Factor VIII binds to phospholipid membranes and to von Willebrand factor (vWF) via its second C domain, which has lectin homology. The crystal structure of the C2 domain has prompted a model in which membrane binding is mediated by two hydrophobic spikes, each composed of a pair of residues displayed on a β-hairpin turn, and also by net positive charge and specific interac- tions with phospholipid-derivatives. To test this model, we prepared 16 factor VIII mutants in which single or multiple amino acids were changed to alanine. Mutants at Arg2215, Arg2220, Lys2227, Lys2249, Gln2213, Asn2217, and multiple amino acids were changed to alanine. Mutants at Arg2209, Lys2227, Trp2313, and Phe2196/Thr2197 had specific activities that were >70% of the wild type. Mutants at Arg2209, Lys2227, Trp2313, and Arg2220 were degraded within the cell. Hydrophobic spike mutants at Met2199/Phe2200, Leu2251/Leu2252, and Met2199/Phe2200/Leu2251/Leu2252 (4-Ala) exhibited 43, 59, and 91% reduction in specific activity in the activated partial thromboplastin time assay. In a phospholipid-limiting factor Xa activation assay, these mutants had a 65, 85, and 96% reduction in specific activity. Equilibrium binding of fluorescent, sonicated phospholipid ves- icles to mutants immobilized on Superose beads was measured by flow cytometry. The affinities for phospholipid were reduced —20, —30, and —35-fold for 2199/2200, 2251/2252, and 4-Ala, respectively. A dimeric form of mature vWF bound to immobilized factor VIII and the same mutants, but the affinities of the mutants were reduced —5, 10, and —20-fold, respectively. In a competition, solution phase enzyme-linked immunosorbent assay, plasma vWF bound factor VIII and the same mutants with the affinities for the mutants reduced —5, —5, and —50-fold, respectively. We conclude that the two hydropho- bic spikess are constituents of both the phospholipid-binding and vWF-binding motifs. In plasma, vWF appa- rently binds the inherently sticky membrane-binding motif, preventing nonspecific interactions.

Factor VIII is a phosphatidyl-l-serine (PS) binding cofactor (1, 2) for the vitamin K-dependent serine protease, factor IXa, that also binds to PS-containing membranes (3, 4). The mem- brane-bound factor VIIIa-factor IXa complex cleaves the zymo- gen, factor X, to factor Xa, which is then responsible for catalyzing prothrombin activation (5). The importance of this enzyme complex is illustrated by hemophilia, a disease in which a deficiency of either factor VIII (hemophilia A) or factor IX (hemophilia B) leads to life-threatening bleeding. Factor IXa gains more than 100,000-fold greater efficiency in activating factor X by assembling with factor VIIIa on a PS-containing membrane than when free in solution (6). We have recently found that the predominant effect of PS-containing membranes on the factor VIIIa-factor IXa complex is to increase the kcat by more than 1000-fold (7). These membranes also increase the affinity of factor IXa for factor VIIIa and for factor X. The central importance of the membrane binding function of factor VIII motivates studies to define the membrane-binding motif.

Factor VIII, with Mw 280,000, is homologous to another procoagulant protein, factor V, in amino acid sequence (8–10) and function, as a membrane-bound enzyme cofactor (5, 11–13). The proteins share a repeating domain structure of A1-A2-B- A3-C1-C2 in which the A domains are homologous with ceru- loplasmin, the B domain is unique to each protein, and the C domains are homologous with discoidin I, a phospholipid-bind- ing lectin (14), and with a murine milk fat globule membrane protein (15). Both factor VIII and factor V bind with high affinity to phospholipid membranes via the “light chain” composed of the A3-C1-C2 segment (16). However, factor VIII requires more PS per binding site than factor V (13), and current evidence implicates different domains in membrane binding. While binding of factor V is apparently mediated by both the A3 domain (17) and the C2 domain (18) is influenced by glycosylation within the C2 domain (19), binding of factor VIII is apparently mediated by the C2 domain (20, 21) and is inde- pendent of glycosylation. Recent publication of two crystal structures for the C2 domain of factor V (22) and a single structure for the C2 domain of factor VIII (23) has provided the basis for a model membrane-binding mechanism. Both C2 do- mains display two hydrophobic spikes at the tips of protruding β-hairpin turns. These hydrophobic spikes are hypothesized to

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puriﬁcation kits were purchased from Qiagen (Valencia, CA). 

Plasmid DNA was transfected into COS-1 cells by the DEAE-dextran method as previously described (29). Conditioned medium was harvested at 64 h post-transfection in the presence of 10% fetal bovine serum.

**Protein Puriﬁcation**

Puriﬁed FVIII WT, BDD-VIII, Met2199/Phe2200, Leu2251/Leu2252, and Met2199/Phe2200/Leu2251/Leu2252 proteins were obtained from 200 ml of conditioned medium from transiently transfected COS-1 cells by immunoaffinity chromatography (33), yielding 1500–3000 ng/purification. The proteins eluted into the ethylene glycol-containing buffer (34) were dialyzed and then concentrated against a polyethylene glycol (Mr, ~15,000–20,000)-containing buffer and stored at −70 °C.

**Measurement of Speciﬁc Activity**

Factor VIII activity was measured by an activated partial thromboplastin time clotting assay on an MLA Electra 750 ﬁbrinometer by the manufacturer's instructions (Diagnostica Stago, Inc., Parsippany, NJ).

**Phospholipid Vesicles**

Phospholipid vesicles were synthesized by sonication in a bath sonicator (Laboratory Supplies Co., Hicksville, NY) under argon until the suspension was visually clear. Phospholipid concentration was determined by phosphorous assay (35). Vesicles were used fresh, or 1-ml aliquots were quick frozen in liquid nitrogen, stored at −80 °C, and thawed at 37 °C.

**Factor Xase Assay**

Preliminary experiments were performed with normal human factor VIII to identify conditions under which the rate at which factor X was cleaved by the factor Xase complex was linearly related to the concentration of phospholipid vesicles with 4% PS. At the concentration selected, 0.15 µM phospholipid, the rate of factor Xa cleavage was ~20% of the maximum rate with saturating phospholipid (data not shown).

Factor Xase activity was measured with a two-step amidolytic substrate assay. Phospholipid was mixed with 4 mM factor IXa and 130 nM factor X in 0.15 M NaCl, 0.2% (w/v) bovine serum albumin, 50 mM Tris-HCl, pH 7.8. Phospholipid vesicles had compositions (PS/PE/PC) of 15:20:65 (PS/PE/PC) of 15:20:65–70 °C. After 5 min at 25 °C, the reaction was stopped by diluting the mixture 1.08 with 16 mM EDTA, and factor Xa activity was determined immediately in a thermostatted kinetic microtiter plate reader (Molecular Devices, Menlo Park, CA) at 25 °C using 0.1 mM S-2765 (Diapharma Group, Inc., West Chester, OH).

**Preparation of Factor VIII Mutants**

Mutagenesis was performed within the mammalian expression vector pMT(31). The vector plasmid containing the wild type factor VIII cDNA sequence was designated FVIII WT. The plasmid containing a B-domain-deleted (BDD) FVIII was designated BDD-VIII.

Factor VIII and mutants were immobilized at ﬁnal concentrations of 1.5 mM, 1 unit/ml, and 0.1 unit/ml. After 5 min at 25 °C, the reaction was stopped by diluting the mixture 1.08 with 16 mM EDTA, and factor Xa activity was determined immediately in a thermostatted kinetic microtiter plate reader (Molecular Devices, Menlo Park, CA) at 25 °C using 0.1 mM S-2765 (Diapharma Group, Inc., West Chester, OH).

**Puriﬁcation of Factor VIII Mutants**

Factor VIII and mutants in tissue culture supernatant were concentrated 10–20-fold using Centricon 100 microconcentrators (Millipore Corp., Bedford, MA). CaCl2 was added to a 0.35 m concentration (to separate factor VIII and vWF), and particulate matter was removed by sedimentation at 100,000 × g for 1 h at room temperature. The clarified factor VIII solution was loaded over a microcolumn of F-8 coupled to cyanogen bromide-activated Superose beads. The total volume of Superose for each column was 10 ml, and the column was prepared by injecting the medium into 0.2-ml internal diameter tubing where the column formed above a 10-µm frit (Alltech). Factor VIII was loaded onto the column at a flow rate of 20–50 µl/min, delivered by a syringe pump. The immobilized factor VIII was washed with Tris-buffered saline plus 0.01% Tween 80, and the medium was removed by reversing the ﬂow through the frit. Alternately, factor VIII and mutants were immobilized.
on Superose beads by incubating the beads with concentrated medium and removing the bound factor VIII on the beads by sedimentation.

**Phospholipid Vesicles Binding to Factor VIII**

Aliquots containing ~8000 F-8-Superose microspheres with bound factor VIII were incubated at various concentrations of NBD-labeled phospholipid vesicles in 100 μl of Tris-buffered saline, 1.5 mM CaCl₂, 0.2% (w/v) bovine serum albumin. The composition of the vesicles (PS/PE/PC/NBD-PC) was 4:20:75:1. After 30 min, the samples were diluted to 500 μl in the same buffer within 30 s of reading the fluorescence/microsphere by flow cytometry with a Becton Dickinson FacScan within 30 s of dilution. Data were acquired with all channels in log mode, and binding curves were analyzed as the geometric mean fluorescence/microsphere. Microspheres were detected and distinguished from background noise based upon the characteristic forward and side light scatter characteristics. Binding data were analyzed using the equation,

\[ F = F_0 + F_{\text{max}}(PL/K_D + PL) = PL \times k_f(1) \]

(Eq. 1)

where \( F \) is the measured fluorescence/Superose bead, \( F_0 \) is the fluorescence for Superose bead in the absence of NBD-labeled vesicles, \( F_{\text{max}} \) is the maximum fluorescence with saturating concentrations of NBD-labeled vesicles, and \( K_D \) is the dissociation constant for phospholipid (PL) binding to factor VIII. The term \( k_f \) is the measured fluorescence per concentration unit of free phospholipid vesicles due to unbound phospholipid and any nonspecific binding of phospholipid vesicles to control Superose beads lacking factor VIII. We assumed that the fraction of bound phospholipid could be neglected (the nominal concentration of immobilized factor VIII was 0.2–0.4 nm). The variable \( K_D \) was determined using nonlinear least squares analysis with the software Graphpad Prism 3.0. \( F_{\text{max}} \) was determined by nonlinear least squares analysis for wild type factor VIII, for which saturable binding was clearly evident (Fig. 4). \( F_{\text{max}} \) for each of the mutants was assigned based upon the quantity of ΔPro vWf binding sites detected/Superose microsphere relative to wild type factor VIII (see below).

**ΔPro vWf Binding to Factor VIII**

ΔPro vWf was labeled with fluorescein maleimide as previously described for factor VIII (36). Aliquots containing ~8000 F-8-Superose microspheres with bound factor VIII were incubated with various concentrations of fluorescein-labeled ΔPro vWf in 100 μl of Tris-buffered saline with 1.5 mM CaCl₂. After 60 min, samples were diluted to 500 μl less than 30 s prior to evaluation of fluorescence/microsphere determined by flow cytometry. Data were analyzed according to Equation 1 except that ΔPro vWf was substituted for PL, \( K_D \), referred to the dissociation constant for binding to ΔPro vWf, and \( k_f \) referred to the fluorescence related to free ΔPro vWf plus nonspecific binding of ΔPro vWf to F-8-Superose microspheres lacking factor VIII.

**Normalization of Binding Data**

The quantity of immobilized factor VIII was estimated by incubation with fluorescein-labeled ESH8, a mAb that binds to the C2 domain of factor VIII but does not interfere with binding to vWf or to phospholipid vesicles (37). Antibody binding curves were generated as described for ΔPro vWf, above. The apparent affinity of ESH8 for wild type factor VIII and the mutants were equivalent, as predicted. The maximum fluorescence, with an ESH8 concentration of 80 nM, was used as the denominator to normalize binding curves for ΔPro vWf to the quantity of immobilized factor VIII bound.

**Plasma vWf Binding to Factor VIII in Solution**

The affinity of factor VIII for plasma vWf was measured in a competition ELISA. Factor VIII, M/F 2199/2200, or L/L 2251/2252 at 1 unit/ml was coated in 1/1000 dilution in 96-well plates overnight at 4°C. The wells were washed, and the bound factor VIII was detected with an antibody ESH8, followed by horseradish peroxidase-conjugated goat anti-mouse antibody. The wells were developed with p-nitrophenyl phosphate reagent (Sigma).

The competition binding data were interpreted as binding data by assuming that all factor VIII that was not bound to ESH4 was bound to vWf and that binding to vWf was an equilibrium binding process. The ELSA data were subtracted from a constant value equivalent to the maximum colorimetric intensity to generate upright binding curves. The data from two experiments were combined, and the combined curves were fitted by nonlinear least squares analysis using Graphpad Prism 3.0 software on a Macintosh computer.

**RESULTS**

We altered amino acids of the C2 domain of factor VIII based upon their hypothetical function in membrane binding (22, 23, 39) (Table I, Fig. 1). The proposed functions that we considered were membrane-penetrating hydrophobic spikes (Met²¹⁹⁹/Phe²²⁰⁰ and Leu²¹⁵¹/Leu²¹⁵²), phospho-1-serine binding motifs (Phe²¹⁹⁷/Thr²¹⁹⁷, Glh²²¹³, Asn²²¹⁷, Arg²²³⁹, and Arg²³³⁹), hydrophobic membrane contact (Val²²²³ and Tyr²³¹³) ionic membrane contact (Arg²²¹⁵, Arg²²²⁰, Lys²²²⁷, and Lys²²⁴⁹), and dispersion of positive net charge (Arg²²⁰⁹ and Lys²²²⁷). In addition, three residues implicated in maintenance of overall C2 domain stability were mutated (Glu²²⁰⁷/Gln²²⁰⁹/Asp²³¹⁰) (40). These residues were changed to alanine singly or in groups, as indicated. The sequence of cDNA coding for each mutant was confirmed by DNA sequencing of the entire C2 domain. The initial constructs were expressed transiently from COS-1 cells as B-domain-deleted factor VIII in defined medium.

Secretion of factor VIII mutants was evaluated in an ELISA assay and in a factor VIII functional assay. Specific activity was evaluated compared with B-domain-deleted factor VIII expressed in parallel with each batch of mutants. Four of the mutants, R,²²⁰⁹A, K,²²²⁷A, W,²³ⁱ₃A, and D,²³₂₀A, were present at <5% of wild type factor VIII in the tissue culture supernatant. Pulse-chase experiments utilizing metabolic labeling with [³⁵S]methionine indicated that these mutants were translated into protein products that were degraded prior to secretion in the medium (data not shown). These results suggest that these four residues influence the overall folding of the C2 domain. One of the mutants, R,²²⁰⁹A, had more than 3-fold greater activity than control B-domain deleted factor VIII. However, subsequent evaluation indicated that this mutant had been underdetected in the ELISA assay (data not shown).

Our evaluation strategy was to identify amino acids whose alteration resulted in at least 50% loss of activity in a factor Xase assay configured so that phospholipid was limiting and stereo-selective binding of factor VIII to phosphatidyl-1-serine was critical for function (41). In the course of these experiments, we found that the B-domain-deleted factor VIII had 30–40% lower activity in the phospholipid-limited factor Xase assay than wild type factor VIII. Therefore, a number of the mutations were reevaluated as mutants of wild type factor VIII as indicated in Table I. None of the mutants evaluated in both B-domain-deleted and wild type factor VIII differed relative to the relevant control factor VIII. Of the original mutants, only the two pairs corresponding to the hydrophobic spikes met the criteria for <50% activity with limiting phospholipid (Fig. 2). Based upon this data, we prepared a mutant in which both pairs of amino acids constituting hydrophobic spikes were mutated to alanine (Met²¹⁹⁹/Phe²²⁰⁰ and Leu²¹⁵¹/Leu²¹⁵², subsequently referred to as 4-Ala). This mutant was not evaluated in the initial set of experiments, but it was subsequently evaluated in more detail by comparison with wild type factor VIII and the mutants of individual hydrophobic spikes (Fig. 3).

The activity of the hydrophobic spike mutants was evaluated relative to wild type factor VIII in two factor Xase assays. In one assay, the phospholipid concentration was 2 μM with sonicated vesicles of 15% PS, 20% PE, and 65% PC. Under these conditions, the assay is saturated with phospholipid when wild type factor VIII is utilized. However, the mutants exhibited 20, 65, and 95% reduction in activity for the 2199/2200, 2251/2252,
Hydrophobic Spikes of Factor VIII Bind Membranes and vWf

Table I

| Mutant       | Amino acid(s) → alanine | Rationale           | Relative secretion | Relative specific activity |
|--------------|-------------------------|---------------------|--------------------|---------------------------|
| 2196/2197<sup>WT</sup> | Phe/Thr              | Phos-L-Ser          | 0.4                | 1.0                       |
| 2199/2200<sup>WT</sup> | Met/Phe              | Hydrophobic spike   | 1.4                | 0.57                      |
| 2199/2000/2251/2252<sup>WT</sup> | Met/Phe/Leu/Leu | Hydrophobic spike   | 1.7                | 0.09                      |
| 2200<sup>WT</sup> | Arg                    | Pos surface         | <0.05             |                           |
| 2213<sup>WT</sup> | Glu                    | Phos-L-Ser          | 1.2                | 1.1                       |
| 2215<sup>WT</sup> | Arg                    | Pos interacting    | 0.8                | 0.99                      |
| 2217<sup>WT</sup> | Asn                    | Phos-L-Ser          | 1.2                | 1.56                      |
| 2220<sup>WT</sup> | Arg                    | Phos-L-Ser          | 0.3                | 1.49                      |
| 2222<sup>WT</sup> | Val                    | Hydrophobic surface | 0.9                | 1.41                      |
| 2227<sup>WT</sup> | Lys                    | Pos interacting    | <0.05             |                           |
| 2249<sup>WT</sup> | Lys                    | Pos interacting    | 0.9                | 0.91                      |
| 2251<sup>WT</sup> | Leu                    | Hydrophobic spike   | 0.1                |                           |
| 2251/2252<sup>WT</sup> | Leu/Leu              | Hydrophobic spike   | 1.7                | 0.41                      |
| 2319<sup>WT</sup> | Trp                    | Hydrophobic surface | <0.05             |                           |
| 2329<sup>WT</sup> | Arg                    | Phos-L-Ser          | 0.9                | 1.16                      |
| 2327/2329/2330<sup>WT</sup> | Glu/Glu/Asp | Antigen integrity  | 0.7                |                           |

Secretion and specific activity of factor VIII mutants

| Wild type | B-domainless |
|-----------|--------------|
| Control   | 65 ± 32      | 11.4 ± 4.9 |
| Control   | 28 ± 14      | 10.0 ± 3.5 |

<sup>a</sup> Superior “WT” indicates that the mutant was in wild type factor VIII. Other mutants were prepared in B-domainless factor VIII. 2199/2200 and 2251/2252 were prepared in both B-domainless factor VIII and wild type factor VIII. Data in this table and Fig. 2 are from B-domainless factor VIII preparations, while data in Fig. 3 and Table II are from wild type preparations.

<sup>b</sup> Phos-L-Ser, homologous residues of the factor V C2 domain are hypothesized to provide a phospho-L-serine binding motif (22); Hydrophobic spike, two pairs of hydrophobic residues, MF and LL, are solvent-exposed hydrophobic protuberances that are hypothesized to penetrate the phospholipid bilayer (23); Pos surface, a ring of positively charged residues are hypothesized to contribute net affinity to the phospholipid surface (23); Pos interacting, positively charged residues positioned to form ion pairs with phosphate or carboxylate moieties in the proposed membrane-phospholipid bilayer (23); Antigen integrity, residues necessary to maintain integrity for antibodies that inhibit phospholipid binding in patients (40).

<sup>c</sup> Secretion indicates factor VIII antigen accumulating in tissue culture supernatant after 36 h relative to the relevant wild type or B-domainless factor VII control.

<sup>d</sup> Factor VIII activity was measured in an activated partial thromboplastin time assay with factor VIII-deficient plasma. The phospholipid concentration in this assay is >0.2 mM.

<sup>e</sup> Pulse-chase experiments confirmed intracellular degradation without secretion.

and 4-Ala mutants relative to activity of the same mutants in a factor VIII assay with excess phospholipid (Fig. 3). (The activated partial thromboplastin time assay utilized a platelet factor 3 reagent with a proprietary phospholipid mixture in excess of 200 μM). When the FS content was reduced to 4% and the phospholipid concentration was reduced to 0.15 μM, the relative activity exhibited by the mutants was further reduced by 70, 85, and 93%, respectively. The activity of 4-Ala yielded a signal less than 2-fold above the background rate of chromogenic substrate development when no factor Xa is added so that this value lacks certainty.

We measured the affinity of the three factor VIII mutants for phospholipid vesicles utilizing a novel flow cytometry assay (Fig. 4). The mutants were extracted from tissue culture supernatant using microcolumns of F-8-Superose as described under "Experimental Procedures." The Superose beads, with bound factor VIII, were removed by reversing the flow through the columns. Equilibrium binding of sonicated phospholipid vesicles of 4% FS, 20% PE, and 1% NBD-PC to the factor VIII on the Superose beads was evaluated by flow cytometry. To normalize the fluorescence signal to the quantity of immobilized factor VIII for each mutant, the fluorescence signals were divided by the fluorescence from the highest concentration of fluorescent-labeled ΔPro vWF that bound to factor VIII on the same beads (Fig. 5). ΔPro vWF is a dimeric form of mature vWF produced by expression of preprotease-deleted vWF that binds with high affinity to wild type factor VIII (42). The results indicated saturable binding of phospholipid vesicles to wild type factor VIII with half-maximal binding at a phospholipid concentration of ~25 μM (Table II). In contrast, the mutants had much lower affinities and did not exhibit clear evidence of saturation at phospholipid concentrations up to 54 μM. These results confirm that the affinity of factor VIII for phospholipid membranes is reduced when either of the hydrophobic spikes are removed and when both spikes are absent. The fluorescence for the mutants at a phospholipid concentration of 27 μM exceeded fluorescence of control microspheres lacking factor VIII by 0.5–3-fold, indicating that phospholipid binding occurred but was of lower affinity than for wild type factor VIII.

Because the phospholipid and vWF binding motifs of factor VIII overlap, we also evaluated the effect of the hydrophobic spike mutations on affinity for vWF. In the first assay, we measured the affinity of fluorescent-labeled ΔPro vWF for immobilized factor VIII on Superose beads (Fig. 5). ΔPro vWF bound saturably to wild type factor VIII with a KD of 6.0 nM, consistent with our prior experiments (Fig. 5, inset; Table II) (43). Affinity of ΔPro vWF for the 2199/2200 mutant was reduced ~5-fold. The affinity of the 2251/2252 mutant was reduced more than 10-fold. The affinity of the combined mutant 4-Ala was reduced >20-fold.

The ratio of ΔPro vWF binding sites to mAb ESH8 epitopes on Superose Beads was 3–5-fold lower for wild type factor VIII than for these three mutants (compare Fig. 5, inset) maximum value with mutants in Fig. 5 (main figure)). The pattern was consistent in two experiments, with different preparations of mutants. It is possible that vWF has more binding sites for the mutants than for wild type factor VIII. We view this alternative is unlikely. Alternatively, the C2 (lectin) domain of factor VIII may also bind to Sepharose, thereby interfering with binding to vWF and phospholipid. Indeed, low affinity binding to Sepharose has been observed for both wild type factor VIII and isolated, recombinant C2 (data not shown). Mutation of the hydrophobic spike amino acids may decrease affinity for Sepharose, increasing the likelihood that the C2 domain of an immo-
bilized factor VIII molecule would be available for binding to vWF or phospholipid.

To confirm that the hydrophobic spike mutants have a reduced affinity for vWF, we measured the affinity for purified plasma vWF using a competition, solution phase ELISA. vWF displaced factor VIII and the three mutants from mAb ESH4 (Fig. 6). The asymptote approached by the dissociation curves was not 0.2 rather than 0. This suggests that the overlap between the epitope of ESH4 and the vWF contact site is incomplete, so that a low affinity binding of factor VIII to ESH4 remains possible in the presence of vWF. The apparent \( K_D \) for wild type factor VIII binding to vWF was 0.52 nM, consistent with prior reports (Table II). The affinities of both the 2199/2200 and the 2251/2252 mutants were reduced more than 5-fold. The affinity of 4-Ala was reduced more than 50-fold. These results confirm that the hydrophobic spikes from the factor VIII C2 domain are constituents of binding sites for both phospholipid and vWF.

The combined data for experiments from two preparations of mutants, for both phospholipid and vWF binding, were subjected to nonlinear, least squares curve fitting (Table II). The

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**Fig. 1.** Space-filling display of the factor VIII C2 domain crystal structure (23) with mutated residues identified by coloring according to the CPK scheme. C2 is oriented so that the hydrophobic spikes (Met2199/Phe2200 and Leu2151/Leu2152) are at the bottom. Labels are located at the \( \alpha \)-carbon of each residue. Side B is rotated \( \sim 180^\circ \) about the vertical axis, and the top is rotated \( \sim 20^\circ \) into the page, relative to side A.

**Fig. 2.** Relative activity of factor VIII mutants with limiting phospholipid. Concentrated tissue culture supernatant containing factor VIII was incubated with thrombin, factor IXa, and factor X for 5 min in the presence of limiting and saturating phospholipid vesicles for 5 min. The reaction was stopped by the addition of 16 mM EDTA, and the quantity of factor Xa formed was measured by monitoring the rate of cleavage of chromogenic substrate S2765. Limiting phospholipid vesicles were composed of PS/PE/PC (4:20:76) at 0.15 \( \mu \)M phospholipid. Saturating phospholipid vesicles were composed of PS/PE/PC (15:20:65) at 2 \( \mu \)M final phospholipid concentration. Displayed data are limiting/saturating phospholipid activity for each divided by limiting/saturating phospholipid activity for wild type factor VIII. Values displayed are mean \( \pm \) S.D. for duplicate experiments from at least two separate COS cell transfections for each mutant.

**Fig. 3.** Relative activity of hydrophobic spike mutants with limiting and saturating phospholipid. Tissue culture supernatant containing mutants prepared in full-length factor VIII were assayed as described under Fig. 2. Results displayed indicate the activity of mutants versus wild type factor VIII with saturating and limiting phospholipid.
FIG. 4. Affinity of phospholipid vesicles for hydrophobic spike mutants of factor VIII. Wild type factor VIII and mutants 2199/2200, 2251/2252, and 4-Ala were purified from tissue culture supernatant using microcolumns of anti-factor VIII Superose, as described. Immobilized factor VIII on Superose beads was incubated with various concentrations of sonicated phospholipid vesicles of PS/PE/PC/NBD-PC (4:20:75:1) for 30 min. Bound phospholipid vesicles were measured by flow cytometry. Values were corrected for the quantity of fluorescence from phospholipid vesicles associated with Superose beads lacking factor VIII. The quantity of bound phospholipid vesicles was normalized to the quantity of δPro vWF that bound to aliquots of the same immobilized factor VIII preparation (see rationale under “Results”). Curves indicate the best fit values for wild type factor VIII (solid line) and mutants 2199/2200 (solid line), 2251/2252 (dashed line), and 4-Ala (dotted line) obtained with the Prism software package 2.0 (GraphPad Software; parameters displayed in Table II). Displayed results are from a single experiment representative of two experiments from separate COS cell transfections.

FIG. 5. Affinity of δPro vWF for hydrophobic spike factor VIII mutants. Wild type factor VIII and mutants 2199/2200, 2251/2252, and 4-Ala were immobilized on microcolumns of anti-factor VIII Superose, as described. Immobilized factor VIII on Superose beads was incubated with fluorescein-labeled δPro vWF, at various concentrations, for 30 min prior to evaluation by flow cytometry. Values were corrected for the quantity of fluorescence from fluorescein-δPro vWF associated with Superose beads lacking factor VIII. Curves indicate the best fit values for wild type factor VIII (solid line) and mutants 2199/2200 (solid line), 2251/2252 (dashed line), and 4-Ala (dotted line) obtained with the Prism software package 3.0 parameters displayed in Table II. Displayed results are mean ± S.D. from two experiments from separate COS cell transfections. Inset, binding of fluorescein δPro vWF to wild type factor VIII.

assumptions utilized are detailed in the footnotes to Table II. Together, the results indicate that the removal of either hydrophobic spike causes a 20-35-fold decrease in affinity for phospholipid, and removal of both spikes causes a decreased affinity of 35-fold. The removal of either spike causes a 5-20-fold reduction in affinity for vWF, whereas removal of both causes a >35-fold decrease.

| Table II Effect of hydrophobic spike mutations on affinity for phospholipid vesicles and vWF |
|---------------------------------|-----------------|-----------------|
| Factor VIII mutant              | Phospholipid (nm) | ΔPro vWF (nm) | vWF (nm)      |
| Wild type                       | 6.6 ± 1.6        | 6.0 ± 2.8       | 0.52 ± 0.12   |
| 2199/2200                       | 150 ± 51         | 34 ± 13         | 9.3 ± 8.7     |
| 2251/2252                       | 231 ± 24         | 108 ± 27        | 8.1 ± 6.4     |
| 2199/2200/2151/2152             | >250             | >200            | >95 ± 38      |

a KD ± S.D. of fit. Normalized binding curves from two experiments were averaged and fitted by nonlinear, least squares analysis. The phospholipid concentration was divided by 2500, the ratio of phospholipid monomers/sonicated vesicle (47), to obtain the dissociation constant. The KD values for the mutants were obtained by assuming the maximum phospholipid binding, at saturation, would be equivalent to wild type factor VIII. If the phospholipid binding data were analyzed using the quantity of ESH8 fluorescence to normalize to binding rather than ΔPro vWF, the results indicated a decrease in affinity with the mutants in the same rank order. However, the estimated decrease in affinity for the mutants would range from 4- to 16-fold rather than the larger decreases implied by Fig. 4 and Table II.

b KD ± S.D. of fit. Displacement curves from two experiments were averaged and fitted by nonlinear, least squares analysis. The concentration of vWF was analyzed as the vWF subunit concentration, assuming a Mr of 220,000.

FIG. 6. Affinity of hydrophobic spike factor VIII mutants for plasma vWF. Wild type factor VIII and mutants 2199/2200, 2251/2252, and 4-Ala were immobilized on microcolumns of factor VIII Superose, as described. Immobilized factor VIII on Superose beads was incubated with varying concentrations of purified plasma vWF for 60 min prior to placement in microtiter wells coated with mAb ESH4. Factor VIII aliiquots were incubated with varying concentrations of purified plasma vWF for 60 min prior to placement in microtiter wells coated with mAb ESH4. Because vWF competes for the ESH4 epitope, it leads to decreased bound factor VIII. Displayed values are normalized to the maximum signals measured for each type of factor VIII in the absence of added vWF. The quantity of factor VIII used was 1 unit/ml for wild type factor VIII, 2199/2200, and 2151/2152 and 3 units/ml for 4-Ala. Displayed results are representative of two such experiments, each performed in duplicate. The molar concentration of vWF subunits, indicated on the abscissa, was obtained by dividing the vWF concentration by the molecular weight of a single vWF subunit.

DISCUSSION

We evaluated the importance of 19 amino acid residues within the factor VIII C2 domain for their importance in binding to membranes containing phosphatidyl-l-α-amine and to vWF. Only four amino acids, constituting the two hydrophobic spikes Met2199/Phe2200 and Leu2151/Leu2152, met our criteria for phospholipid binding with >50% loss of activity under conditions where the stereoselective interaction with PS was critical. The mutants had >20-fold reduced affinity for phospholipid vesicles in direct membrane binding studies. The compound mutant, with all four residues changed to Ala, had >25-fold reduced affinity for phospholipid. These same four residues caused 18- and 16-fold reduction in affinity for von Willebrand...
factor when mutated as pairs of 2199/2200 and 2251/2252 and 200-fold reduced affinity for the compound mutant. We interpret these results to indicate that the two hydrophobic spikes are constituents of the binding motif for both phospholipid membranes and for vWF.

Mutations at residues 2199/2200 and 2251/2252 could plausibly affect the C2 domain conformation rather than removing moieties that participate in membrane binding. The best evidence that this is not the case comes from the recently described crystal structure of the C2 domain in complex with mAb B02C11 (44). The antibody was obtained from a patient, and it inhibited normal factor VIII phospholipid binding and vWF binding, thus implying that it recognizes the native conformation of the factor VIII C2 domain (45). The structure of the C2 domain in the antibody-C2 cocrystal is very nearly the same as the previously published C2 crystal structure. However, the 2199/2200 hydrophobic spike was rotated 90° compared with the prior structure. We interpret this to indicate that the C2 domain does not undergo a global conformational change; rather, the β-hairpin turn that displays hydrophobic spike 2199/2200 is flexible. Thus, the membrane-binding and vWF binding conformation of factor VIII probably displays the two hydrophobic spikes that, although flexible, participate directly in binding.

The C2 domain of factor V also displays two hydrophobic spikes (22). The first spike, composed of Trp2063/Trp2064, is homologous to the Met2199/Phe2200 spike of factor VIII. Site-directed mutagenesis studies, similar to those in this report, indicate that this spike is engaged in phospholipid binding (24). The loss in specific activity for this mutant, when phospholipid was limiting, was 65% versus 80% for factor VIII in this study. In contrast, the second hydrophobic spike, composed of Leu2116, did not have clear phospholipid-binding importance. These studies indicate a parallel functional importance for the first hydrophobic spike. The second hydrophobic spike of factor V is composed of a single leucine residue, and mutation to alanine did not cause a clear deficit in phospholipid binding. However, because the threshold for clear detection in both studies was a 50% loss in phospholipid-dependent activity, it remains possible that both spikes have homologous function and that decreased hydrophobic surface of the second factor V spike simply causes a smaller loss in activity.

Because mutation of the individual positively charged residues listed in Table I did not cause a 50% loss in phospholipid-dependent activity, it is likely that none form a critical interaction with PS. Substitution of phosphatidyl-t-serine for PS causes >95% loss of activity for native factor VIII under similar conditions. This implies that factor VIII contains at least one critical site that forms interactions with the amine, the carbonyl, and the phosphate moieties of phospho-t-serine (1). Because isolated C2 binds to the same membranes with equivalent affinity, the phospho-t-serine binding motif is apparently localized within C2. Our results indicate that none of the residues investigated, with the possible exception of the hydrophobic spikes, provide critical interactions with PS. Identification of residues that make critical salt bridges or hydrogen bonds with phospho-t-serine will require further studies.

The hydrophobic spikes of the factor VIII C2 domain are excellent candidates to contribute to the “stickiness” of intact factor VIII, which binds to glass, plastic, dialysis tubing, and agarose (data not shown). The observation that these residues contribute to vWF interaction suggests that in the factor VIII-vWF complex, the spikes are protected from contact with other molecules, thus reducing the capacity for nonspecific interactions. In the course of these studies, we found that the 2251/2252 mutant was lost in dialysis tubing at only one-quarter the rate of wild type factor VIII (data not shown). All of the hydrophobic spike mutants accumulated well in conditioned medium despite exhibiting reduced affinity for vWF (compare Table I and Table II). These data suggest that the clearance of secreted factor VIII from tissue culture supernatant, which is obviated by the presence of vWF, is also mediated, in part, by the hydrophobic spikes.

The hydrophobic spikes investigated in these studies make a major contribution to membrane binding. The protruding nature of the spikes suggests that they penetrate to the core of a phospholipid bilayer. As such, it is anticipated that their chief contribution would be via hydrophobic interactions with the phospholipid acyl chains. Indeed, the necessity to accommodate such protrusions may help to account for the requirement for phosphatidylethanolamine (2) and unsaturated phospholipid acyl chains (46) for phospholipid bilayers to efficiently bind factor VIII. The smaller dimension of ethanamolamine versus choline allows for decreased lateral pressure in the interfacial layer of the membrane, possibly potentiating penetration of hydrophobic spikes. The increased cross-sectional dimensions of unsaturated acyl chains also cause reduced lateral pressure at the surface of the membrane, promoting penetration. Thus, the results of this study validate the prediction, based upon the crystal structure of the C2 domain of factor VIII, that the hydrophobic spikes participate in membrane binding, presumably by penetrating the core of the membrane.

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