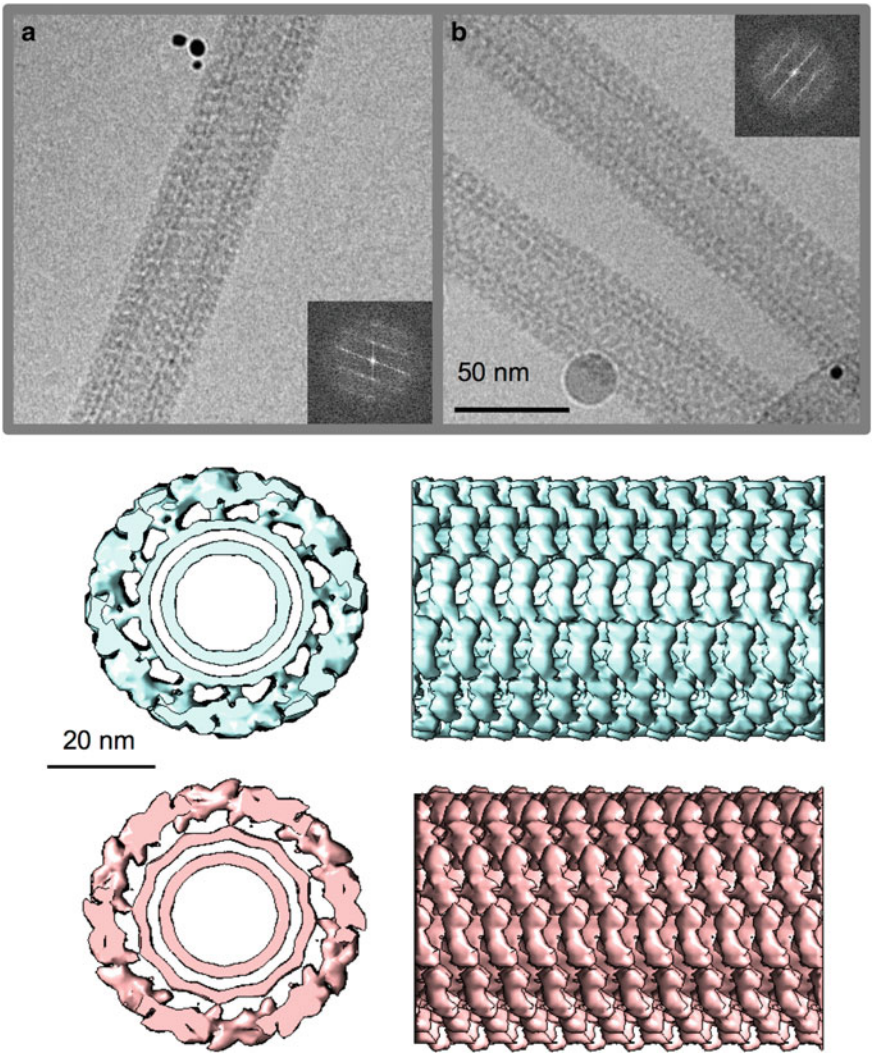


Fig. 2.10 Cryo-EM micrographs of pFVIII (A) and pFVIIIa (B) helically organized on LNT (1024 × 1024 pixels at 1.4 Å/pix resolution). The insets are Fourier transform of the helical tubes (512 × 512 pixels) showing the layer lines corresponding to the helical organization. Helical reconstruction of pFVIII-LNT (in blue) and pFVIII-LNT (in pink) calculated from 951 and 944 helical segments (512 × 512 pixels) respectively, as described in (Dalm et al. 2015)

crystals of plasma derived FVIIIa (unpublished), that there are no significant differences in the oligomeric organization of the proteins after activation. This finding confirmed our hypothesis, that significant domain reorganization of FVIII occurs

upon membrane-binding, not activation. Activation of the pro-cofactor is subtle structurally, optimizing solely the FVIIIa-FIXa interface following proteolysis of FVIII at Arg372.

It is important to note that all crystallographic structural studies of serine protease so far, including FIXa and FXa have been carried out with active site-inhibited constructs lacking the membrane-bounding Gla domain. Our FVIII cyo-EM studies have been focused on the porcine BDD-FVIII construct that has been proven to be more stable than the human analogue. At the same time porcine FVIII has been successfully used for therapies for Hemophilia A, as it has been shown to form active complexes with human FIXa (Lollar et al. 1993). Our working hypothesis for the assembly of the tenase complex is that first the dimeric FVIII-FIXa complex is assembled that requires significant reorganization of the FVIII domain upon membrane-binding followed by dimerization (Dalm et al. 2015). The FVIII activation happens for the membrane-bound FVIII form, restructuring the FVIIIa-FIXa interface to increase singificantly FIXa proteolytic activity (Fig. 2.11).

Conclusions

The prothrombinase complex is the last step of the propagation phase of coagulation impacting thrombin generation and the clotting process as shown in Fig. 2.4 and Table 2.2. For that reason, most pharmaceutical interventions in the last 20 years have been directed towards modulators of the FXa active site. Hence more than 100 crystal structure of FXa with different inhibitors exist in the protein structure data bases (RSCB.org). A finer and refined modulation of the pro-coagulant pathways can be achieved by targeting the co-factors and specifically the FVIIIa/FIXa and the FVIII-FVIII dimer interface within the membrane-bound tenase complex. These studies are however only in an initial stage. Several crystal structures of FVa exist that are not publicly available except for the FVai (Adams et al. 2004), and a structure of a soluble, not membrane-bound prothrombinase complex, has been resolved by X-ray crystallography (Lechtenberg et al. 2013; Csencsits-Smith, Grushin and Stoilova-McPhie 2015). The exact mechanism of the FVa-FXa interaction is not yet fully characterized that makes it difficult to target with novel therapeutics. The situation is more complex with the FIXa and tenase complex, as the FVIIIa form is highly unstable and has not so far been structurally resolved with any of the existing direct structural approaches: X-ray crystallography, cryo-EM and NMR. In our lab, we conducted cryo-EM studies of FVIII bound to nanodiscs and tested our constructs in hemophilic A mice followed by a proposal for nanodrugs based on our design for treatment of Hemophilia A. Present achievement in both the nanotechnology and Cryo-EM fields will soon lead to our full understanding of the FVIII and FV membrane-bound complexes assembly and regulation.

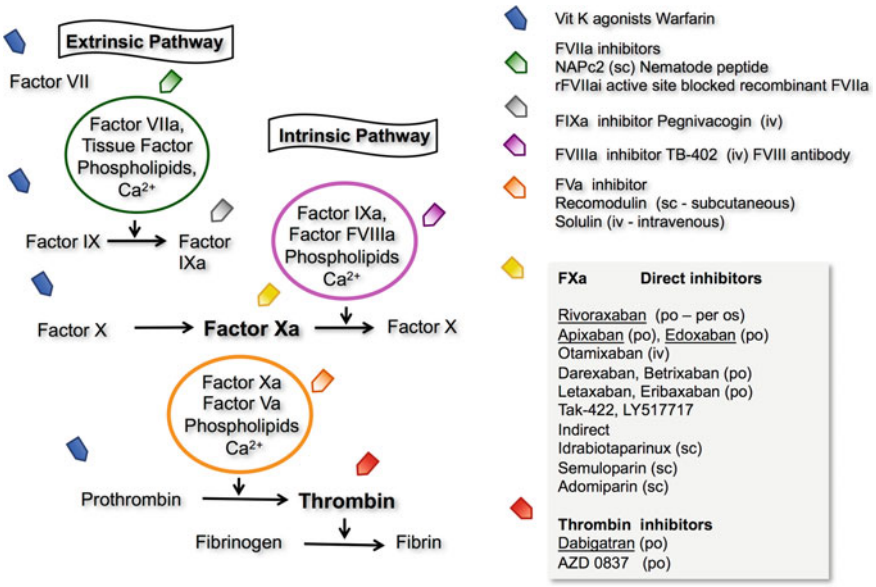


Fig. 2.11 Oral anticoagulant (non-Vitamin K Antagonist, NOAC) targeting the three membranefigound complexes in the pro-coagulant pathways of the clotting process. So far mainly the serine proteases and specifically FXa have been the target for modulation of the coagulation process (Fig. 2.11). These new pharmaceutics, known as oral anticoagulant (po) or non-vitamin K antagonists (NOAC) can inhibit a single protease, are sufficiently small to reach the active site and their binding is reversible. The dosage and side effects of these oral coagulants however, can be severe. Resolving the structural basis and mechanism of FVIII and FV co-factor functions within the membrane-bound tenase and prothrombinase complexes will open avenues for pharmaceuticals interventions at a new level of specificity and efficacy, providing efficient cures for coagulation disorders (Hoffman and Monroe, 2017). In this chapter, we have focused on the FVIII and the tenase (FVIII/FIXa) membrane bound complex assembly and macromolecular organization. The protocols developed for these studies can be extended to the prothrombinase (FVa/FXa) complex and the FVa and FVIIIa anti-coagulant membrane-bound complexes with APC. Resolving the functional structure of the components of these complexes in a membrane environment and at atomic resolution by cryo-EM will help elucidate their function. This will benefit not only drug discovery, but will advance our scientific understanding of structure-function relarionship at the membrane interface. It will open novel avenues for membrane-based nanotechnology designs that can modulate protein activity though the assembly of intricate macromolecular complexes

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