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REVIEW

Molecular defects in coagulation Factor VIII and their impact on Factor VIII function

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Molecular defects in Factor VIII (FVIII), such as haemophilia A-related mutations or denaturative conformational changes, may affect the stability of FVIII as well as its interactions with physiological activators, von Willebrand Factor, phospholipid, or conformationally sensitive antibodies. We summarize the contemporary assays which allow identification of impaired functional interactions of FVIII that cause a reduction or loss of its cofactor activity and/or increased immunogenicity. These assays can potentially be used for detection of molecular defects in FVIII and elucidation of the function impaired by these defects.

Key words: conformational changes, Factor VIII, haemophilia A, mutations, phospholipid binding, surface plasmon resonance.

Structure of Factor VIII and its function in haemostasis

Factor VIII (FVIII) is an essential component of the intrinsic pathway of the blood coagulation cascade where it serves as a cofactor for a serine protease, activated Factor IX (FIXa). Proteolytically activated FVIII (FVIIIa) increases the rate of FIXa-catalysed conversion of Factor X (FX) into its active form (FXa) by several orders of magnitude [1,2]. In turn, FXa participates in the conversion of zymogen prothrombin to thrombin, the key enzyme of the coagulation cascade. Assembly of the FVIIIa/FIXa complex (Xase complex) occurs on phospholipid (PL) membranes predominantly provided by activated platelets at the sites of coagulation [3]. The role of the PL surface is to concentrate components of the Xase complex, direct their interactions from three- to two-dimensional space, and provide the optimal mutual orientation of complex components [4,5].

Initiation of blood coagulation is ascribed to the extrinsic, tissue factor-dependent pathway, in which small amounts of activated FIX and FX are generated, whereas the intrinsic pathway dramatically amplifies the coagulation events triggered by the tissue factor-dependent pathway by catalysing FX activation approximately 50-fold more efficiently [6]. This powerful amplification of the coagulation burst via the FVIII-dependent intrinsic pathway is an ultimate requirement for the normal coagulation process. Deficiency or functional defects in the FVIII molecule result in the most common inherited sex-linked bleeding disorder, haemophilia A, which affects 1 in 5000 males [7]. Based on the residual activity of FVIII in plasma, haemophilia A is categorized as severe (< 1% of normal activity), moderate (1–5%) or mild (5–30%).

The FVIII molecule (~300 000 molecular weight, 2332 amino acid residues) consists of three homologous A domains, two homologous C domains and a unique B domain, which are arranged in the following order: A1–A2–B–A3–C1–C2 (Fig. 1). Prior to its secretion into plasma, FVIII is processed

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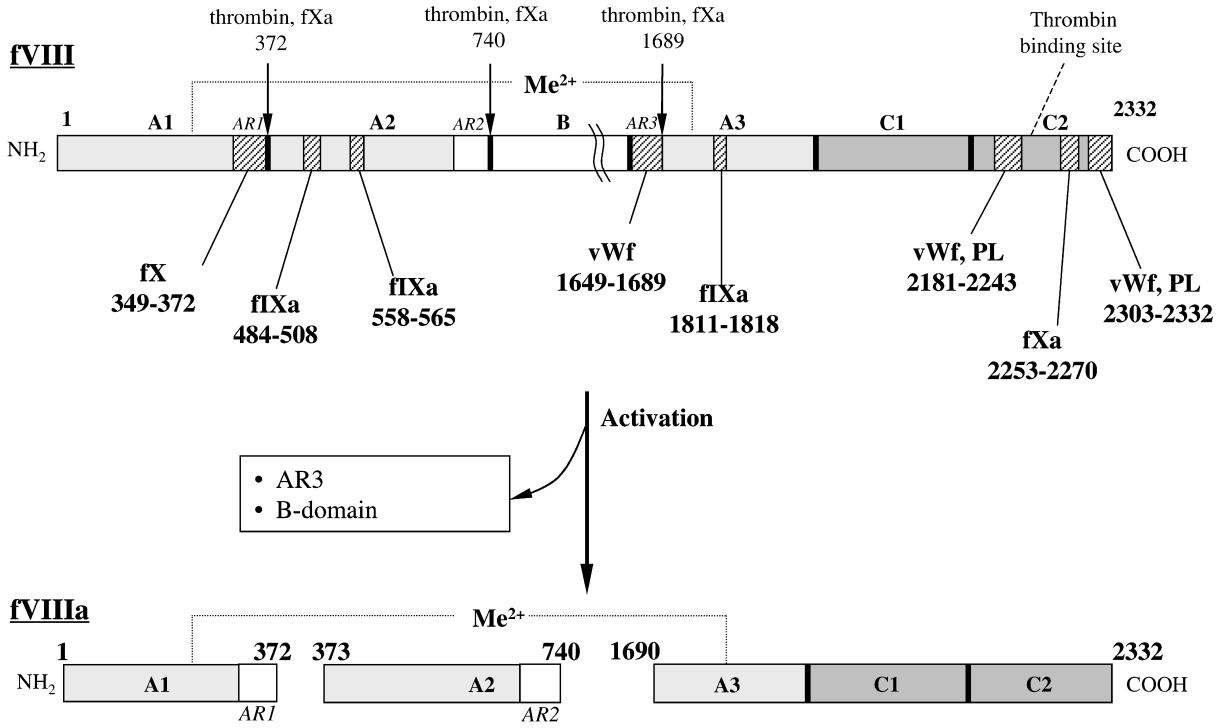


Fig. 1 Factor VIII (FVIII) structure and sites involved in its major interactions. Non-activated FVIII is shown as a multidomain structure, in which A1 and A3 subunits are non-covalently linked via a divalent metal ion bridge (dotted line) and three A domains are flanked by acidic regions (AR1, AR2 and AR3). The regions of FVIII involved in the binding of von Willebrand factor (vWf), activated Factor IX (FIXa), Factor X (FX), activated FX (FXa) and phospholipid (PL) are shown as hatched boxes. Activation of FVIII by cleavage after Arg372, Arg740 and Arg1689 (shown by arrows), by thrombin or FXa, leads to release of the B domain and AR3. Activated FVIII is a heterotrimer consisting of A1, A2 and A3–C1–C2 domains, in which A1 and A3 domains retain the metal ion linkage, and the stable A1/A3–C1–C2 dimer is weakly associated with the A2 subunit through electrostatic interactions.

intracellularly into a series of metal ion-linked heterodimers produced by cleavage at the B–A3 junction and by a number of additional cleavages within the B domain. These cleavages generate the heavy chain (HCh), consisting of the A1 (amino acids 1–372), A2 (amino acids 373–740) and B domains (amino acids 741–1648), and the light chain (LCh), composed of the A3 (amino acids 1690–2019), C1 (amino acids 2020–2172) and C2 (amino acids 2173–2332) domains. The C-terminal portions of the A1 (amino acids 337–372) and A2 (amino acids 711–740) domains and the N-terminal portion of the LCh (amino acids 1649–1689) contain a high number of negatively charged residues and are called acidic regions (AR1, AR2 and AR3, respectively).

Major interactions of Factor VIII in the intrinsic pathway of blood coagulation

Complex formation with von Willebrand Factor (vWf) is required for maintenance of the normal FVIII level in plasma. FVIII binds to vWf with high affinity, K_d ~0·4 nM [8]. The binding to vWf prevents premature formation of the Xase complex prior to activation of FVIII and protects FVIII from inactivation by activated protein C, FIXa and FXa [4]. Three regions within the LCh of FVIII are directly involved in binding to vWf (Fig. 1): AR3 (amino acids 1649–1689); and two C2-domain regions (amino acids 2181–2243, 2303–2332) [8,9]. AR3 not only directly participates in vWf binding, but is also required for maintaining an optimal conformation of the C2 domain for binding to vWf.

At the site of the coagulation event, FVIII is activated by two major physiological activators – thrombin and FXa. Both proteases cleave FVIII after Arg372 and Arg740 within the HCh, and after Arg1689 within the LCh, producing A1, A2, and A3–C1–C2 fragments, generating heterotrimeric activated FVIII (FVIIIa) (Fig. 1). The cleavage after Arg1689 results in removal of AR3 and release of FVIIIa from vWf [8]. In addition, this cleavage is required for generation of the maximal cofactor activity of FVIIIa [9]. An important thrombin-binding site responsible for thrombin-catalysed cleavage after Arg1689 in the LCh is assigned to the C2 domain of FVIII, although this site has not yet been precisely mapped [10]. The FXa-binding site was localized to the C2-domain amino acids 2253–2270, based on the inhibition of the FXa/FVIII interaction by overlapping synthetic peptides [11].

In the assembled intrinsic Xase complex, the cofactor activity of FVIIIa is provided by its three essential interactions:

with PL membranes; with the enzyme FIXa; and with the substrate FX. Regarding the interaction of FVIII with PL, the C2 domain of the LCh of FVIII is entirely responsible for its high-affinity binding [12,13]. Two PL-binding sites were mapped to the C2 amino acids 2181–2243 and 2303–2332, which overlap with vWf-binding sites [13–15]. Resolution of the crystal structure of the human FVIII C2 domain revealed the presence of three hydrophobic 'feet' formed by the side-chains of Met2199/Phe2200, Val2223 and Leu2251/Leu2252, which are proposed to penetrate the membrane bilayer [16,17]. Additionally, four basic amino acids (Arg2215, Arg2220, Lys2227 and Lys2249), which lie underneath the 'feet', may stabilize FVIII/PL binding by electrostatic interaction with negatively charged PL. The predictions based on the X-ray structure of the C2 domain were confirmed by the demonstration that Ala mutations of the amino acids comprising the hydrophobic 'feet' – Met2199/Phe2200 and Leu2251/Leu2252 – dramatically reduced the affinity of FVIII for PL [18]. It is noteworthy that, in parallel with their effect on the FVIII/PL interaction, these mutations led to a reduction of FVIII affinity for vWf, indicating that two hydrophobic 'feet' are also important contributors in the FVIII interaction with vWf.

The high-affinity interaction between FVIIIa and FIXa (K_d ~15 nM) is mainly provided by amino acids 1811–1818 within the A3 domain of the LCh of FVIII [19]. The A2 domain of the HCh also interacts directly with FIXa via two regions: 558–565 [20] and 484–508 [21]. Interestingly, these A2 sites become exposed only upon cleavage at Arg372 within the HCh by either thrombin or FXa [22]. Although the affinity of the isolated A2 subunit for FIXa is rather low (K_d ~300 nM), this interaction defines the cofactor activity of FVIIIa, as the A2 domain amplifies the enzymatic activity of FIXa by modulating its active site [23]. Involvement of the A2 and the LCh sites in FVIIIa interaction with FIXa stabilizes FVIIIa within the Xase complex, where FIXa serves as a bridge linking the A2 subunit and the A1/A3–C1–C2 heterodimer [5]. This stabilization is critical, considering the short half-life of isolated or membrane-bound FVIIIa (2·1 min) owing to spontaneous dissociation of the A2 subunit [24,25]. Interaction of FVIIIa with the substrate, FX, in the assembled Xase complex occurs via amino acids 336–372 of AR1 (Fig. 1) [26,27].

Therefore, the normal functioning of FVIII requires it to interact with physiologically important ligands, including vWf, PL, FIXa, FX, thrombin and FXa. Molecular defects in FVIII, such as haemophilia A-related mutations or denaturative conformational changes potentially occurring in the process of FVIII manufacture, may affect one or more of these key FVIII interactions. Below, we summarize the contemporary assays allowing identification of impaired functional interactions of FVIII that cause a reduction or loss of its cofactor activity and/or lead to an increased immunogenicity. These assays can potentially be used for detection of molecular defects in FVIII and elucidation of the impaired function.

Detection of conformational changes in the C2 domain

Physiological changes within the C2 domain upon FVIII activation have been previously reported [8,12,28]. These changes occur upon removal of AR3 and lead to a 10-fold increase in the affinity of FVIIIa binding to PL membranes [12]. This was measured in a surface plasmon resonance (SPR)-based assay using either a phosphatidylserine (PS)/phosphatidylcholine (PC) (25/75) monolayer formed on a hydrophobic alkanethiol-coated HPA biosensor chip (Biacore, Uppsala, Sweden) or immobilized intact vesicles PS/PC/phosphatidylethanolamine (PE) (4/76/20) with a composition close to that of membranes of activated platelets [12]. While immobilized intact vesicles represent a good model of physiological membranes in their FVIII-binding properties, they do not withstand repetitive binding-regeneration steps in a SPR-based assay [29]. An improved SPR-based assay was developed recently. This assay employs a flexible PL bilayer formed on the surface of a biosensor chip (L1) coated with lipophilically modified dextran [30]. The FVIII-binding properties of the bilayer formed on the L1 chip proved to be identical to those of intact vesicles, and this PL surface is stable during repeated binding/regeneration cycles, which makes this assay superior in comparison to that employing an HPA chip.

SPR-based FVIII-PL binding assays employing PS/PC monolayers formed on HPA chips have been extensively used for characterization of the conformational status of the C2 domain of FVIII in the course of the industrial manufacture of therapeutic FVIII concentrates [31–33]. The necessity of controlling the conformational status of the C2 domain emerged from the reported abnormally high formation of inhibitory anti-FVIII antibodies with C2-domain specificity, which developed in patients with haemophilia A who were treated with FVIII concentrates prepared from plasma pools with elevated levels of coagulation markers [34,35]. It was demonstrated that samples of FVIII concentrates associated with an increased immunogenicity had altered ability to bind to an HPA-supported PS/PC monolayer [31,33]. The above studies suggest that SPR-based FVIII/PL-binding assays can be used as a criterion for evaluation of the conformational status of the C2 domain.

As the PL-binding sites within the C2 domain of FVIII overlap with the binding sites for vWf [36], and the binding to vWf is strongly dependent on the conformation of the C2 domain [8], testing of FVIII binding to vWf is another approach for investigating the conformation of C2. The SPR-based assay for measuring kinetics of FVIII binding to vWf has been described previously and was applied to

confirm the identity of the properties of recombinant B-domain-deleted FVIII and its plasma-derived analogue [8,37]. An attractive microassay for quantitative measurement of FVIII binding to either PL vesicles or vWf has been recently reported [18]. In this assay, FVIII is preconcentrated and purified from contaminants and vWf on beads with immobilized anti-FVIII antibody from small amounts of unpurified material with a low concentration of FVIII. Subsequently, the binding of immobilized FVIII to fluorescently labelled PL vesicles or fluorescently labelled vWf can be accurately quantified by flow cytometry. This assay has been successfully applied for characterization of vWf and PL binding of FVIII mutants expressed at low levels [18]. It can also be potentially used for characterization of the FVIII component in therapeutic FVIII concentrates without preliminary FVIII isolation by conventional methods.

Another approach to detect conformational changes within the C2 domain employs conformationally sensitive anti-C2 domain antibodies as probes in SPR-based [8,28] or quantitative immunoprecipitation assays [33]. In SPR-based assays, conformationally sensitive monoclonal anti-C2 domain antibodies, NMC-VIII/5 (epitope within the C2 domain amino acids 2170–2327 [38]) or ESH8 (epitope 2248–2285 [39]), were covalently immobilized on a carboxymethyldextran-coated biosensor chip via amino groups [8,28]. The physiologically relevant conformational changes within the C2 domain, induced by FVIII activation, were detected with this assay, as evidenced by a difference of approximately 10-fold between the affinities of non-activated and activated FVIII derivatives for immobilized antibody [8,28]. These SPR-based assays may appear applicable for evaluating the C2 conformational status in therapeutic FVIII concentrates. Indeed, the use of NMC-VIII/5 antibody proved to be effective in probing the C2 conformation by a competitive immunoprecipitation assay in samples of FVIII concentrates prepared from abnormal plasma pools [33]. As samples of FVIII concentrates associated with increased immunogenicity demonstrated a reduced binding to NMC-VIII/5 antibody and also had an impaired binding to the PS/PC monolayer, probing of the C2 conformational status with conformationally sensitive antibodies is another useful, practical criterion [33]. The described approaches should be used in combination with the full spectrum of high-resolution analytical techniques, including size-exclusion chromatography, one- and two-dimensional electrophoretic analysis, analysis of protease peptide maps and immunochemical tests, comprehensively summarized in several publications [40–42].

Detection of molecular defects impairing Factor VIII activation

It was recently reported that the C2 domain of FVIII contains the binding sites for two major FVIII physiological activators – thrombin and FXa [10,11]. As the C2 domain is potentially susceptible to conformational changes during manufacturing procedures, the ability of FVIII to bind thrombin or FXa may also be impaired. This can be tested using recently developed SPR-based assays, in which the binding of FVIII or its derivatives to anhydro-thrombin or anhydro-FXa, immobilized on a carboxymethyldextran-coated Biacore chip (CM5) is measured [10,11]. The catalytically inactive derivatives of thrombin or FXa are prepared by converting the active site serine to dehydroalanine, as described previously [43]. Performance of this binding assay may be helpful in explaining the results reported by Raut *et al.* [31], who found that FVIII concentrate samples with impaired PL binding (indicative of altered C2 conformation) also showed reduced cleavage by thrombin at Arg1689. Considering that cleavage at Arg1689 requires thrombin binding to the C2 domain [11], the reduced thrombin cleavage at Arg1689 may be related to defects in thrombin interaction with the conformationally altered C2 domain of FVIII. The described SPR-based binding assays could complement the classical analysis of FVIII activation by electrophoresis [28,44,45]. While electrophoretic analysis reveals defects in proteolysis of FVIII by thrombin or FXa, the binding assays may reveal a reason for such abnormality.

Another situation when defective activation of FVIII was observed is related to mutations at the thrombin and FXa cleavage sites (amino acids 372 and 1689). Indeed, it was reported that mild-to-moderate haemophilia A is caused by mutations of Arg372 to His [46] or to Cys [47], of Ser373 to Leu [48] or to Pro [49], and of Arg1689 to Cys [50] or to His [51]. While the defects in thrombin cleavage at a given specific site(s) can be detected by conventional electrophoretic analysis, enzyme-linked immunosorbent assay (ELISA) may be beneficial for serial screening of FVIII samples. Such an ELISA has been developed for detecting the defect in thrombin cleavage of FVIII in which Arg1689 was mutated to Cys [52]. This assay uses an anti-C2 domain antibody for FVIII capture, and the thrombin-catalysed cleavage within the LCh is detected with monoclonal antibody NMC-VIII/10 (which recognizes AR3), which is removed upon activation. Although an ELISA for detecting mutations at the other activation site in FVIII (amino acid 372 within the HCh) has not been reported, it can be developed in an analogous manner using a pair of antibodies directed against A1 and A2.

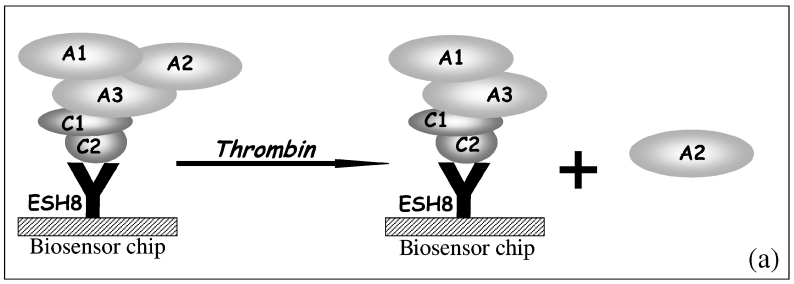
Mutations in A domains increasing instability of activated FVIII

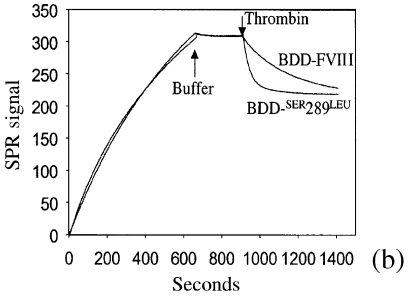
Thrombin-activated FVIII (FVIIIa) is a heterotrimer (A1/A2/A3–C1–C2), in which the A2 subunit is weakly associated with the A1 and A3–C1–C2 subunits via ionic interactions. Retention of A2 is required for normal stability of FVIIIa and dissociation of A2 correlates with FVIIIa inactivation. A phenotype of patients with haemophilia A has been described

whose plasmas showed a discrepancy between their FVIII activities determined in one- and two-stage activity assays [53–55]. In these plasma samples, the FVIII clotting activity determined in a two-stage assay is at least twofold lower than the activity value in a one-stage assay. A critical difference between one- and two-stage assays is that the latter includes a first stage incubation step of several minutes duration. A model of FVIII based on its homology to ceruloplasmin [56,57] predicts that the three A domains of the activated FVIII heterotrimer form a pseudo-threefold axis with interdomain interfaces between A1–A2, A1–A3 and A2–A3. The reported patient mutations with one-stage/two-stage activity discrepancy are all missense point mutations and lie at or near the interdomain axis. Molecular characterization of these mutations demonstrated that their resulting FVIII molecules all have an increased rate of A2 dissociation from the A1/A3–C1–C2 subunits following thrombin cleavage. The mutant FVIIIa, owing to its inherent instability, would therefore exhibit reduced FXa generation in the two-stage assay where there is a prolonged incubation period during the first stage. We developed an SPR-based assay to monitor the real-time kinetics of A2 dissociation upon thrombin activation of FVIII captured by anti-C2 monoclonal antibody ESH8 covalently immobilized on a CM5 chip [58,59]. The principal scheme of the assay is shown in Fig. 2. The A2 dissociation kinetic curves are used to determine the kinetic constant (k_{diss}) and to calculate the half-life of FVIIIa as ln $2/k_{diss}$. This assay was applied to elucidate whether haemophilia A-associated mutations within the A2 subunit result in an increased instability of FVIIIa owing to a faster dissociation of A2. This assay allows determinations to be performed without preliminary purification of FVIII and, because the capturing ESH8 antibody binds to the FVIII/vWf complex [28], testing of plasma samples can also be performed. In addition, this assay can be used to verify that interactions between A domains within the FVIII molecule are not altered by manufacturing procedures.

Detection of limiting concentration of FVIII by advanced clotting assay

The routine assays for measuring FVIII activity do not allow determination of less than 0·01 IU/ml of FVIII (1% of normal level). However, some molecular defects of FVIII frequently result in very low levels of FVIII (less than 1% of normal level), as in haemophilia A patients with a severe form of the disease. Recently, a highly sensitive one-stage clotting assay for serial sample analysis of very low levels of FVIII activity has been developed [60]. While this assay is principally similar to the conventional one-stage activated partial thromboplastin time clotting analysis, it measures the clotting time, not by arbitrary decrease in light transmission, but by quantitative waveform analysis of the clotting kinetics registered in an automated coagulation analyser. This device registers the complete kinetics of decrease in light transmission during the clotting event, calculates the second derivatives at each time-point and determines the end-point of the clotting event (clotting time) as the time at which the second derivative is minimal. A significantly higher precision in determination of the clotting time allows determination of approximately 10-fold lower FVIII concentrations in comparison with the conventional clotting analysis. A higher sensitivity of the





Mutation	Domain	Reference
Arg531His*	A2	[59]
Ala284Glu*	A1	[59]
Ser289Leu*	A1	[59], [62]
Glu272Lys	A1	[62]
His1755Tyr	A3	[62]
His1954Leu	A3	[62]
Arg1966Gln	A3	[62]
Glu1682Lys	AR3	[62]
Arg698Leu*	A2	[63]
Arg698Trp*	A2	[63]
Cys329Ser*	A1	[64]
Gly1948Asp*	A3	[64]

(c)

Fig. 2 Assessment of the stability of activated Factor VIII (FVIII). Panel (a) shows the general scheme of a surface plasmon resonance (SPR)-based assay for monitoring A2 dissociation upon activation of FVIII. FVIII is captured by anti-C2 domain monoclonal antibody ESH8, covalently immobilized on a biosensor chip. Following FVIII activation by thrombin, the kinetics of A2 dissociation is measured by SPR. Panel (b) shows typical kinetic curves of FVIII binding to immobilized monoclonal antibody ESH8, and real-time kinetics of A2 dissociation upon thrombin activation of the reference B domain-deleted FVIII (BDD-FVIII) and FVIII with an A1 mutation, which results in reduced retention of the A2 subunit. The curves are reproduced from Pipe *et al.* [59]. Panel (c), haemophilia A-related mutations on the interface of the A domains of FVIII, which lead to increased instability of FVIIIa. For FVIII mutants designated by a star, measurements of A2 dissociation were performed in SPR-based assay, as described above for panel (b).

newly developed assay makes it advantageous in monitoring FVIII levels in patients with severe form of haemophilia A. This assay may also be useful in rigorous monitoring of the progress of haemophilia A gene therapy in animal models, and potentially in humans, as well as in detecting low levels of recombinant haemophilia A-related FVIII C2-domain mutants characterized by defective trafficking [18,61]. In addition, deviation of the clotting kinetics of a FVIII sample of interest from that of the reference FVIII sample may serve as an indicator of molecular defects in the FVIII molecule.

Concluding remarks

The normal functions of FVIII require its interactions with physiologically important ligands including vWf, PL, FIXa, FX, thrombin and FXa. Molecular defects in FVIII, such as haemophilia A-related mutations or denaturative conformational changes potentially induced in the process of FVIII manufacturing, may affect one or more of these key FVIII interactions. SPR-based assays employing immobilized PL surfaces, immobilized vWf or immobilized conformationally sensitive antibodies, allow evaluation of the conformational status of the C2 domain of FVIII. The latter is important, as conformational alterations within the C2 domain are probably related to an increased immunogenicity of FVIII products. The recently developed microassay using FVIII immobilized on anti-FVIII antibody-coated beads, allows the testing of FVIII binding to PL vesicles or vWf in experimental samples with a low FVIII concentration, without preliminary purification. SPR-based assays employing immobilized anhydro-thrombin or anhydro-FXa can be used for testing FVIII interaction with its two major physiological activators, thrombin and FXa. As both proteases bind to the C2 domain of FVIII, their impaired binding can be an additional indication of the conformational changes within the C2 domain. We also described an SPR-based assay for assessing the stability of activated FVIII by measuring dissociation of the A2 subunit from the FVIIIa heterotrimer (A1/A2/A3–C1–C2). The recently developed improved automated one-stage clotting assay based on the waveform analysis of the clotting event provides an approximately 10-fold higher sensitivity in measuring FVIII activity and is advantageous for monitoring FVIII levels in patients with a severe form of haemophilia A or in experimental samples with very low FVIII concentrations. In summary, the described assays are potentially useful in detecting molecular defects in FVIII and for the elucidation of impaired FVIII function. These assays can complement a wide spectrum of high-resolution analytical techniques, such as chromatographic and electrophoretic analysis, analysis of proteolytic peptide maps, and immunochemical tests classically used for characterization of FVIII preparations.

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