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Surface-exposed Hemophilic Mutations across the Factor VIII C2 Domain Have Variable Effects on Stability and Binding Activities*

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Factor VIII (fVIII) is a plasma glycoprotein that functions as an essential cofactor in blood coagulation. Its carboxyl-terminal "C2" domain is responsible for binding to both activated platelet surfaces and von Willebrand factor. We characterized the effect of 20 hemophilia-associated missense mutations across this domain (that all occur in patients *in vivo*) on its stability and its binding activities. At least six of these mutations were severely destabilizing, and another four caused moderate destabilization and corresponding reductions in both binding functions. One mutant (A2201P) displayed a significant reduction in its membrane binding activity but normal von Willebrand factor binding, while two others (P2300S and R2304H) caused the opposite effect. Several mutations (including L2210P, V2223M, M2238V, and R2304C) displayed near wild-type stabilities and binding activities and may instead affect mRNA splicing or alternative properties or functions of the protein. This study demonstrated that von Willebrand factor and membrane binding activities can be uncoupled and uniquely disrupted by different mutations and that either effect can lead to similar reductions in clotting activity. It also illustrated how a heterogeneous genetic disorder causes diverse molecular phenotypes that result in similar disease states.

Factor VIII (fVIII)[1] is an essential, nonenzymatic cofactor in the intrinsic coagulation pathway where it participates in the proteolytic activation of factor X by the serine protease factor IXa (1–3) (Fig. 1). The full-length, unprocessed fVIII protein consists of 2332 amino acid residues and has the domain structure A1-A2-B-A3-C1-C2 (4–7). The A domains are ~40% identical to each other and to the copper-binding protein ceruloplasmin (8). The C domains are more distantly related to galactose oxidase, which is a member of the discoidin protein fold family (9–12). The B domain has no known structural homologues. Factor VIII is initially processed by proteolytic cleavage events that remove a large portion of the B domain, generating a heterodimer that circulates in a tight complex with von Willebrand Factor (vWF) (13). This interaction is essential for maintaining stable levels of factor VIII in circulation (14). Upon vascular injury, further proteolytic processing generates activated factor VIIIa, a heterotrimer that is released from vWF and binds to the membrane surfaces of activated platelets (13). There it recruits factor IXa into a tightly bound complex (15) where the proteolytic activity of factor IXa is enhanced by ~200,000-fold (16).

The carboxyl-terminal 159 amino acids of fVIII (and also of the homologous coagulation factor V) comprise its C2 domain, which is largely responsible for binding to vWF and to platelet membrane surfaces (10, 17, 18). This latter interaction is dependent on the transient, specific exposure of phosphatidylserine (PS) head groups on the outer leaflet of activated platelet membranes (18–21). The vWF and membrane binding activities of the C2 domain appear to be competitive and mutually exclusive (18, 19, 22, 23). The C2 domain of factor VIII has also been shown to participate in binding to factor Xa and thrombin, further illustrating its importance (24, 25). Additionally many surface epitopes for both alloimmune and autoimmune antibodies as well as monoclonal antibodies are located within the C2 domain, indicating that the C2 domain might be an antigenic "hotspot" (22, 26–29).

A deficiency in factor VIII clotting activity leads to a common bleeding disorder, hemophilia A, which affects 1 in 5000 males worldwide. Hemophilia A is an X-linked disorder of variable severity that is due to mutations in the factor VIII gene, which is 187 kilobases long and contains 26 exons (6, 7). Hemophilic mutations may cause defects in factor VIII expression, secretion, and/or half-life in circulation. Alternatively some hemophilic mutations can generate stable but dysfunctional factor VIII. An international data base of point mutations that are associated with hemophilia A lists 50 unique missense mutations within the C2 domain that all have been observed *in vivo* and are associated with variable severity of disease symptoms (30). Comparison of the specific clotting activity in the plasma of these patients with their circulating factor VIII antigen level (as well as observing their incidence of antibody inhibitor development) can aid in identifying the effect of different hemophilic mutations. Unfortunately these data have been uniformly reported for only a minority of these patients.

The crystal structure of the factor VIII C2 domain has been solved to 1.5-Å resolution and in complex with the Fab fragment from a patient-derived antibody inhibitor at 2.0-Å resolution (11, 31). These structures show an eight-stranded β-sandwich topology indicative of the discoidin protein fold family. The membrane-binding surface of the domain consists of two protruding β-hairpin turns that display four solvent-exposed hydrophobic residues (Met-2199, Phe-2200, Leu-2251, and Leu-2252) and an adjacent ring of positively charged basic residues (Arg-2215, Arg-2220, Lys-2227, and Arg-2320). The inhibitory antibody, which blocks binding of the C2 domain to

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[1] The abbreviations used are: fVIII, factor VIII; vWF; von Willebrand factor; PS, phosphatidylserine; ELISA, enzyme-linked immunosorbent assay; TBS, Tris-buffered saline; BSA, bovine serum albumin; mut, mutant; wt, wild type.

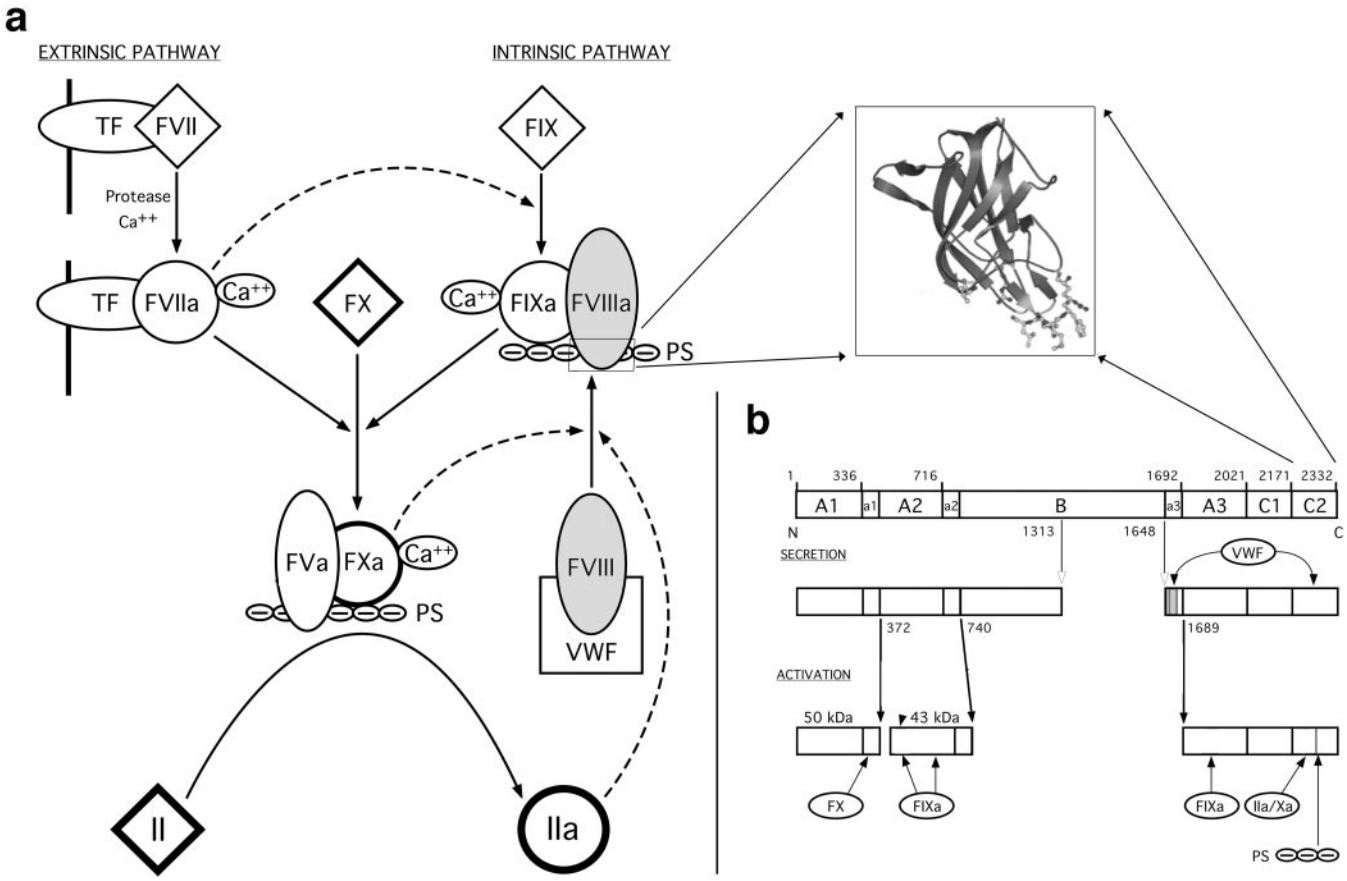

FIG. 1. **Factor VIII and the coagulation cascade.** *a*, the blood coagulation cascade consists of two pathways (extrinsic and intrinsic) that are initiated by the exposure of tissue factor (*TF*) or PS groups of activated platelet membranes to circulating protein factors, respectively. Factor VIII is a large plasma glycoprotein that acts as an initiator and regulator of the intrinsic pathway (5). Upon proteolytic activation by either factor Xa or thrombin, activated factor VIII (factor VIIIa) dissociates from vWF, associates with the factor IXa serine protease, and directs the localization of the resulting complex to the membrane surface of activated platelets via an interaction with its carboxyl-terminal C2 domain (structure in *inset*) (45). The membrane-bound factor VIIIa-factor IXa complex functions to proteolytically activate factor X (3), which then activates thrombin (factor II). *b*, domain structure of factor VIII. Factor VIII is synthesized as a single polypeptide chain of 2332 residues (2). Based on sequence homology, factor VIII has the domain structure A1-A2-B-A3-C1-C2 as described in the text (4, 6, 46). Membrane association is primarily accomplished through the C2 domain; its deletion completely abrogates binding of factor VIII to platelet surfaces (17). Crystal structures of the factor VIII C2 domain (*inset*) indicate its probable membrane-binding surface (11, 31). *F*, factor.

both membrane surfaces and to vWF, completely sequesters this region from solvent, indicating that it may be directly involved in both binding activities.

High resolution crystal structures of the factor VIII C2 domain also provide an opportunity to analyze the position and role of each hemophilic missense mutation within this region of the protein (32). Approximately 50% of these missense mutations display greater than 10% surface exposure. While these solvent-exposed mutations may cause a destabilization or misfolding event in the factor VIII C2 domain, it seems plausible that some might disrupt the ability of factor VIII to bind either activated platelet surfaces and/or vWF, causing a loss in procoagulant function.

In this study, the position and surface exposure of hemophilic mutations localized to the factor VIII C2 domain were reassessed. Mutations that have greater than 10% surface exposure were produced and subsequently analyzed for their effects on solubility during protein expression and purification, protein stability, PS binding, and vWF binding. A total of 20 unique mutations at 13 different amino acid positions were characterized. Based on these results, disease-associated mutations in the fVIII C2 domain were segregated into those that disrupt structure and stability, those that uniquely affect either membrane binding or vWF association, and those that cause defects in fVIII expression or function that are independent of the biophysical properties of the isolated C2 domain. The analysis serves as a detailed example of the dissection of a genetic molecular disorder where multiple structure-function phenotypes are correlated with the same disease state through different but related effects on protein activity.

MATERIALS AND METHODS

Reagents—A factor VIII C2 domain-specific monoclonal antibody, ESH-8, was purchased from American Diagnostica, Inc. (Greenwich, UK). An Fc-specific, goat anti-mouse IgG that is conjugated with alkaline phosphatase was purchased from Sigma. 96-well Maxisorp plates were purchased from Nunc. Phosphatidyl-L-serine was purchased from Avanti Polar Lipids, Inc. (Alabaster, AL). Bovine serum albumin (fraction V powder) and *p*-nitrophenyl phosphate were purchased from Sigma. Purified von Willebrand factor was a generous gift from M. Jacquemin (Leuven, Belgium). BL21(DE3), AD-494™ and Rosetta-gami™ cell lines were purchased from Novagen (Madison, WI). TALON™ metal affinity resin was purchased from Clontech.

Structural Analysis of Hemophilic Mutations—Characterization of hemophilic mutations localized to the factor VIII C2 domain was performed as described previously (32). Solvent accessibility of all side chains from the 1.5-Å crystal structure of the C2 domain was tabulated from the program NACCESS. These values ranged from 0% (completely buried, core residues) to 100% (completely solvent-exposed) as compared with the same residue in an "Ala-*X*-Ala" tripeptide in a fully extended conformation (33). Residues that displayed values between 10 and 100% in this analysis (Fig. 2*A*) were considered to be partially to fully solvent-exposed and targeted for subsequent biochemical studies on the assumption that a subset of such mutations might display unique deficiencies in function, apart from deficiencies of core stability,

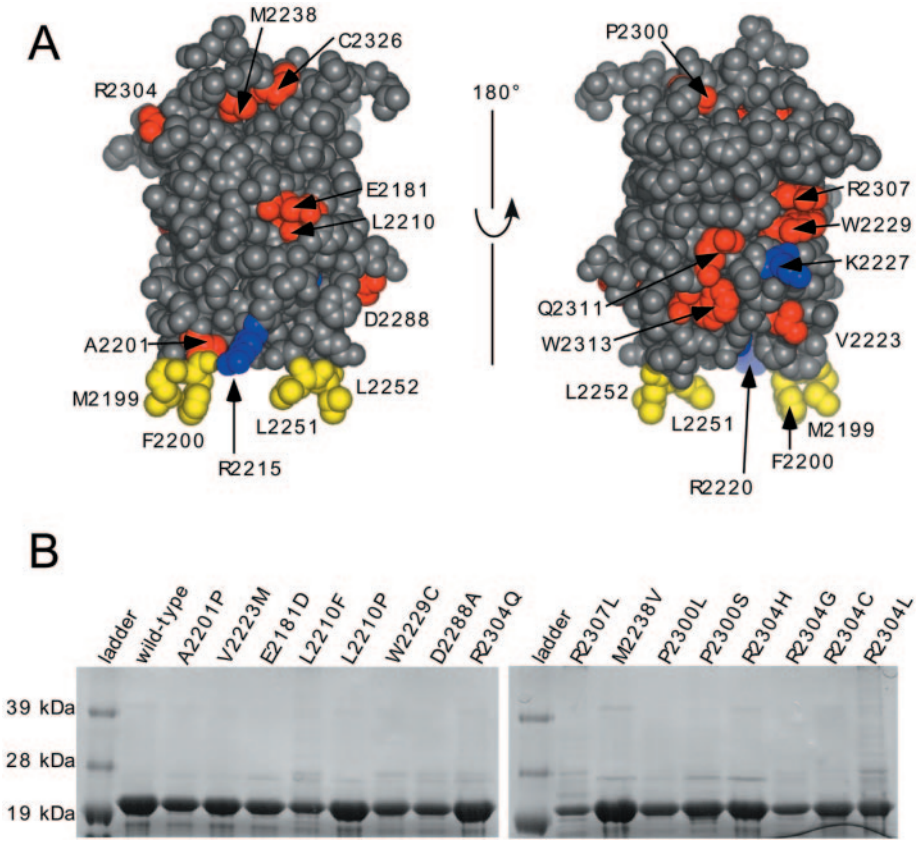

FIG. 2. **Solvent-exposed hemophilic mutations localized to the factor VIII C2 domain.** *A*, van der Waals surface space-filled model of the factor VIII C2 domain. Solvent-exposed hemophilic mutations are shown in *red*. Hydrophobic and basic residues involved in the putative membrane binding surface are shown in *yellow* and *blue*, respectively. *B*, Coomassie-stained SDS-polyacrylamide gel of soluble C2 domain mutants. 16 of 20 hemophilic mutations are soluble (not shown: R2307G, Q2311P, W2313R, and C2326S).

that illustrate the relative significance of such functions in coagulation and hemostasis. Site-directed mutagenesis of the factor VIII C2 domain was done using the Stratagene QuikChange® kit following the standard protocols supplied by the manufacturer. High pressure liquid chromatography-purified oligonucleotides for site-directed mutagenesis were ordered from Operon (Qiagen).

C2 Domain Expression and Purification—A construct containing the factor VIII C2 domain (residues 2171–2332) was subcloned into a pET15b plasmid (Novagen) using the restriction enzymes NdeI and BamHI (New England Biolabs) and transformed into one of three cell lines (BL21(DE3), AD-494, and/or Rosetta-gami). Ten-ml cultures were grown overnight from a single colony at 37 °C in Luria Broth (LB) medium containing 35 mg/liter chloramphenicol and 100 mg/liter ampicillin (as well as 25 mg/liter kanamycin for AD-494 and 25 mg/liter tetracycline for Rosetta-gami). One-liter cultures were inoculated with 10 ml of overnight cultures and grown to an A_{600} of 0.8–1.0. Protein expression was induced by the addition of 1 mM isopropyl-thio-β-D-galactosidase. Inductions were grown for 16–20 h at 16 °C with shaking.

Cells were lysed by sonication in 300 mM NaCl, 20 mM Tris-HCl (pH 7.0), 10 mM imidazole, 0.01% (v/v) Triton X-100, 100 μM phenylmethylsulfonyl fluoride, and 2.5% (v/v) glycerol. Lysates were centrifuged at 16,000 rpm in an SS-34 rotor (Sorvall) for 30 min. The supernatant was applied to TALON metal affinity resin at 4 °C for 1.5–2.0 h. TALON resin was pelleted from the supernatant by centrifugation in a tabletop centrifuge at 1500 rpm at 4 °C. The resin was washed with 20 column volumes of 300 mM NaCl, 20 mM Tris-HCl (pH 7.0), 10 mM imidazole, and 2.5% (v/v) glycerol. The resin was then washed with 20 column volumes of 150 mM NaCl, 20 mM Tris-HCl (pH 7.0), 10 mM imidazole, and 2.5% (v/v) glycerol. The protein-resin slurry was then applied to a gravity low column (Bio-Rad). The pure fraction was eluted in 150 mM NaCl, 20 mM Tris-HCl (pH 8.0), 150 mM imidazole, and 2.5% (v/v) glycerol and subsequently dialyzed into 150 mM NaCl, 20 mM Tris-HCl (pH 7.4), and 2.5% (v/v) glycerol. Purified factor VIII C2 domain mutants (Fig. 2*b*) were concentrated to between 0.5 and 5 mg/ml (dependent on yield and solubility).

CD Measurements of Folding and Stability—The stabilities of the recombinant factor VIII C2 domain and soluble hemophilic mutants were measured by chemical denaturation using circular dichroism as described previously (34). CD spectra on the wild-type domain (Fig. 3) and on the mutants were collected on an Aviv 62A DS spectrometer. All protein samples were diluted in 1× phosphate-buffered saline. Far-UV CD wavelength scans (200–260 nm) (Fig. 3) at varying protein concentrations (5–20 μM) were collected using a 1-mm pathlength cuvette. Protein denaturation due to guanidine HCl titration was followed by change in ellipticity at 220 nm (Fig. 4 and Table I). Measurements were carried out using a 1-cm pathlength cuvette and a Microlab titrator (Hamilton) for guanidine HCl mixing. To obtain the Gibbs free energy of unfolding (ΔG_U), chemical denaturation curves were fit by non-linear least-squares analysis using a linear extrapolation model as described previously (35, 36). For each mutant, three independent denaturation

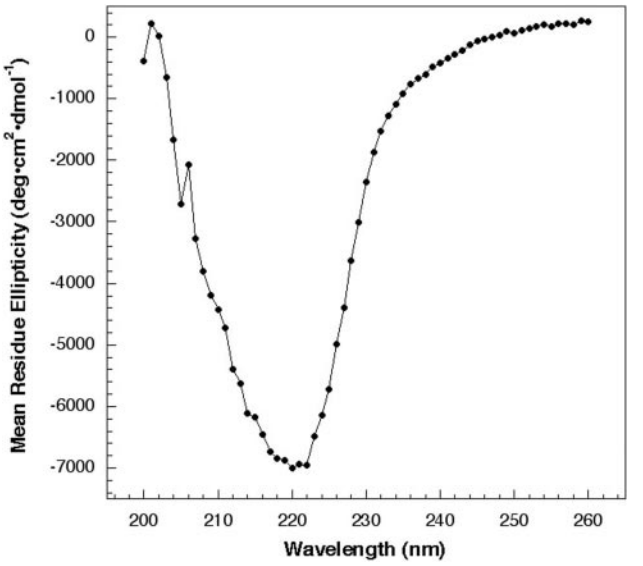

FIG. 3. **Circular dichroism spectrum of wild-type factor VIII C2 domain.** The CD spectrum of the wild-type C2 domain shows the expected signal for the wild-type secondary structure content. The signal recorded at 220 nm was used during chemical denaturation experiments. *deg*, degrees.

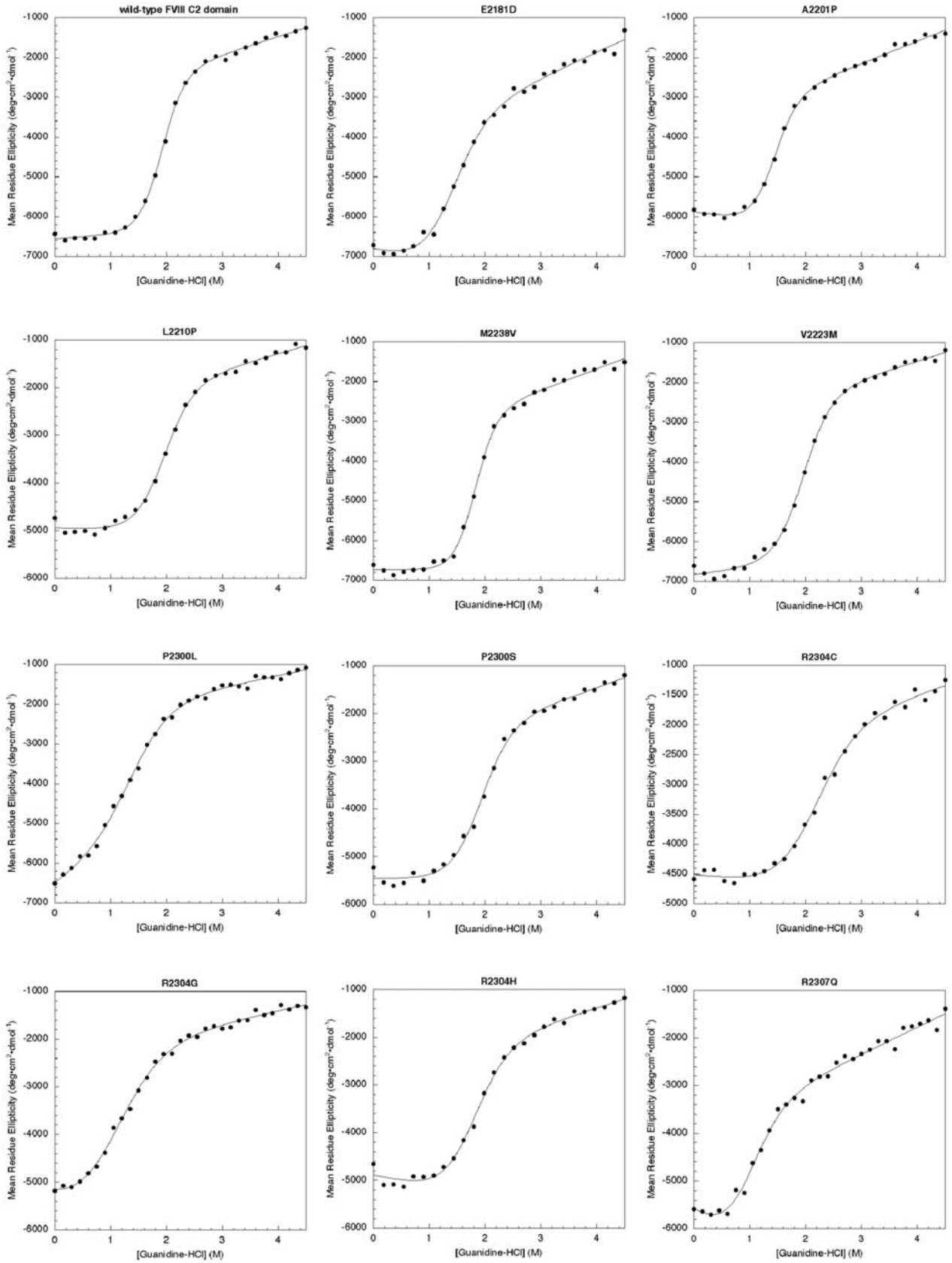

FIG. 4. **Chemical denaturation of factor VIII C2 domain hemophilic mutants.** The guanidine HCl-induced denaturation of each mutant indicates two-state, cooperative unfolding. Ellipticity was measured as a function of denaturant concentration at 220 nm. The Gibbs free energy of unfolding was determined by a non-linear least-squares analysis using a linear extrapolation model. *deg*, degrees.

experiments were performed, and standard deviation values of ΔG were calculated. $\Delta\Delta G$ values (Table I, final column) correspond to the relative difference in the free energy change (ΔG) of unfolding between the wild-type and mutant domains; a negative value indicates destabilization of the mutant with the extent of destabilization proportional to the magnitude of $\Delta\Delta G$.

TABLE I
Summary of experimental results

Mutation	SE[a]	Solubility[b]	PS binding[c]	VWF binding[c]	$\Delta G_{(N-U)}$[c]	$\Delta\Delta G_{(wt \rightarrow mut)}$[c]
	%		%	%	*kcal/mol*	
Wild type		Yes	100 ± 10	100 ± 9	−4.58 ± 0.44	0.00
E2181D	52.6	Yes	84 ± 2	113 ± 13	−2.67 ± 0.51	−1.91
A2201P	35.7	Yes	41 ± 10	123 ± 17	−1.77 ± 0.43	−2.81
L2210F	10.2	Low	84 ± 34	134 ± 6		
L2210P	10.2	Yes	91 ± 2	89 ± 34	−4.32 ± 0.49	−0.26
V2223M	66.8	Yes	105 ± 7	98 ± 1	−5.48 ± 0.36	+0.90
W2229C	25.3	Low	137 ± 36	105 ± 16		
M2238V	11.4	Yes	94 ± 1	84 ± 24	−5.57 ± 0.55	+0.99
D2288A	52.1	Low	112 ± 6	138 ± 10		
P2300L	36.8	Yes	41 ± 14	26 ± 16	−2.22 ± 0.45	−2.36
P2300S	36.8	Yes	101 ± 1	37 ± 1	−4.15 ± 0.58	−0.43
R2304C	40.6	Yes	93 ± 1	117 ± 31	−3.47 ± 0.60	−1.11
R2304G	40.6	Yes	13 ± 4	23 ± 11	−1.49 ± 0.38	−3.09
R2304H	40.6	Yes	81 ± 2	32 ± 7	−3.40 ± 0.52	−1.18
R2304L	40.6	Low	20 ± 9	26 ± 7		
R2307G	18.7	No				
R2307Q	18.7	Yes	54 ± 21	49 ± 2	−1.90 ± 0.63	−2.68
R2307L	18.7	Low	20 ± 9	14 ± 2		
Q2311P	27.0	No				
W2313R	21.1	No				
C2326S	42.7	No				

[a] Relative solvent exposure as calculated by the program NACCESS.
[b] Qualitative estimate of partitioning into soluble *versus* insoluble fractions based on electrophoretic analyses of whole cell lysate, soluble fractions, and pellets. Yes, ~80–100% soluble fraction; low, 10–80%; no, <10%.
[c] All biophysical binding and stability measurements were made in triplicate (at each protein concentration across the linear range of the ELISA assays and at a single protein concentration for the CD denaturation studies). The ± values in the table represent the standard deviation of the final calculated values for relative PS and VWF binding assays (calculated from nine separate measurements for each mutant distributed across three separate protein concentrations) and ΔG of unfolding (three separate measurements at a single protein concentration for each mutant). The derivation of ΔG and $\Delta\Delta G$ values of unfolding is described under "Materials and Methods."

PS-binding Enzyme-linked Immunosorbent Assay (ELISA)—The interactions between soluble factor VIII C2 domain mutants and PS were measured using a solid phase ELISA that was adapted from methods described previously (28, 37). 96-well Maxisorp plates were incubated with 100 μl of 5 μg/ml PS dissolved in methanol and dried overnight at room temperature or for 2 h at 37 °C. Plates were then blocked with 200 μl of 2% (w/v) bovine serum albumin in Tris-buffered saline (BSA-TBS). 100 μl of each soluble C2 domain mutant was serially diluted to concentrations between 5 and 50 nM in BSA-TBS and were incubated on the PS-coated plates for 60 min at 37 °C with shaking. These protein concentrations correspond to the linear range of the assay dose response before saturation of the membrane surface by the C2 domain becomes observable. Plates were manually rinsed three times with TBS between all incubation steps. Subsequent to the C2 domain incubation, 100 μl of the C2 domain-specific monoclonal antibody ESH-8 diluted 1:1000 in BSA-TBS was added. 100 μl of an alkaline phosphatase-conjugated goat anti-mouse antibody was diluted 1:4000 in BSA-TBS and then added as the secondary antibody. The C2 domain mutants that had bound to the PS-coated plate were detected by the addition of 200 μl of *p*-nitrophenyl phosphate followed by absorbance measurements at 405 nm using a microplate reader.

The relative binding activities of the point mutants described under "Results" compared with the wild-type C2 domain (Fig. 5 and Table I) correspond to the average of the change in signal in the ELISA assay across the range of protein concentrations. Similar to the wild-type domain, the mutants all display a linear dose response in the assay across these concentrations. All measurements were made in triplicate at all protein concentrations, allowing calculation of the standard deviation of relative binding activities using at least nine separate measurements distributed across three protein concentrations for each mutant.

VWF-binding ELISA—A factor VIII C2 domain-vWF binding assay using the C2 domain-specific monoclonal antibody (ESH-8) was carried out to detect the vWF binding abilities of hemophilic mutations localized to the C2 domain. Briefly 96-well Maxisorp plates were incubated with 50 μg/ml plasma purified vWF diluted in 100 mM $NaHCO_3$ (pH 9.5) overnight at 4 °C. VWF-immobilized plates were subsequently washed once with 150 mM NaCl, 20 mM Tris-HCl (pH 7.4), 0.2% BSA, and 250 mM $CaCl_2$ and then twice with 150 mM NaCl, 20 mM Tris-HCl (pH 7.4), 0.2% BSA, and 2.5 mM $CaCl_2$ (wash buffer). Plates were then blocked with 200 μl of BSA-TBS and 2.5 mM $CaCl_2$. 100 μl of each soluble C2 domain mutant was serially diluted between 20 and 1.25 nM in BSA-TBS and 2.5 mM $CaCl_2$ and then incubated on the vWF-immobilized plates for 60 min at 37 °C with shaking. As described above for the membrane binding assay, these protein concentrations correspond to the linear range of the assay dose response before saturation of the signal becomes observable. Plates were washed between all incubations three times with 200 μl of wash buffer. The bound C2 domain was detected as in the PS-binding ELISA protocol (see above).

The relative binding activities of the point mutants described under "Results," relative to the wild-type C2 domain (Fig. 5), correspond to the average of the change in signal in the ELISA assay across the range of protein concentrations. Similar to the wild-type domain, the mutants all displayed a linear dose response across these concentrations. All measurements were made in triplicate at all protein concentrations, allowing calculation of the standard deviation of relative binding activities using at least nine separate measurements distributed across three protein concentrations for each mutant.

Dynamic Light Scattering Analysis of Protein Solution Behavior—Three of the mutants studied (L2210F, W2229C, and D2288A) partitioned into the soluble fraction of cell lysate during expression and displayed normal binding activities toward both membranes and vWF (Figs. 2 and 5) but were not expressed in sufficient amounts to permit quantitative CD denaturation assays (which requires multimilligram quantities of protein). The oligomeric solution behavior of these mutants and the wild-type domain were analyzed by dynamic light scattering (38) using a Dynapro-800 instrument (Proterion Inc.) to determine whether they display abnormal levels of aggregation that might lead to elevated, artifactual binding signal in the ELISA assays.

RESULTS

Surface Accessibility of Hemophilia-associated Mutations—A total of 50 unique missense mutations were localized to the C2 domain of factor VIII. Of these, 20 mutations at 13 different residues have surface exposures of greater than 10% as reported by the program NACCESS (Fig. 2 and Table I). The calculated surface exposure values are shown for all positions in the second column of Table I. Further inspection of these mutations indicated that they occur somewhat non-uniformly across the protein surface. There is a relative dearth of mutations that occur at the putative membrane-binding surface (only A2201P and V2223M reside close to the solvent-exposed hydrophobic β-hairpin turns), and many of the mutations cluster into pairs across the protein surface. The following residues

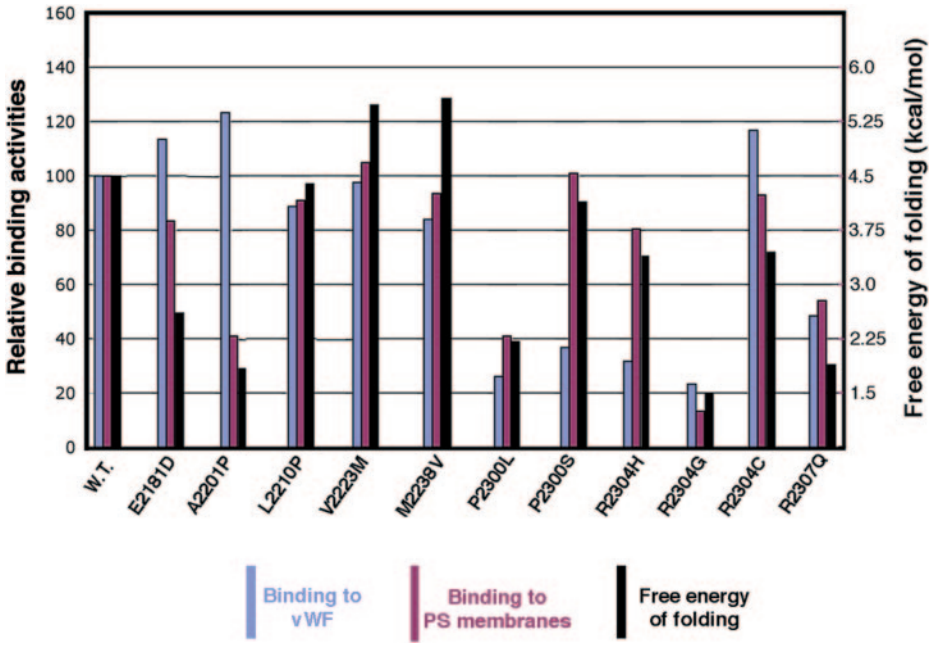

FIG. 5. **Relative stabilities and binding activities of surface-exposed C2 domain point mutants.** Relative levels of measured binding activities and thermal stabilities were measured as described under "Materials and Methods." Only those 11 constructs that could be assayed for their folding stability and for PS membrane and vWF binding activities are shown. Those constructs that did not express efficiently enough to allow determination of folding stability are described in the text. Note that while many constructs demonstrate highly correlated patterns of both destabilization and loss of binding activities, some constructs display uncoupling of the two binding activities and/or differential effects on stability and function. In particular, membrane binding activity of A2201P is uniquely reduced but displays wild-type vWF binding; in contrast P2300S and R2304H display reduced vWF binding but wild-type membrane binding activities.

in the C2 domain crystal structure make van der Waals contacts to each other: Glu-2181 and Leu-2210, Met-2238 and Cys-2326, Arg-2307 and Trp-2229, and Gln-2311 and Trp-2313. Finally three positions with one or more hemophilic mutations (D2288A, P2300S/L, and R2304C/G/H/L are significantly solvent-exposed (>35%), occur in loop regions, and are usually associated with measurable clotting activity and particularly mild hemophilic phenotypes.

Expression and Solubility—To examine the binding and stability of the solvent-exposed hemophilic mutations that localize to the factor VIII C2 domain, an *Escherichia coli*-based expression and metal affinity purification scheme was devised (39). The AD-494 and Rosetta-gami cell lines (cells that are deficient in a thioredoxin reductase gene) were utilized to properly express and fold the C2 domain, which contains one disulfide bond. Histidine-tagged C2 domain was purified by metal affinity chromatography and tested for PS binding. The wild-type C2 domain bound specifically to PS surfaces but did not bind specifically to uncharged phosphatidylcholine surfaces, indicating that the protein is properly folded (39).

Attempts were made to express and purify all 20 missense mutants. Of these constructs, at least six appeared to be severely compromised on the basis of their expression behavior and solubility. Four of these (R2307G, Q2311P, W2313R, and C2326S) partitioned entirely into the insoluble fraction of the cell lysate and were not studied further. Another two (R2304L and R2307L) exhibited very low expression levels, purification yields, and negligible binding activities and therefore are almost certainly structurally destabilized.

An additional three mutants (L2210F, W2229C, and D2288A) were also poorly expressed and recovered in low yields but displayed wild-type binding activities. Those three mutants were analyzed for aggregation behavior using quasielastic (dynamic) light scattering and were found to behave similarly to the wild-type C2 domain, forming relatively monodisperse solutions of individual monomers at concentrations approaching 1 mg/ml. Therefore, the effects of these three mutations on fVIII function appear to be independent of its intrinsic membrane and vWF binding functions.

Correlation of Mutational Effects on Domain Stability and Individual Binding Activities—The remaining 11 mutants of the C2 domain behaved well enough during expression and purification to permit accurate determinations of their Gibbs free energy of folding and also of their relative membrane and vWF binding activities (Table I and Figs. 3–5). Chemical denaturation experiments were performed using guanidine hydrochloride titrations and CD spectroscopy to determine the relative stability of each mutant. The wild-type C2 domain was subjected to a wavelength scan to determine the extent of its secondary structure from its associated CD spectra. As expected from a mostly β-sheet protein, a minimum value was observed at 220 nm (Fig. 3). This wavelength was used to follow subsequent unfolding during guanidine hydrochloride titration experiments where denaturant was titrated from 0 to 4.5 M in 30 incremental injections.

All 11 mutations that were analyzed displayed cooperative unfolding, indicative of a simple two-state unfolding process (Fig. 4). Analyses of these mutants allowed calculation of their free energy change of unfolding (ΔG) and the difference in these values relative to the wild-type domain ($\Delta\Delta G$) as described under "Materials and Methods." These values are shown for each mutant in the final two columns of Table I. Seven of these mutations were destabilizing, while four mutations (L2210P, V2223M, M2238V, and P2300S) displayed stabilities that are similar to or superior to the wild-type domain. Of the seven destabilizing mutations, five (E2181D, A2201P, P2300L, R2304G, and R2307Q) gave reductions in folded stability of greater than 1.5 kcal/mol ($\Delta\Delta G_{(wt \rightarrow mut)}$ = −1.9, −2.8, −2.4, −3.1, and −2.7 kcal/mol, respectively). Two mutations (R2304C and R2304H) were less destabilizing ($\Delta\Delta G_{(wt \rightarrow mut)}$ = −1.1 and −1.2 kcal/mol, respectively).

The relative abilities of these 11 hemophilic mutant C2 domains to bind either PS- or vWF-coated surfaces were examined using an ELISA method for detection of protein-protein and protein-membrane interactions as described under "Materials and Methods." Of these mutants, one (A2201P, which is located near a β-hairpin structure on the putative membrane-binding surface) was deficient in its membrane binding activity but displayed normal vWF binding activity. This mutation was moderately destabilizing ($\Delta\Delta G_{(wt \rightarrow mut)}$ = −2.8 kcal/mol). A second mutation (E2181D) also displayed moderate destabilization and reduction of only its membrane binding function, but the effect was not as pronounced as for A2201P.

In contrast, two different mutations (P2300S and R2304H)

displayed near wild-type stability and membrane binding activity but were deficient in vWF binding. Both of these mutations caused a slight to moderate destabilization but a reduction of relative vWF binding to 30–40% of wild-type activity.

Finally three mutations in this analysis (P2300L, R2304G, and R2307Q) displayed loss of both binding activities that are well correlated with the extent of structural destabilization for each construct. In direct contrast, four mutations (L2210P, V2223M, M2238V, and R2304C) displayed little or no significant decrease in their stability or in either binding activity, indicating that those mutations may act either upstream of protein function (for example, by interfering with splicing of the full-length factor VIII message) or on alternative functions or properties of the fVIII protein (such as the interactions between the C2 domain and the rest of the protein or the association of fVIII with factor IX or thrombin).

Four sets of mutations were clustered at common positions (L2210F or -P; P2300L or -S; R2304C, -G, -H, or -L; and R2307G, -Q, or -L). Of these sets of mutations, only those at residue 2210 displayed a somewhat common effect (a significant reduction in the expression and recovery of soluble material). Mutations at the other three positions each yielded diverse molecular phenotypes of destabilization and loss of binding functions.

DISCUSSION

In this study, we reassessed the surface-exposed hemophilic mutations that localize to the factor VIII C2 domain. The rationale for concentrating on these residues was that they might display greater diversity of molecular dysfunction than mutations found in the protein core (which primarily affect domain folding and stability) and thereby facilitate a comparative study of diverse molecular defects that cause a heterogeneous genetic disorder such as hemophilia A. Also of interest was whether extensive biophysical studies on an isolated structural domain that can be manipulated extensively and efficiently in a bacterial system can be used to generate information that is relevant to the full-length, biologically active parental molecule.

An international data base of point mutations that are associated with hemophilia A (30) lists known mutations within the fVIII protein, including missense substitutions localized to the C2 domain. This data base provides a useful (although incomplete) tabulation (for each mutation) of the relative measurable level of factor VIII-dependent clotting activity, of circulating factor VIII antigen, of whether antibody inhibitors are induced by fVIII replacement therapy, and of the range of disease symptoms associated with the mutation (mild to severe). An observation of low clotting activity combined with measurable circulating fVIII and an absence of inhibitor development is generally interpreted as a dysfunctional but structurally stable fVIII protein. Conversely observation of low clotting activity combined with low or absent circulating fVIII and the development of inhibitors is generally interpreted as indicative of a structurally misfolded or unstable protein.

Of the four hemophilic mutations (R2307G, Q2311P, W2313R, and C2326S) that exhibited complete insolubility in protein expression trials, presumably due to either a misfolding event or severe destabilization of the protein structures, three are associated with at least one incidence of severe hemophilic symptoms and have less than 1% of factor VIII-specific clotting activity in circulation (only W2313R is not associated with severe symptoms and has 2% specific clotting activity) (30). Two additional mutations (R2304L and R2307L) were poorly expressed and/or recovered, were not amenable to performing chemical denaturation experiments, and displayed poor binding activity against both membranes and vWF. The behavior of those constructs also appeared to be caused by significant destabilization of the protein fold and was found to correspond to mutations that yield variable circulating protein levels and disease severity.

Three mutations (L2210F, W2229C, and D2288A) were also poorly expressed but displayed wild-type aggregation behavior and binding activities. Two of these mutants (L2210F and D2288A) were found to correspond to reduced circulating protein but mild disease states and an absence of antibody inhibitors. In contrast, the third (W2229C) causes variable disease symptoms and is associated with inhibitor development. It is possible that at least the first two of these mutants may display poor trafficking or secretion, leading to reduced levels of still functional factor VIII. These observations and hypothesis are in agreement with previous studies on additional hemophilic missense mutations elsewhere in the protein structure that appear to affect the secretion of functional factor VIII molecules, thus reducing the serum concentration of factor VIII antigen (40–42).

Of particular interest to us were those mutants that appeared to display unique, uncoupled deficiencies in their relative binding activities toward PS membranes or vWF. The A2201P mutation, which exhibited poor membrane binding activity (but was fully active in the vWF assay), is associated with mild disease phenotype, a high level of circulating antigen (36% relative to wild type), and a lack of antibody inhibitors. Thus, the phenotype of this mutation seems appropriate for a dysfunctional but still secreted and circulating protein factor. Previous observations have shown that the complete deletion of Ala-2201 in full-length factor VIII causes a loss in both PS and vWF binding, supporting the observation that the mutant A2201P also causes loss in PS binding (43). The HamSters data base lists the Ala to Pro point mutation at this position as causing a deficiency in vWF binding; however, given its high level of circulating antigen (which would not be observed if the protein was defective in associating with vWF) and the results reported here, this entry may be in need of revised annotation. A second mutation (E2181D) that displayed a similar but less pronounced molecular phenotype also is associated with mild hemophilia symptoms and retention of significant, but reduced, clotting activity.

In comparison, two mutations that we observed to be stable but deficient only in vWF binding function (P2300S and R2304H) are associated with variable and mild disease, respectively. Like the A2201P mutation that specifically perturbed membrane binding activity, these mutants are associated with reduced but still significant clotting activity. No circulating protein levels are reported for either mutant. While vWF binding has not been previously reported for either mutation, a related lesion (P2300L) is reported to be deficient in vWF binding.

A variety of mutations that displayed significant destabilization (including E2181D, A2201P, P2300L, R2304G, and R2307Q with values of $\Delta\Delta G_{(\mathrm{wt} \rightarrow \mathrm{mut})}$ between -1.9 and -3.1 kcal/mol) had correlated defects in binding to PS and/or vWF surfaces. While these mutations showed defects in at least two of the primary functions of the factor VIII C2 domain, each mutation has detectable factor VIII-specific clotting activity and antigen levels (30). This suggests that while these mutations were destabilized within the isolated C2 domain, the entire factor VIII molecule is, to some extent, properly synthesized and secreted to circulation. Indeed all of these mutations only cause mild or moderate hemophilic symptoms. While these C2 domain mutations appeared to not function as well as wild type, they still provide some degree of procoagulant activity, thus preventing severe bleeding complications that are associ-

ated with cases of no detectable factor VIII-specific clotting activity.

Therefore, most or all mutations that are associated with severe disease symptoms and many with moderate or heterogeneous defects either exhibit compromised expression or severe destabilization in this study. In contrast, mutations that cause more moderate levels of destabilization or unique patterns of inhibition of binding functions appear to correlate with less severe disease. Additional analyses from other laboratories (42–44) have shown that various combinations of alanine mutations among the solvent-exposed hydrophobic residues across the membrane-binding surface of the C2 domain cause a significant loss in the ability of factor VIII to bind to PS and vWF. This also supports the hypothesis that this region is important for PS and vWF interaction (44).

Finally some mutations did not affect binding or stability of the C2 domain in the studies reported here. These mutations might exhibit defects in other activities that cannot be easily tested for the isolated domain, such as binding to factor Xa or thrombin (24, 25). Interactions between the C2 domain and adjacent domains within factor VIII (*i.e.* C1 and/or A3 domains) might also be disrupted. In addition, the mutation V2223M (which is localized to the putative membrane-binding surface) had no defects in PS and vWF binding or stability. It has been reported that this mutation might cause a splicing defect in the factor VIII gene (30) rather than a direct, significant defect in the structure and function of the C2 domain. Interestingly valine at position 2223 in the factor VIII protein sequence is not strictly conserved as this residue is an alanine in canine factor VIII and in the factor V C2 domain of three different species.

In conclusion, we systematically constructed each surface-exposed hemophilic mutation and performed tests for solubility, PS and vWF binding, and protein stability. Our observations indicate that in most cases, these mutations caused a destabilization of the C2 domain structure. The degree of destabilization clearly has implications for the severity of the hemophilic phenotype. Most mutations that rendered the C2 domain completely insoluble are associated with severe forms of hemophilia A. Mutations that resulted in limited solubility or severe destabilization as measured by chemical denaturation also had defects in PS and vWF binding but are associated with mild and/or moderate hemophilic symptoms. Finally these observations may have implications for the development of novel, recombinant factor VIII therapies. While it is apparent that the binding of factor VIII to its various protein and membrane targets must be conserved or enhanced, it may also be important to take protein stability and solubility into account.

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