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Topology of factor VIII bound to phosphatidylserine-containing model membranes

Vivek S. Purohit[a], Karthik Ramani[a], Ramesh S. Kashi[b], Manzer J. Durrani[b], Timothy J. Kreiger[b], Sathyamangalam V. Balasubramanian[a,*]

[a] The Department of Pharmaceutical Sciences, University at Buffalo, State University of New York, 541 Cooke Hall, Amherst, NY 14260-1200, USA
[b] Baxter Healthcare Corporation, Bioscience Division, Duarte, CA 91010-2923, USA

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Abstract

Factor VIII (FVIII), a plasma glycoprotein, is an essential cofactor in the blood coagulation cascade. It is a multidomain protein, known to bind to phosphatidylserine (PS)-containing membranes. Based on X-ray and electron crystallography data, binding of FVIII to PS-containing membranes has been proposed to occur only via the C2 domain. Based on these models, the molecular topology of membrane-bound FVIII can be envisioned as one in which only a small fraction of the protein interacts with the membrane, whereas the majority of the molecule is exposed to an aqueous milieu. We have investigated the topology of the membrane-bound FVIII using biophysical and biochemical techniques. Circular dichroism (CD) and fluorescence studies indicate no significant changes in the secondary and tertiary structure of FVIII associated with the membranes. Acrylamide quenching studies show that the protein is predominantly present on the surface of the membrane, exposed to the aqueous milieu. The light scattering and electron microscopy studies indicate the absence of vesicle aggregation and fusion. Binding studies with antibodies directed against specific epitopes in the A1, A2 and C2 domains suggest that FVIII binds to the membrane primarily via C2 domain including the specific phospholipid binding epitope (2303–2332) and may involve subtle conformational changes in this epitope region.
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1. Introduction

Factor VIII (FVIII) is an important plasma glycoprotein involved in the blood coagulation cascade. Deficiency of FVIII causes hemophilia A, an X-linked genetic bleeding disorder [1]. FVIII is a heterodimeric, multidomain protein of approximately 280,000 Da molecular weight consisting of a heavy chain and a light chain (Fig. 1a). It is synthesized as a 2351-residue single chain precursor protein consisting of A1–A2–B–A3–C1–C2 domains. A 19-residue signaling peptide is cleaved prior to the translocation of the protein into the endoplasmic reticulum. The protein is further cleaved to form the heavy chain (A1–A2) and light chain (A3–C1–C2) in the Golgi [2–5]. The cleaved protein consists of multiple polypeptides with molecular weight ranging from 70 to 230 kDa. The amino acid sequence indicates that 230 and 70 kDa peptides represent the carboxy (heavy chain) and amino (light chain) terminals, respectively (Fig. 1).

In the coagulation cascade, FVIII upon activation by thrombin, forms activated FVIII (FVIIIa) in which the FVIII molecule is cleaved into heavy (A1–A2 domains) and light (A3–C1–C2 domains) chains. Both FVIII and FVIIIa are known to bind to phosphatidylserine (PS)-rich membranes of the platelets via the C2 domain [6,7] with similar binding characteristics [8], but FVIIIa displays higher affinity [9,10].

The structure of the C2 domain has been solved by X-ray crystallography where two hydrophobic beta hairpin

Abbreviations: FVIII, factor VIII; PS, phosphatidylserine; DMPC, dimyristoyl phosphatidylcholine; PBT, phosphate buffer with Tween 20; PB, phosphate buffer; CD, circular dichroism; aPTT, activated partial thromboplastin time; TB, Tris buffer

* Corresponding author. Tel.: +1-716-645-2842x245; fax: +1-716-645-3693.

E-mail address: svb@acsu.buffalo.edu (S.V. Balasubramanian).

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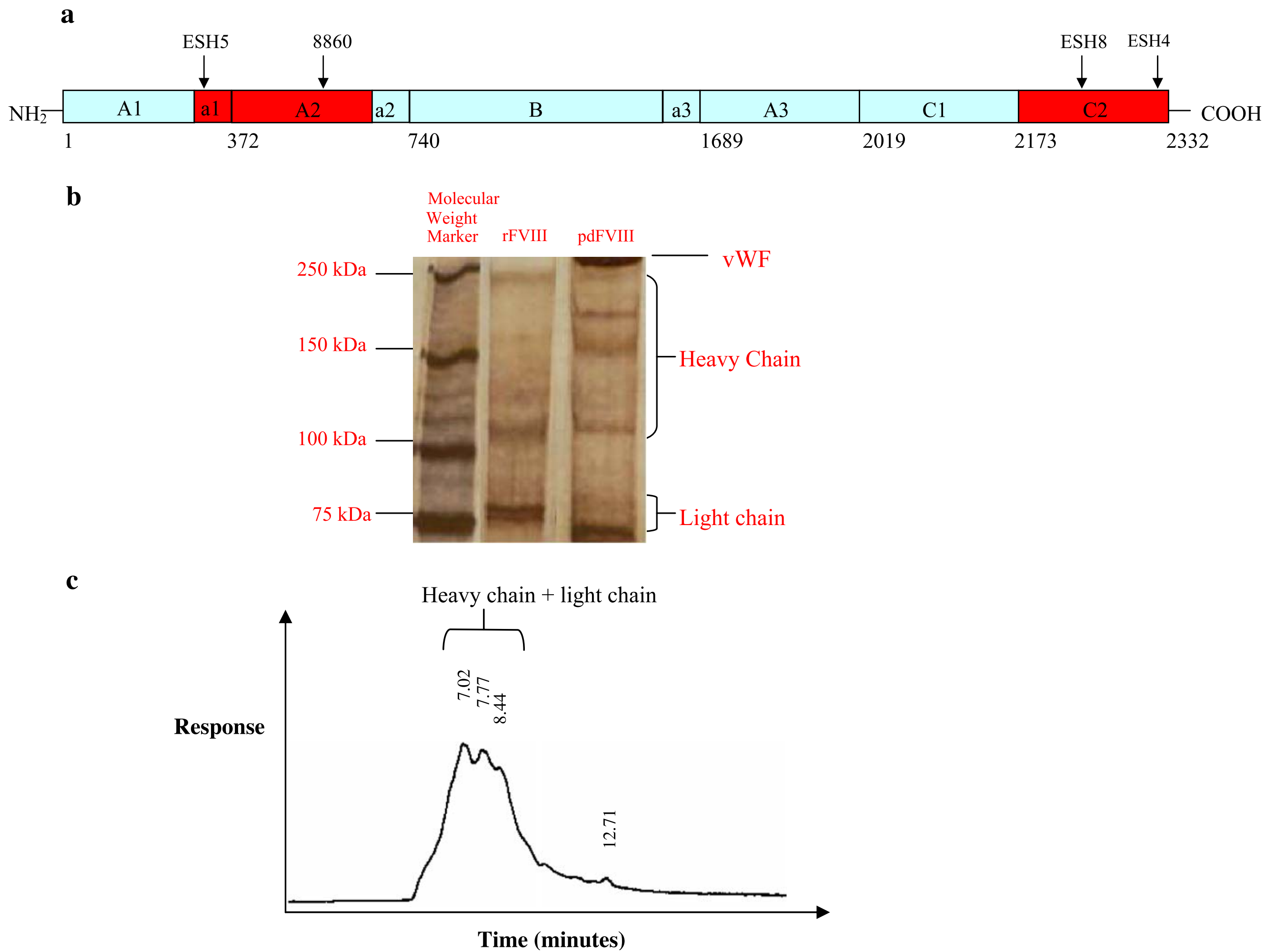

Fig. 1. (a) Schematic representation of the domain structure of FVIII and the monoclonal antibodies recognizing different epitopes. (b) SDS-PAGE of silver-stained plasma-derived FVIII (pdFVIII) and recombinant FVIII (rFVIII). (c) Size exclusion chromatogram of recombinant FVIII. vWF—von Willebrand factor.

turns (Met2199–Phe2200 and Leu2251–Leu2252) have been proposed as the membrane binding epitopes [11]. Site-directed mutagenesis studies have confirmed the involvement of the above four amino acid residues [6]. Recently, based on 1.5-nm resolution, 2-D electron crystallography studies, it has been proposed that four hydrophobic loops in the C2 domain (2222–2227, 2196–2201, 2313–2315 and 2249–2255) are involved in binding to membranes [8]. In this model, the involvement of 2313–2315 has been suggested in addition to two hydrophobic hairpin turns proposed by Pratt et al. [11]. Further, this model suggests that a major part of the protein is not involved in the interaction with the membranes (Fig. 2). Since the binding of FVIII to activated platelet surfaces is essential for its activity [12], understanding the conformation and topology of FVIII bound to membranes would shed light on the structure–function relationship of this multidomain protein. In order to investigate the molecular topology of FVIII bound to membranes, we utilized biophysical and biochemical techniques and the results suggest that FVIII binds to PS-containing membranes primarily via the C2 domain with only subtle changes in the conformation of the protein.

2. Materials and methods

2.1. Materials

The monoclonal antibodies ESH4, ESH5 and ESH8 were obtained from American Diagnostica (Greenwich, CT) and 8860 was a gift from Baxter (Duarte, CA). Avidin alkaline phosphatase conjugate and IgG-free bovine serum albumin was obtained from Sigma (Saint Louis, MO). Recombinant FVIII was expressed either in Chinese hamster ovary (CHO) cell line or in Cos-7 cells and was chromatographically purified as described previously [13–16]. The protein was characterized by SDS-PAGE (Fig. 1b) and high performance size exclusion chromatography (HP-SEC) (Fig. 1c). The recombinant protein thus obtained is similar to the plasma-derived FVIII as shown in Fig. 1b, where both display multiple polypeptide bands in the range of 230–70 kDa

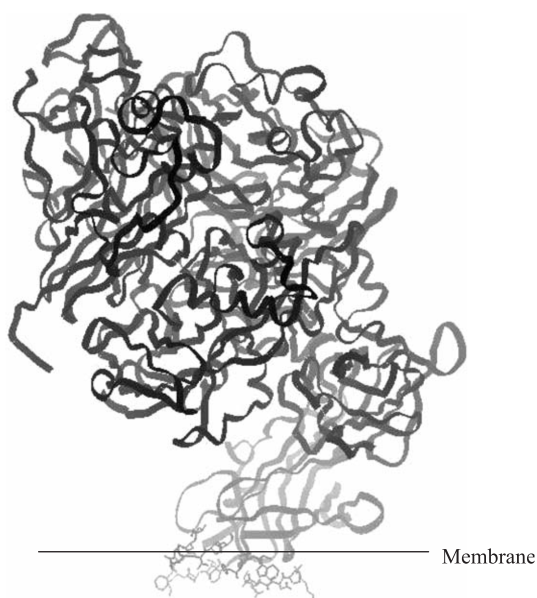

Fig. 2. The proposed molecular model for membrane-bound FVIII. The figure was reconstructed using Rasmol (Ver. 2.7.2.1) based on the coordinates available at http://europium.csc.mrc.ac.uk.

[17]. Dimyristoyl phosphatidylcholine (DMPC) and brain phosphatidylserine (BPS) were obtained from Avanti Polar Lipids (Alabaster, AL) and were used without further purification. *p*-Nitrophenyl phosphate disodium salt was obtained from Pierce (Rockford, IL). All other buffer salts and solvents used in the study were obtained from Fisher Scientific (Fairlawn, NJ) and were used without further purification.

2.2. Biological activity assay of FVIII

The clotting activity was determined by one-stage activated partial thromboplastin time (aPTT) assay using micronized silica as activator (Organon Teknika Corporation, Durham, NC) and FVIII-deficient plasma (Biopool International, Ventura, CA) as the substrate [18]. The assay was performed using COAG-A-MATE coagulation analyzer (Organon Teknika). FVIII was added to FVIII-deficient plasma and the clotting time was monitored. The activity and concentration of the protein was obtained using calibration curve generated using lyophilized reference standards (6th International Standard, NIBSC, Hertfordshire, UK).

2.3. SDS-PAGE and HP-SEC

Silver-stained sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels (SDS-PAGE) were run according to the procedure of Laemmli (Biorad, Hercules, CA) [19]. HP-SEC was performed using Biosep SEC S4000 4.6 × 300 mm analytical column. Chromatograph comprised of a Waters 510 HPLC Pump, Rheodyne injector with a 50-μl sample loop and Hitachi F1050 fluorescence detector. Excitation and emission were set at 285 and 330 nm, respectively, to monitor the elution of the protein. Gel filtration was carried out under isocratic conditions at a flow rate of 0.4 ml/min using an aqueous buffer consisting of 5 mM Tris, 5 mM $CaCl_2$ and 300 mM NaCl, pH 7.4.

2.4. Preparation of membrane-associated FVIII

Membrane vesicles were prepared by rehydrating a thin film of appropriate molar ratios of DMPC and BPS (70:30) with Tris buffer (TB) (25 mM Tris, 5 mM $CaCl_2$ and 300 mM NaCl, pH = 7.0). The thin film was prepared by mixing DMPC and BPS in chloroform in a small round bottom flask or kimax tube and evaporating the solvent in a Rotaevaporator (Buchi R-200, Fischer Scientific, NJ). The membrane vesicles were sized by extruding through a 200-nm cutoff polycarbonate membrane in a high-pressure extruder (Lipex Biomembranes Inc., Vancouver, Canada) at a pressure of ∼ 250 psi. The sized membrane vesicles were associated with FVIII (150 μg) by incubating at 37 °C with gentle swirling for ∼ 30 min. The molar ratio between the lipid and the protein was maintained at 10,000:1 (5.35 mM: 0.535 μM) for all experiments, respectively. Use of sized membrane vesicles with a fixed size to charge ratio ensured reproducible association of FVIII to membrane vesicles. In this isolated system, it is assumed that the location of the protein on the inner leaflet of the vesicles is minimal. The membrane-associated FVIII was analyzed immediately or within 24 h after storage at 4 °C.

2.5. Separation of free protein from membrane-bound

The free protein was separated from the membrane-bound protein by dextran density gradient ultracentrifugation [20]. Briefly, the membrane vesicle/protein mixture (0.5ml) was mixed with 1 ml of 20% (w/v) dextran in TB and placed in a 5 ml polypropylene tube with 3 ml of 10% (w/v) dextran, followed by 0.5 ml buffer (0% dextran) layered over it. The gradient column was ultracentrifuged at 45,000 rpm for 30 min in Beckman SW 50.1 rotor. The membrane-associated protein floated to the interface of 0% dextran buffer and 10% dextran layer leaving the unassociated protein in the bottom dextran layer. The percentage of activity associated with the membranes was determined by the one-stage aPTT method [18].

2.6. Determination of particle size of membrane vesicles

Particle size of the membrane vesicles were determined by dynamic light scattering using a Nicomp Model CW 380 particle size analyzer (Particle Sizing Systems, Santa Barbara, CA) both before and after association with the protein. The instrument was calibrated using 0.258-μm standard latex beads. All measurements were carried out at a temperature of 23 °C with viscosity and refraction

index set at 0.933 cP and 1.333, respectively. Data was fitted to an intensity weighted Gaussian curve to determine the distribution.

2.7. Circular dichroism (CD) studies

CD spectra were acquired on a JASCO-715 spectropolarimeter calibrated with d-10 camphor sulfonic acid. Samples were scanned in the range of 255–208 nm for secondary structural analysis using a 2 mm quartz cuvette and typically, the protein concentration used was 20–22 μg/ml. CD spectra of the protein in the presence and absence of membrane vesicles were corrected by subtracting the spectra of buffer and protein-free liposomes. Multiple scans were acquired and averaged to improve the signal quality.

2.8. Fluorescence spectroscopy and acrylamide quenching

Emission spectra of FVIII and membrane-associated FVIII were obtained using PTI fluorometer (QuantaMaster, Photon Technology International, Lawrenceville, NJ). The samples were excited at 280 nm and the emission spectrum was obtained from 300 to 400 nm. A slit width of 4 nm was used on both the excitation and emission paths.

For fluorescence quenching experiments, the samples were excited at 280 nm and the emission was monitored at 335 nm for FVIII and 333 nm for membrane-bound FVIII. A small shoulder observed at ~ 310 nm in the spectrum of membrane-associated FVIII was due to Raman scattering. This was confirmed by exciting the sample at 265 nm, which resulted in the disappearance of the shoulder. Studies were carried out at 20 °C using a slit width of 4 nm on the excitation and emission paths. In order to minimize the contribution due to inner filter effect for samples containing acrylamide, an "I-shaped" cuvette with two different path lengths was used to acquire the fluorescence emission spectra [21]. The protein concentration was typically 5 μg/ml in TB. Quenching was monitored following successive addition of aliquots of 5 M acrylamide stock solution. The data were analyzed according to the classical Stern–Volmer relationship [21].

2.9. Sandwich ELISA

Sandwich ELISA utilizes an immobilized antibody against a specific epitope as a capture antibody and a different, non-overlapping antibody as the probe antibody, which is biotinylated. The probe antibody recognizes epitopes that are different from capture antibody. The advantages of the sandwich ELISA over simple antibody capture ELISA is that this technique allows both the membrane-bound and the free protein to be in solution rather than bound to surface of the ELISA plate. In the latter case, membrane-bound FVIII may have different binding characteristics compared to free FVIII and the amount of FVIII localized on the plate can be a confounding variable. The sandwich ELISA assumes that the protein–membrane interaction is stronger than antibody-protein binding.

Nunc-Maxisorb 96-well plates were coated with ESH4/8860 antibody by incubating 50 μl/well solution of the antibody at a concentration of 5 μg/ml in carbonate buffer (0.2 M, pH = 9.4) overnight at 4 °C. The plate was then washed 10 times with 100 μl of phosphate buffer containing 0.05% Tween 20 (PBT consisting of 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, 0.14 mM NaCl, 2.7 mM KCl, and 0.02% NaN_3). The remaining nonspecific protein binding sites on the adsorptive surface of the plastic were blocked by incubating 200 μl of blocking buffer consisting of 1% bovine serum albumin in phosphate buffer (PB consisting of 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, 0.14 mM NaCl, and 2.7 mM KCl) for 2 h at room temperature. The plates were washed 10 times with PBT and 50 μl of 100 ng/ml of FVIII or membrane-associated FVIII in blocking buffer was added and incubated at 37 °C for 1 h. The plates were washed 10 times with PBT and incubated with 50 μl of biotinylated ESH8/ESH5 at 1 μg/ml concentration and 50 μl of a 1:1000 dilution of avidin-alkaline phosphatase conjugate, both in blocking buffer at room temperature for 1 h. The plates were washed 10 times with PBT and 100 μl of 1 mg/ml *p*-nitrophenyl phosphate solution in diethanolamine buffer (consisting of 1 M diethanolamine, 0.5 mM $MgCl_2$ and 0.02% NaN_3) was added. The plates were incubated at room temperature for 30 min and the reaction was quenched by adding 100 μl of 3 N NaOH. Absorbance was read at 405 nm using a plate reader.

3. Results and discussions

3.1. Association of FVIII with membranes

The characterization of membrane binding properties of FVIII has been the focus of several studies [22–24]. Bardelle et al. [23] have shown that the binding of FVIII to membrane is biphasic with a surface adsorption step followed by specific binding. The dissociation also occurs in a biphasic manner. It has been shown that the binding of FVIII is dependent on the concentration of lipid [23,25]. In larger vesicles (>200 nm lipid particles) approximately 29 lipid molecules are involved per protein. In vitro binding studies have shown that the binding saturates at a lipid composition of ~ 30% PS [23,24]. Therefore, based on previous studies, the membrane-bound protein was obtained using a lipid composition of DMPC/BPS (70:30) and the protein–lipid mixture was equilibrated for 30 min to promote specific interaction between protein and membrane, as described in Section 2.4. The fraction of protein bound to membranes was separated from the free, and possibly surface-adsorbed FVIII, using gradient centrifugation. It was found that 45.2% (± 16.8, $n = 16$) of the activity was associated with the membrane fraction that floated based on the aPTT and spectroscopic assay. The conformation and

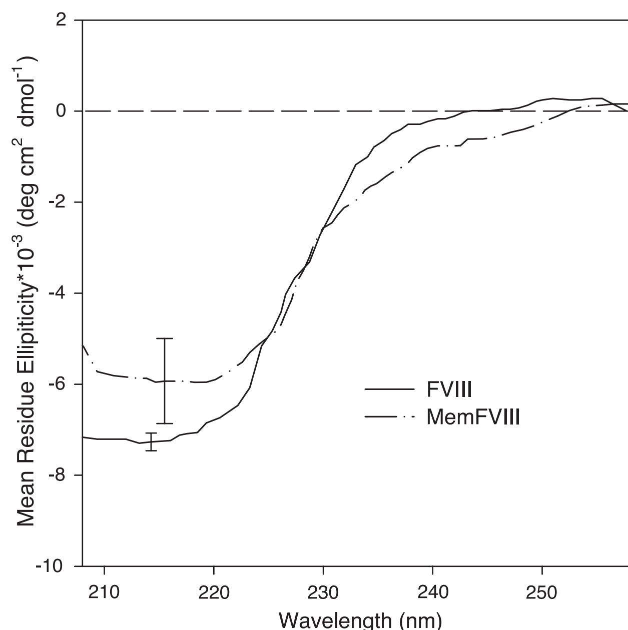

Fig. 3. Secondary structure of FVIII associated with membranes (MemFVIII): the far UV CD spectra of free and membrane-bound FVIII was acquired in the range of 255–208 nm. Typically, the concentration of the protein used was ~ 20–22 μg/ml and the path length of quartz cuvette was 2 mm. The vertical bars indicate the noise level in the spectral measurement.

topology of the protein was carried out using floated fractions of the centrifugation gradient and the concentration of the protein was corrected based on activity assay of the membrane-bound fraction.[1]

3.2. Conformation of membrane-associated FVIII

The secondary and tertiary structural changes of membrane-bound FVIII were studied by CD and fluorescence spectroscopy, respectively.

Far UV CD (255–208 nm) was used to monitor the changes in the secondary structure of the free protein and protein associated with membranes (Fig. 3). The CD spectrum acquired for free FVIII showed a broad negative band at 215 nm, indicating that the protein exists predominantly in the β-sheet conformation. The addition of membrane vesicles did not result in significant changes in the spectral characteristics. Apart from small changes in intensity, a similar broad negative band was also observed for the FVIII bound to membrane that was separated from free protein. Overall, the data suggests that the membrane binding does not result in significant alteration(s) in the secondary structure of the protein. The small decrease in intensity could be due to variation in estimation of concentration of membrane-bound protein based on activity assay. It is known that determination of activity by one-stage aPTT assay is sensitive to phospholipid concentration [26].

Tertiary structural changes in the protein were investigated by fluorescence spectroscopy (Fig. 4). The emission spectrum of membrane-free FVIII showed an emission maximum of 335 nm [27] and the addition of PS-containing membranes resulted in a small blue shift in the emission maxima to 333 nm that is accompanied by a small increase in intensity. If the binding of the protein to membrane vesicles were mediated by substantial intercalation or encapsulation of hydrophobic domains, the spectral properties would indicate a pronounced blue shift associated with a substantial increase in quantum yield. However, modest changes in the emission spectrum indicate that there are only minimal conformational changes and the majority of the protein in the membrane-bound form is in an environment comparable to free protein.

3.3. Acrylamide quenching studies of membrane-bound FVIII

The molecular topology consistent with little conformational change is that the contact between the protein and

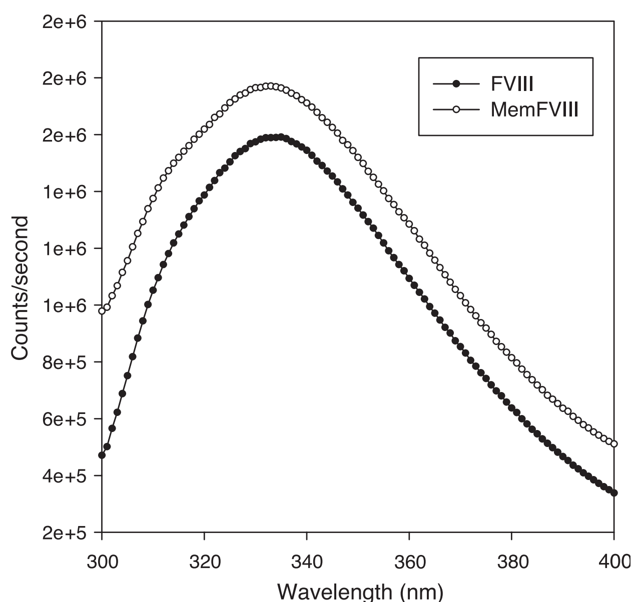

Fig. 4. Tertiary structure of FVIII associated with membrane (MemFVIII): fluorescence emission spectra of free membrane-associated FVIII was acquired in the range of 300–400 nm. The excitation monochromator was set at 280 nm. The protein concentration used was 5 μg/ml.

[1] The centrifugation step may interfere with the membrane binding equilibrium. Centrifugation step depletes the available free protein and possibly the protein that is adsorbed to the membrane surface. The protein-vesicle complex in the floated fraction may reestablish the equilibrium by generating a small fraction of free FVIII. In order to determine the contribution of centrifugation, the biophysical and biochemical studies were carried out before and after the centrifugation step as control. In general, the results indicated that the conformation and topology was similar before and after centrifugation step except that the % of protein associated with the membrane decreased as a result of centrifugation.

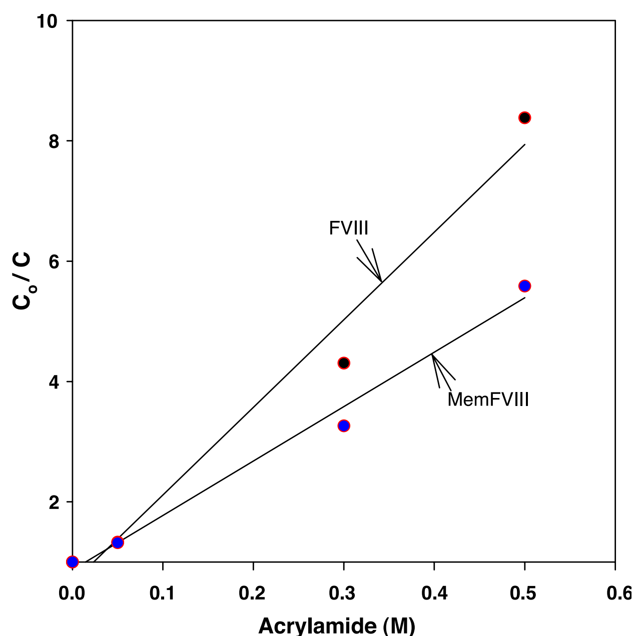

Fig. 5. Acrylamide quenching of FVIII and membrane-bound FVIII (MemFVIII): Stern–Volmer plots were obtained by plotting C_0/C vs. Q. The samples were excited at 280nm and the emission was monitored at 335 nm (FVIII) and 333 nm (MemFVIII). Quenching was achieved by using acrylamide over the concentration range of 0–0.5 M. The protein concentration was typically 5 μg/ml, and an I-shaped cuvette with varying path lengths was used to minimize inner filter effects.

vesicles is mediated by a small fraction of the protein. Based on this observation, it is anticipated that a large fraction of the protein surface is accessible to aqueous medium and membrane provides only modest shielding of the fluorescent residues such as Trp. Therefore, the addition of collisional quenchers would result in comparable loss of Trp fluorescence intensity for both free and membrane-bound FVIII. In order to investigate the accessibility of fluorophores in the protein, collisional quencher acrylamide was added to the membrane-bound FVIII (Fig. 5). As is clear from the figure, the addition of increasing concentration of acrylamide quenches the Trp fluorescence of both free and membrane-bound FVIII. The extent of quenching is slightly lower for the membrane-bound protein, suggesting that only a small fraction of the protein molecule is inaccessible to quenchers. The modified Stern–Volmer plot was used to evaluate the number of Trp residues accessible to collisional quenchers (data not shown). The preliminary data indicates that the fraction of accessible Trp residues decreases by approximately 5–10%. FVIII has 36 Trp residues and the semi-quantitative estimation based on quenching data indicates that probably two to four Trp residues may be inaccessible to the quencher as a result of membrane binding. It is appropriate to mention here that, the above interpretation assumes that all 36 Trp residues spanning the various domains of FVIII contribute equally to the intrinsic fluorescence of the protein.

3.4. Membrane fusion and aggregation

It has been well documented that PS-containing vesicles fuse and aggregate in the presence of Ca^{2+} ions [28]. In order to investigate whether vesicle fusion or aggregation influence the determination of molecular topology of membrane-bound FVIII as our buffer system contained 5 mM Ca^{2+}, we performed dynamic light scattering and electron microscopic studies. The size of the vesicles was measured before and after the addition of protein. The vesicles formed after extrusion showed that the distribution of particle size is Gaussian with a mean particle diameter of 223.73 nm (Table 1). The chi-squared values indicated the particle distribution is homogeneous. The addition of protein did not result in significant change (two-sample t-test, P-value = 0.8785) in the particle size suggesting that membrane fusion and aggregation is minimal. This is further confirmed by negative stain electron microscopic studies (data not shown). The absence of vesicle fusion may be due to the presence of large excess of PC and NaCl and is consistent with the observations of Duzgunes et al. [28]. A critical ratio of 0.35 of Ca bound per molecule of PS (Ca/PS) was reported to be necessary for the Ca^{2+}-mediated destabilization and vesicle fusion. However, this destabilization was found to be dependent on the bulk Na^{+} concentration. High Na^{+} concentration (as is the case here) inhibits Ca^{2+}-induced fusion of PS vesicles by reducing the critical ratio of Ca/PS. Similarly, presence of PC at >50 mol% was also found to interfere with Ca^{2+}-induced destabilization.

3.5. Sandwich ELISA

Based on the models proposed by X-ray [11] and electron diffraction studies [8], it has been proposed that the protein binds to membranes via C2 domain. The spectral studies were consistent with this model in which there are minimal or no conformational changes upon binding of

Table 1
Particle size analysis of PS-containing membrane vesicles in the presence and absence of FVIII

Trial	Membrane vesicles				FVIII-associated membrane vesicles			
	Mean size	Standard deviation	Chi-square	Distribution	Mean size	Standard deviation	Chi-square	Distribution
1	221.2	102.9	0.43	Gaussian	213.5	102.7	2.440	Gaussian
2	225.4	101.4	0.89	Gaussian	266.7	136.8	1.840	Gaussian
3	224.6	94.8	0.59	Gaussian	200.7	83.50	1.861	Gaussian

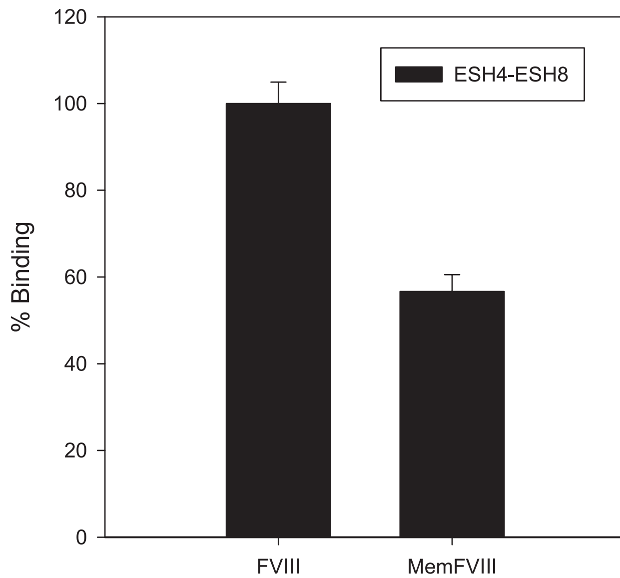

Fig. 6. Antibody binding to FVIII and membrane-associated FVIII (MemFVIII) as determined by sandwich ELISA: binding of ESH4 (C2 domain 2303–2332) and ESH8 (C2 domain 2248–2285).

FVIII to membranes. In order to determine the participation of C2 domain in membrane binding, we performed sandwich ELISA, a functional assay to probe the specific domains and amino acids that participate in membrane interaction (Fig. 6).

Sandwich ELISA is a screening technique that uses antibody binding to probe epitope sites that are not involved in membrane binding. In general, the free epitopes will be available for antibody binding, whereas the membrane-associated epitopes are shielded and will not be accessible. The antibodies recognizing different epitopes in different domains of the protein were used. As negative controls, sandwich ELISA using 8860-ESH5 antibodies, both recognizing heavy chain epitopes, was carried out. 8860 recognizes an epitope in the A2 domain, whereas ESH5 recognizes an epitope in the a1 region. Consistent with the model proposed by Stoilova-McPhie et al. [8], the binding of FVIII to membranes did not inhibit its interaction with these antibodies (data not shown). However, the ELISA studies using C2 domain-specific antibodies ESH4/ESH8 (biotinylated) displayed a large (~ 50%) reduction in binding of the membrane-associated FVIII relative to FVIII. (Fig. 6). The ESH8 recognizes epitope 2248–2285, whereas ESH4 antibody recognizes lipid-binding epitope (2303–2332) of the C2 domain (Fig. 1a) [29]. The results indicate that the binding of either or both ESH4 and ESH8 is inhibited by membrane interaction suggesting the participation of 2303–2332 and 2248–2285 in membrane binding. However, a complete inhibition of binding was not observed, probably due to the existence of a small fraction of free protein due to complex association/dissociation process (see footnote 1) or antibody-mediated release of FVIII from membranes.

Saenko et al. [30] have shown ESH8-mediated inhibition of FVIII activity is not due to the reduced binding of FVIII to membranes. This observation implies that the epitope region recognized by ESH8 is not involved in membrane binding. This corroborates with our control sandwich ELISA studies using 8860-ESH8 antibodies, in which no reduction in antibody binding was observed for membrane-associated FVIII (data not shown). However, the putative membrane binding amino acid residues, 2249–2255 in this epitope region, may not be essential for antibody binding.

In conclusion, FVIII interaction with membranes involves minimal conformational change due to the involvement of few amino acids in the C2 domain as the primary contact between the protein and the membrane. These observations are consistent with the model proposed by electron crystallographic and biochemical studies [8,22,23,31]. Additionally, the Stern–Volmer plots and sandwich ELISA provide evidence for the involvement of Trp-2313 localized in lipid binding domain in membrane binding.

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References

[1] P.A. Foster, T.S. Zimmerman, Factor VIII structure and function, Blood Rev. 3 (1989) 180–191.
[2] J.J. Toole, J.L. Knopf, J.M. Wozney, L.A. Sultzman, J.L. Buecker, D.D. Pittman, R.J. Kaufman, E. Brown, C. Shoemaker, E.C. Orr, et al., Molecular cloning of a cDNA encoding human antihaemophilic factor, Nature 312 (1984) 342–347.
[3] G.A. Vehar, B. Keyt, D. Eaton, H. Rodriguez, D.P. O'Brien, F. Rotblat, H. Oppermann, R. Keck, W.I. Wood, R.N. Harkins, et al., Structure of human factor VIII, Nature 312 (1984) 337–342.
[4] W.I. Wood, D.J. Capon, C.C. Simonsen, D.L. Eaton, J. Gitschier, B. Keyt, P.H. Seeburg, D.H. Smith, P. Hollingshead, K.L. Wion, et al., Expression of active human factor VIII from recombinant DNA clones, Nature 312 (1984) 330–337.
[5] R.J. Kaufman, L.C. Wasley, A.J. Dorner, Synthesis, processing, and secretion of recombinant human factor VIII expressed in mammalian cells, J. Biol. Chem. 263 (1988) 6352–6362.
[6] G.E. Gilbert, R.J. Kaufman, A.A. Arena, H. Miao, S.W. Pipe, Four hydrophobic amino acids of the factor VIII C2 domain are constituents of both the membrane-binding and von Willebrand factor-binding motifs, J. Biol. Chem. 277 (2002) 6374–6381.
[7] M. Shima, D. Scandella, A. Yoshioka, H. Nakai, I. Tanaka, S. Kami-

sue, S. Terada, H. Fukui, A factor VIII neutralizing monoclonal antibody and a human inhibitor alloantibody recognizing epitopes in the C2 domain inhibit factor VIII binding to von Willebrand factor and to phosphatidylserine, Thromb. Haemost. 69 (1993) 240–246.

[8] S. Stoilova-McPhie, B.O. Villoutreix, K. Mertens, G. Kemball-Cook, A. Holzenburg, 3-Dimensional structure of membrane-bound coagulation factor VIII: modeling of the factor VIII heterodimer within a 3-dimensional density map derived by electron crystallography, Blood 99 (2002) 1215–1223.

[9] E.L. Saenko, D. Scandella, A.V. Yakhyaev, N.J. Greco, Activation of factor VIII by thrombin increases its affinity for binding to synthetic phospholipid membranes and activated platelets, J. Biol. Chem. 273 (1998) 27918–27926.

[10] E.J. Duffy, E.T. Parker, V.P. Mutucumarana, A.E. Johnson, P. Lollar, Binding of factor VIIIa and factor VIII to factor IXa on phospholipid vesicles, J. Biol. Chem. 267 (1992) 17006–17011.

[11] K.P. Pratt, B.W. Shen, K. Takeshima, E.W. Davie, K. Fujikawa, B.L. Stoddard, Structure of the C2 domain of human factor VIII at 1.5 Å resolution, Nature 402 (1999) 439–442.

[12] M.E. Nesheim, D.D. Pittman, J.H. Wang, D. Slonosky, A.R. Giles, R.J. Kaufman, The binding of 35S-labeled recombinant factor VIII to activated and unactivated human platelets, J. Biol. Chem. 263 (1988) 16467–16470.

[13] Y. Cho, C. Garanchon, R. Kashi, C. Wong, M.J. Besman, Characterization of aggregates of recombinant human factor VIII by size-exclusion chromatography and immunoassay, Biotechnol. Appl. Biochem. 24 (1996) 55–59.

[14] V.S. Purohit, K. Ramani, S.V. Balasubramanian, Evaluation of antigenicity and immunogenicity of liposome associated recombinant antihemophilic factor (rahf), AAPSPharmSci 4 (2002) T2045.

[15] K. Ramani, V.S. Purohit, R. Miclea, S.S. Lakhman, S.V. Balasubramanian, Unfolding of recombinant human factor VIII (rhFVIII): a multi-domain protein, AAPSPharmSci (2003) T2091.

[16] E. Gomperts, R. Lundblad, R. Adamson, The manufacturing process of recombinant factor VIII, recombinate, Transfus. Med. Rev. 6 (1992) 247–251.

[17] M. Griffith, H. Kingdon, S.L. Liu, W. Burkart, In-process controls and characterization of recombinate antihemophilic factor (recombinant), Ann. Hematol. 63 (1991) 166–171.

[18] J. Over, Methodology of the one-stage assay of Factor VIII (VIII:C), Scand. J. Haematol., Suppl. 41 (1984) 13–24.

[19] U.K. Laemmli, Cleavage of structural proteins during the assembly of the head of bacteriophage T4, Nature 227 (1970) 680–685.

[20] T.D. Heath, B.A. Macher, D. Papahadjopoulos, Covalent attachment of immunoglobulins to liposomes via glycosphingolipids, Biochim. Biophys. Acta 640 (1981) 66–81.

[21] J.R. Lakowicz, Principles of Fluorescence Spectroscopy, 2nd ed., Kluwer Academic/Plenum, New York, 1999.

[22] M. Arai, D. Scandella, L.W. Hoyer, Molecular basis of factor VIII inhibition by human antibodies. Antibodies that bind to the factor VIII light chain prevent the interaction of factor VIII with phospholipid, J. Clin. Invest. 83 (1989) 1978–1984.

[23] C. Bardelle, B. Furie, B.C. Furie, G.E. Gilbert, Membrane binding kinetics of factor VIII indicate a complex binding process, J. Biol. Chem. 268 (1993) 8815–8824.

[24] G. Kemball-Cook, T.W. Barrowcliffe, Interaction of factor VIII with phospholipids: role of composition and negative charge, Thromb. Res. 67 (1992) 57–71.

[25] A. Lajmanovich, G. Hudry-Clergeon, J.M. Freyssinet, G. Marguerie, Human factor VIII procoagulant activity and phospholipid interaction, Biochim. Biophys. Acta 678 (1981) 132–136.

[26] M. Mikaelsson, U. Oswaldsson, M.A. Jankowski, Measurement of factor VIII activity of B-domain deleted recombinant factor VIII, Semin. Hematol. 38 (2001) 13–23.

[27] A.O. Grillo, K.L. Edwards, R.S. Kashi, K.M. Shipley, L. Hu, M.J. Besman, C.R. Middaugh, Conformational origin of the aggregation of recombinant human factor VIII, Biochemistry 40 (2001) 586–595.

[28] N. Duzgunes, S. Nir, J. Wilschut, J. Bentz, C. Newton, A. Portis, D. Papahadjopoulos, Calcium- and magnesium-induced fusion of mixed phosphatidylserine/phosphatidylcholine vesicles: effect of ion binding, J. Membr. Biol. 59 (1981) 115–125.

[29] P.A. Foster, C.A. Fulcher, R.A. Houghten, T.S. Zimmerman, Synthetic factor VIII peptides with amino acid sequences contained within the C2 domain of factor VIII inhibit factor VIII binding to phosphatidylserine, Blood 75 (1990) 1999–2004.

[30] E.L. Saenko, M. Shima, G.E. Gilbert, D. Scandella, Slowed release of thrombin-cleaved factor VIII from von Willebrand factor by a monoclonal and a human antibody is a novel mechanism for factor VIII inhibition, J. Biol. Chem. 271 (1996) 27424–27431.

[31] D. Scandella, G.E. Gilbert, M. Shima, H. Nakai, C. Eagleson, M. Felch, R. Prescott, K.J. Rajalakshmi, L.W. Hoyer, E. Saenko, Some factor VIII inhibitor antibodies recognize a common epitope corresponding to C2 domain amino acids 2248 through 2312, which overlap a phospholipid-binding site, Blood 86 (1995) 1811–1819.