THROMBOSIS AND HEMOSTASIS

Solution structure of the major factor VIII binding region on von Willebrand factor

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Key Points
- The high-resolution structure of the complex disulfide-bonded TIL,E′(D′) region of VWF is presented.
- The major factor VIII binding site is localized around a flexible region on the TIL′ domain.

Although much of the function of von Willebrand factor (VWF) has been revealed, detailed insight into the molecular structure that enables VWF to orchestrate hemostatic processes, in particular factor VIII (FVIII) binding and stabilization in plasma, is lacking. Here, we present the high-resolution solution structure and structural dynamics of the D′ region of VWF, which constitutes the major FVIII binding site. D′ consists of 2 domains, trypsin-inhibitor–like (TIL′) and E′, of which the TIL′ domain lacks extensive secondary structure, is strikingly dynamic and harbors a cluster of pathological mutations leading to decreased FVIII binding affinity (type 2N von Willebrand disease [VWD]). This indicates that the backbone malleability of TIL′ is important for its biological activity. The principal FVIII binding site is localized to a flexible, positively charged region on TIL′, which is supported by the rigid scaffold of the TIL′ and E′ domain β sheets. Furthermore, surface-charge mapping of the TIL′E′ structure reveals a potential mechanism for the electrostatically guided, high-affinity VWF FVIII interaction. Our findings provide novel insights into VWF FVIII complex formation, leading to a greater understanding of the molecular basis of the bleeding diathesis type 2N VWD. (Blood. 2014;123(26):4143-4151)

Introduction

The preservation of hemostatic integrity is secured by the activities of von Willebrand factor (VWF). Upon vascular damage, VWF acts as a molecular bridge facilitating the initial adhesion and aggregation of platelets to the site of vessel injury. Furthermore, VWF is the protective carrier of procoagulant factor VIII (FVIII) in plasma, thereby prolonging its half-life and efficiently localizing FVIII to the incipient platelet plug.1,2 The arrest of bleeding is critically dependent on VWF, as exemplified by von Willebrand disease (VWD), the most common inherited bleeding disorder in humans, which results from defective or deficient VWF protein.3

The importance of VWF-FVIII complex formation is illustrated by patients with severe VWD, who have undetectable VWF levels. Not only do these patients have a concomitant deficiency of FVIII, but they also have a considerably shortened survival of intravenously administered FVIII.4 This phenotype, which mimics hemophilia A, is also observed in patients with type 2N VWD, whose VWF harbors mutations that lead to decreased FVIII binding affinity.4 This behavior reflects the dependence of FVIII survival on the formation of VWF-FVIII complexes and illustrates the biological significance of this interaction. Hence, the importance of elucidating the structure of the FVIII binding region on VWF is 2-fold. First, it would provide insights into the mechanism of FVIII binding to VWF and reveal the minimal VWF unit required to stabilize FVIII. Second, analysis of VWD patient mutations against a background of both structural and functional perturbations will provide the link between genetic pathology and clinical phenotype.

Electron microscopy studies have given a first glimpse of the domain arrangement and overall structure of the multidomain VWF protein5 and in particular showed that the trypsin-inhibitor-like (TIL′) E′ domains form a protrusion from the D3 domain, thereby presenting the TIL′E′ domains to the physiological binding partner FVIII. However, the limited resolution of these structures has precluded the elucidation of the details needed to examine the molecular recognition and binding of FVIII to VWF. In addition, high-resolution structure determinations of the topologically complex disulfide-bonded VWF domains have not hitherto been feasible, except for the triplicated A domains,6,7 which are notable for their...
relative scarcity of cysteine residues. Thus, a detailed characterization of the interaction between VWF and FVIII has remained intractable, until now.

The FVIII binding region on VWF has been identified within a tryptic fragment termed SPIII-T4 (residues 767-1031). A recent domain assignment of VWF, based on conserved cysteine signatures,9 reveals that SPIII-T4 is composed of 3 distinct and conserved domains, namely TIL’ (residues 766-827), E’ (residues 829-863), and VWD3 (residues 867-1031). Accumulated lines of evidence suggest that of these regions it is the TIL’ and E’ domains (Figure 1), previously known collectively as D’, that are essential for FVIII binding.3 First, 72% of unique missense mutations that directly affect FVIII binding to VWF (type 2N VWD) are found in domains TIL’ and E’.10 Importantly, the most severe type 2N phenotypes, characterized by the lowest circulating plasma FVIII levels, are due to mutations in TIL’ (Figure 1 and supplemental Figure 1 available on the Blood Web site).3 Second, epitope-mapping studies of potent FVIII binding-blocking monoclonal antibodies (mAbs) mAb-418 and E’11,12 concentrated to 450 μL with final sample concentrations of 0.2 to 0.4 mM for further analyses.

Protein purification

After harvesting, the cells were resuspended in −40 mL of wash buffer (20 mM sodium phosphate, 500 mM NaCl, 40 mM imidazole [pH 7.4]). To the cell suspension was added 1 tablet of Complete Protease Inhibitor (Roche), nonionic and non-denaturing detergent in the form of 0.25% octylphenyl-polyethylene glycol, lysozyme, and DNase (5-10 μg/mL). Cells were broken by French press and subsequently centrifuged, and the cleared lysate (−40 mL) was filtered (0.22 μm) and loaded onto a His-Trap Ni-nitrilotriacetic acid column (5 mL; GE Healthcare) pre-equilibrated with wash buffer. Bound protein was eluted using a linear gradient with increasing imidazole and pooled fractions dialyzed against 20 mM sodium phosphate, 100 mM NaCl (pH 7.4) for TEV proteolysis. TEV protease was added in the ratio of 1 OD560 unit TEV per 5 to 10 OD560 units of TRX-His6-TIL’. The reaction was incubated for 3 hours at room temperature and then at 4°C overnight to complete cleavage. Subsequently, the N-terminal TRX-His6 tag was separated from the cleaved TIL’ E’ fraction on the His-Trap Ni-nitrilotriacetic acid column. The TIL’ E’ was loaded onto a 16/60 Superdex 75 column (GE Healthcare) pre-equilibrated with 20 mM sodium phosphate, 100 mM NaCl (pH 7.4) as a final purification step. Monomeric TIL’ E’ was concentrated to 450 μL with final sample concentrations of 0.2 to 0.4 mM for further analyses.

Materials and methods

Protein expression

The TIL’E’ coding sequence (residues 766-864) was amplified from full-length VWF complementary DNA and cloned into a PET32b+ vector containing an N-terminal thioredoxin (TRX) and His6 tag. The TRX-His6 tag was separated from the TIL’E’ coding sequence by a 31-residue linker (SSGLVPRGSKMKETAAAKFERQHMDSPDLGT) and a tobacco etch virus (TEV) cleavage sequence (ENLYFQG)._leaving residues GAMG as the sole sources of carbon and nitrogen, respectively. For the 10% fractionally labeled TIL’E’ used for stereospecific assignment of the Val and Leu side-chain methyl groups, the M9 medium was supplemented with 10% U-13C-glucose and 90% unlabeled glucose. Cells were grown to an optical density (OD) at 600 nm of ~0.6, and subsequent overexpression for 16 hours at 16°C was induced by the addition of 0.4 mM isopropyl-beta-D-thiogalactopyranoside.

Protein purification

After harvesting, the cells were resuspended in −40 mL of wash buffer (20 mM sodium phosphate, 500 mM NaCl, 40 mM imidazole [pH 7.4]). To the cell suspension was added 1 tablet of Complete Protease Inhibitor (Roche), nonionic and non-denaturing detergent in the form of 0.25% octylphenyl-polyethylene glycol, lysozyme, and DNase (5-10 μg/mL). Cells were broken by French press and subsequently centrifuged, and the cleared lysate (−40 mL) was filtered (0.22 μm) and loaded onto a His-Trap Ni-nitrilotriacetic acid column (5 mL; GE Healthcare) pre-equilibrated with wash buffer. Bound protein was eluted using a linear gradient with increasing imidazole and pooled fractions dialyzed against 20 mM sodium phosphate, 100 mM NaCl (pH 7.4) for TEV proteolysis. TEV protease was added in the ratio of 1 OD560 unit TEV per 5 to 10 OD560 units of TRX-His6-TIL’E’; the reaction was incubated for 3 hours at room temperature and then at 4°C overnight to complete cleavage. Subsequently, the N-terminal TRX-His6 tag was separated from the cleaved TIL’ E’ fraction on the His-Trap Ni-nitrilotriacetic acid column. The TIL’ E’ was loaded onto a 16/60 Superdex 75 column (GE Healthcare) pre-equilibrated with 20 mM sodium phosphate, 100 mM NaCl (pH 7.4) as a final purification step. Monomeric TIL’ E’ was concentrated to 450 μL with final sample concentrations of 0.2 to 0.4 mM for further analyses.

Isotope-labeled 15N and 13C,15N samples were prepared in 20 mM sodium phosphate, 100 mM NaCl, 1% D2O at pH 7.4 for nuclear magnetic resonance (NMR) spectroscopy. Samples used for backbone residual dipolar coupling (RDC) measurements were prepared by adding PFI bacteriophage (ASLA Biotech, Latvia)14 (−5 mg/mL) or pentactenyl-2,4-bis(2-nitrobenzoic acid) which reacts with a free sulfhydryl group to 15N,13C,15N samples of pm3 E8 (Sigma-Aldrich) (3% wt/wt C12E5, r = 0.94),15 where r is the molar ratio of C12E5 to n-hexanol, with corresponding deuterium quadrupolar splittings in PFI and C12E5/n-hexanol of −1.5 Hz and 8 Hz, respectively.

Mass spectrometry

Recombinant TIL’E’ (10 μM) was buffer exchanged into 100 mM ammonium acetate (pH 7.0) for mass spectrometry (MS) experiments, which were carried out on a Syapt HDMS (Waters) mass spectrometer. A 2.5-μL aliquot of the protein sample was delivered to the mass spectrometer by means of nanoelectrospray ionization using gold-coated capillaries (prepared in-house).

Ellman’s reagents

Formation of all disulfide bonds was verified by an Ellman’s assay with 5,5’-dithio-bis(2-nitrobenzoic acid) which reacts with a free sulfhydryl group to
yield 2-nitro-5-thiobenzoic acid, which was detected spectrophotometrically at 412 nm. The Ellman’s assay yielded ~0.1 mol free -SH per mol TIL’E’, thus confirming that essentially all cysteine residues are engaged in disulfide-bond formation.

**Binding of FVIII to plasma-derived VWF and recombinant TIL’E’ fragments**

The potential FVIII binding partners (plasma-derived VWF, TIL’E’, reduced and alkylated TIL’E’) were coated directly onto a 96-well plate and incubated with an increasing concentration of FVIII. After equilibration and washing steps, bound FVIII was monitored with murine anti-FVIII and secondary anti-mouse horseradish peroxidase antibodies. Blanks were subtracted from the absorbance measurements, and 3 experiments were performed in duplicate. Details are given in supplemental Methods and materials.

**NMR experiments**

NMR experiments were recorded at 298 K on 500 MHz, 600 MHz, and 700 MHz spectrometers. Resonance assignments were obtained from a standard suite of triple-resonance 3-dimensional experiments (supplemental Methods and materials), and distance restraints were extracted from $^{15}$N- and $^{13}$C-edited nuclear Overhauser effect (NOE) spectroscopy spectra. RDCs were measured using aligned samples prepared in C$_{12}$E$_{5}$/n-hexanol$^{15}$ (N-H$^\alpha$, H$^\beta$-C$, N$-C$, C$^\beta$, C$^\gamma$-P, and C$^\delta$-H$^\delta$) and Pf1 bacteriophage$^{14}$ (N-H$^\alpha$N). Backbone $^{15}$N relaxation rates ($R_1$, $R_2$, and $\langle H \rangle$ $^{15}$N steady-state heteronuclear NOEs) were measured at 600 MHz using standard methods$^{17}$ and analyzed according to the Model-Free formalism.$^{18}$ Relaxation dispersions were measured using a transverse relaxation optimized spectroscopy—selected Carr-Purcell-Meiboom-Gill (CPMG) experiment$^{19}$ at 500 MHz and 700 MHz and analyzed in terms of a 2-site exchange model.

**Structure calculations**

The solution structure of TIL’E’ was calculated from the experimental NMR restraints using the programs CYANA$^{20}$ and Xplor-NIH$^{21}$ with the standard scripts provided. The structures shown are those from the Xplor-NIH calculation. In total, 954 unique distance restraints were obtained, of which 353 are long range (between residues that are >5 residues apart in the primary sequence); details are given in supplemental Methods and materials.

**Results**

**Recombinantly produced TIL’E’ is correctly folded and disulfide bonded**

Based on predictions of the FVIII binding site on VWF (Figure 1 and supplemental Figure 1), the TIL’ and E’ domains (residues 766-864), which are located just after the Furin cleavage site (residues 763-764) in the primary sequence of VWF, were selected for structural characterization. Previous homology modeling$^9$ predicted that all cysteine residues in TIL’E’ form disulfide bridges, of which 3 have been confirmed experimentally: Cys767-Cys808, Cys776-Cys804, and Cys810-Cys821 (Figure 1).$^{22}$ Thus, isotopically labeled TIL’E’ for NMR structural and dynamic studies was obtained by overexpression in an E coli strain engineered to facilitate cytoplasmic disulfide-bond formation.$^{13}$ Correct pairing of the 16 cysteine residues, and hence correct folding of TIL’E’, was critically dependent on the expression of these domains downstream of an N-terminal thioredoxin tag, yielding an initial translation product that bears resemblance to the native VWF prodomains, which contain oxidoreductase-like sequences.$^{23}$

Recombinant TIL’E’ was analyzed by size-exclusion chromatography (supplemental Figure 2), electrospray-ionization MS (supplemental Figure 3), and Ellman’s assay, which together confirmed that TIL’E’ was homogeneous, monomeric, and contained a negligible amount of free thiol groups. Compelling evidence that the TIL’E’ protein is well folded is provided by the very narrow charge-state distribution of the native MS profile (supplemental Figure 3) and the wide $^{1}$H chemical-shift dispersion in the $^{1}$H-$^{15}$N heteronuclear single quantum coherence (HSQC) NMR spectrum (Figure 2). Closer inspection of the $^{1}$H-$^{15}$N HSQC spectrum in Figure 2 revealed a heterogeneous distribution of peak intensities, providing the first indication that the TIL’ and E’ domains possess differential flexibility.

**Recombinant TIL’E’ binds to FVIII and mAb-418**

The best reporter of TIL’E’ function is binding to FVIII; this is dependent upon the folded TIL’E’ adopting its biologically active
correctly folded and functional, the NMR resonances covering 78% of resonance broadening due to micro-to-millisecond chemical exchange processes in this region of the protein (see below). The reorientation of resonance broadening due to limited NOE density, all predicted cysteine pairs came into close proximity. Therefore, all disulfide bonds included as constraints in the structure calculation, resulting in the solution structure of VWF TIL’E’ shown in Figure 4.

TIL’ has a long dominating loop, whereas E’ is all β sheet

The global fold of TIL’E’ shown in Figure 4 is crescent shaped, as has been suggested from electron microscopy imaging. The structure clearly shows that TIL’ and E’ form independently folding domains, confirming the previous domain assignment separating D’ into the 2 distinct domains. Salient features of domain TIL’ (residues 766-827) are the limited regions of secondary structure (~70% loop; 26% NMR assignments, verification of disulfide-bond topology, and structure of the TIL’E’ domains

After establishing that the recombinantly produced TIL’E’ was both correctly folded and functional, the NMR resonances covering 78% of the TIL’E’ 1H, 13C, and 15N backbone nuclei were assigned using standard triple-resonance NMR experiments (Figure 2). The unassigned nuclei are predominantly located between residues 781 and 805 of the TIL’ domain (supplemental Figure 5), a consequence of resonance broadening due to micro-to-millisecond chemical exchange processes in this region of the protein (see below). The resonances of side-chain 1H and 13C nuclei were subsequently assigned using 3-dimensional carbon total correlated spectroscopy (CC-TOCSY)-based experiments to correlate the backbone resonances with those of the side chains, giving a total assignment of 89% of the TIL’E’ nuclei. 15N- and 13C-edited NOE spectroscopy experiments were used to obtain 954 unique interresidue distance restraints (supplemental Table 1; supplemental Figure 6) for structure determination. In addition, 108 backbone dihedral-angle restraints (supplemental Table 2) were derived from the TIL’E’ chemical shifts, and 361 vector-orientation restraints were collected by measurement of multiple RDCs in 2 alignment media (supplemental Table 3).

The disulfide-bonding pattern shown in Figure 1 was verified by cross-disulfide NOEs corresponding to Cys1-Hβ-Cys42, Hββ, where Cys1-Cys42 represents a predicted disulfide bond between the iβ and jβ cysteine residues in the domain sequence (supplemental Figure 7). This permitted the unambiguous assignment of 4 disulfide bridges, including Cys792-Cys827 in TIL’ and Cys829-Cys851, Cys849-Cys858, and Cys846-Cys863 in E’ that had been predicted but not previously biochemically assigned (Figure 1 and supplemental Figure 7). The overall disulfide-bonding topology was further confirmed by exclusion of the corresponding constraints from preliminary structure calculations. With the exception of the Cys788-Cys799 linkage and to some extent the Cys776-Cys804 linkage in the TIL’ domain, which were poorly defined due to limited NOE density, all predicted cysteine pairs came into close proximity. Therefore, all disulfide bonds were included as constraints in the structure calculation, resulting in the solution structure of VWF TIL’E’ shown in Figure 4.
β strand) and lack of a significant hydrophobic core, such that 5 conserved disulfide bonds constitute the major stabilizing force constraining the fold (Figure 4). TIL’ is formed of 2 short β sheets, β1-β2 (residues 772–775:806–809) and β3-β4 (residues 814–817:820–823), that form a scaffold bearing a long 30-residue loop, the β1-to-β2 loop. This loop encompasses an 8-residue insertion between the second and third conserved TIL’ cysteine residues as compared with structural homologs of TIL’ (supplemental Figure 1). The second antiparallel β sheet, formed of strands β3 and β4, is connected by a reverse turn forming a small-hairpin structure. There is a single turn of a 3_10 helix (residues 824–826) at the C terminus of TIL’. TIL’ belongs to the Ascaris protease inhibitor family, defined by a 10-cysteine signature and a conserved protein fold,33 with the closest structural homolog being a chymotrypsin inhibitor domain (Protein Data Bank accession number 1CCV) with a Z score of 3.6, as calculated by the DALI server.34 In contrast, Figure 4 shows that the greater proportion of E’ is formed of a triple-stranded antiparallel β sheet formed of strands β3 (residues 839–844), β4 (residues 847–852), and β5 (residues 855–858). Additionally, the N terminus contains a short double-stranded β1:β2 sheet (residues 829–831:834–836). The relative positions of the 2 β sheets are restrained by the E’ Cys829-Cys851 disulfide bond. The 6-cysteine E’ domain was previously suggested to be a member of the fibronectin I-like superfamily of proteins35 and thus similar to the von Willebrand factor type C (VWC) module. Supplemental Figure 8 shows an overlay of the structure of the E’ domain solved here by NMR spectroscopy and the first subdomain of a full VWC module. The 2 domains shown are structurally highly similar with a backbone root-mean-square deviation (RMSD) of 1.3 Å, thus highlighting the structural similarities between the E’ domain and the VWC module.

The family of the 10 lowest-energy structures of TIL’E’ is well defined, with an average backbone pairwise RMSD of 1.12 Å, and corresponding RMSDs for the TIL’ and E’ domains in isolation of 1.37 Å and 0.34 Å, respectively (supplemental Figure 9). The backbone RMSD of the TIL’ domain excluding the long β1-to-β2 loop is 0.81 Å, indicating that this loop dominates the structural heterogeneity of TIL’ and alluding to its intrinsic dynamic character (supplemental Figure 10). The 10 lowest-energy structures have 73.1% of residues in the most-favored regions in the Ramachandran plot, 22.3% in the additionally allowed regions, 3.4% in the generously allowed regions, and 1.2% in disallowed regions.

**TIL’ is strikingly dynamic on at least 2 timescales**

The conformational sampling of a protein is often essential to its biological activity,36 and hence the dynamic characterization of TIL’E’ is of great importance for elucidating the mechanism by which FVIII is stabilized in plasma. The structural dynamics of TIL’E’ were quantified experimentally by 15N nuclear spin relaxation experiments.17 Analysis

![Figure 5](image-url)
of these relaxation data revealed interesting motional trends; most notably, that there is a distinguishable mobility differential between the TIL’ and E’ domains. \(^{1}^H\)\(^{15}\)N nuclear Overhauser effects (hetNOEs; Figure 5A), which report specifically on backbone fluctuations on a pico-to-nanosecond (ps-ns) timescale, clearly show that, with the exception of the very C-terminal residues, the E’ domain is rigid with a uniform distribution of hetNOE values around 0.7. In contrast, several residues in the β1-to-β2 loop of the TIL’ domain show significantly lower NOE values, indicating a comparatively higher degree of flexibility. The amplitudes of the local ps-ns motions were quantified as squared order parameters,\(^{18}\) \(S^2\), according to the model-free analysis of the \(^{15}\)N spin relaxation rates (supplemental Table 4), and confirmed the relative flexibility of the TIL’ β1-to-β2 loop on this timescale.\(^{37}\) The analysis of the \(^{15}\)N relaxation rates includes a determination of the diffusion tensor, \(D\). Of special interest here is the ratio of the parallel and the perpendicular components of the axial diffusion tensor, \(D_{||}/D_{\perp}\), because this ratio reports on the shape of the molecule. Firstly, the ratios obtained separately for the 2 domains are very similar (\(D_{||}/D_{\perp}\) TIL’ = 1.82 ± 0.05 and \(D_{||}/D_{\perp}\) E’ = 1.9 ± 0.1), which indicates that the TIL’ and E’ domains tumble as a single entity with only limited interdomain motion. Secondly, the relatively large values of \(D_{||}/D_{\perp}\) confirm that the overall structure is a highly prolate spheroid, in agreement with the calculated structures (Figure 4).

Backbone dynamics on the broader pico-to-microsecond (ps-μs) timescale were investigated, because ligand binding and dissociation often takes place in this time regime.\(^{36,38}\) The amplitudes of motions over the ps-μs timescale were estimated from backbone chemical shifts\(^{30-32}\) and subsequently included in structure calculations,\(^{21,39}\) together with the restraints listed above, to determine an ensemble of protein conformations that represent, simultaneously, the TIL’ structure and its associated dynamics. Accordingly, this structural ensemble represents a more faithful atomistic description of the conformational variability of TIL’ on a ps-μs timescale. The ensemble representation of TIL’ was systematically cross-validated by a combination of RDCs and side-chain \(^{13}\)C methyl chemical shifts\(^{40-42}\) to give an optimal ensemble size of 10 replicas (supplemental Figure 11). The ensemble representation is shown in Figure 5B and shows that the β1-to-β2 loop is significantly more flexible than the remainder of TIL’.

Although the hetNOEs (Figure 5A) and chemical shifts report on ps-ns and ps-μs dynamics, respectively, structural dynamics on the millisecond timescale can be specifically probed using CPMG relaxation dispersion experiments.\(^{19,44}\) The time constants of many biochemical processes, such as ligand binding and dissociation,\(^{36}\) are on the millisecond timescale, and CPMG experiments were therefore performed on the TIL’ domains to quantify such dynamics (Figure 5C). A first inspection of the CPMG data reveals localization of slower motions to the domain TIL’, particularly in the β1-to-β2 loop (Figure 5C), confirming the existence of a sparsely populated (excited) state with a lifetime in the millisecond range, whereas the E’ domain remains rigid on this timescale. A detailed analysis of the CPMG relaxation dispersions reveals that the ground state of TIL’ exchanges with an excited state; the lifetime of the excited state is approximately 0.5 ms and has a population of 3.5% (supplemental Methods and materials). Excited states often have significant functional roles in molecular recognition and ligand binding, and the existence of the TIL’ excited state is likely to be functionally relevant for the binding of VWF to FVIII.

A summary of the TIL’ dynamics is shown in Figure 5D, from which it is evident that the TIL’ β1-to-β2 loop is strikingly dynamic on at least 2 timescales, whereas the remainder of TIL’
provides a rigid scaffold supporting this conformationally dynamic entity.

**Discussion**

The solution structure and dynamic characterization of TIL' E' allow an unprecedented interpretation of the likely functional consequence of type 2N VWD missense mutations. The greater proportion of the known type 2N missense mutations are distributed in the vicinity of the dynamic TIL' β1-to-β2 loop (Figure 6A). Type 2N mutations that involve a cysteine residue (C788R/Y, Y795C, and C804F in TIL'; C858S/F in E') are associated with aberrant multimerization, poor secretion, and reduced FVIII binding, indicating that correct disulfide-bond formation is critical to the folding and function of VWF (Figure 3). Of the type 2N mutations, 3 account for the majority of cases reported (T791M, R816W, and R854Q), of which R854Q occurs with polymorphic frequency, and all of which are in immediate proximity to the β1-to-β2 loop. However, the most accurate reporters of the FVIII binding site are those mutations that are associated with a more severe type 2N phenotype; that is, E787K, T791M, and R816W. Two of these mutations, T791M and R816W, together with T789P, M800V, R816Q, and H817Q, are clustered around a region of positive charge density on TIL' (Figure 6B-C). Two regions on the FVIII light chain have been implicated in binding VWF, that is, the N-terminal acidic a3 domain and the C-terminal C2 domain. Although these 2 regions are thought to act synergistically in binding VWF, studies show that a3 in isolation binds ∼8 times more strongly to VWF than the C2 domain, with affinities of ∼72 nM and 564 nM, respectively, and the acidic a3 domain is critical to high-affinity binding to VWF. We therefore hypothesize that the positively charged and dynamic region on TIL' E' is the major binding site to the negatively charged FVIII a3 domain. Thus, the deleterious effect of the R816W mutant is likely due to the introduction of a large and hydrophobic side chain and/or loss of positive charge (Table 1). Figure 6 shows that residues R782, G785, and E787 are on the opposite side of the structure yet in close proximity to the β1-to-β2 loop (Figure 6 and supplemental Figure 12), which suggests that the severe type 2N mutation E787K, and mutations R782W and G785E, lead to deficiencies in FVIII binding through a perturbation of the β1-to-β2 loop, the upper portion of which borders the predicted binding region.

In summary, the majority of the noncysteine type 2N mutations (Figure 6) are found in the vicinity of the flexible regions of TIL', thereby localizing the FVIII binding site to this part of the VWF protein. Of the type 2N mutations in TIL'E' involving charged residues, all but one result in loss of positive charge, supporting a predominantly electrostatic interaction between VWF and the FVIII light chain, which is in agreement with the fact that high ionic strength and low pH result in a much lower affinity of the VWF-FVIII complex. Hence, based on the foregoing analyses, we propose that E' and the β sheets of TIL' provide a rigid scaffold for the positively charged region on TIL', which complements the charge distribution of the FVIII light chain to electrostatically guide VWF-FVIII complex formation.

The cysteine-rich N- and C-terminal flanking regions of VWF have been the subject of significant efforts aimed at characterizing the structure and plasticity of function of this unique protein. Superposition of the VWD type 2N missense mutations on the structure of TIL'E', together with the characterization of its structural dynamics, reveals the malleable FVIII binding surface of VWF and represents an important step toward a detailed molecular understanding of these functionally important VWF domains for which atomic-resolution structures have hitherto been unavailable.

**Table 1. Type 2N missense mutations in domains TIL' and E' of VWF**

<table>
<thead>
<tr>
<th>Domain</th>
<th>Mutation</th>
<th>Δ Volume (Å³)*</th>
<th>Position</th>
<th>Predicted effect</th>
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<tbody>
<tr>
<td>TIL'</td>
<td>R782W</td>
<td>+41.1</td>
<td>β1-β2 loop</td>
<td>Loss +ve, polar-to-hydrophobic, structural perturbation</td>
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<td>G785E</td>
<td>+88.1</td>
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<td></td>
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<td>-8.9</td>
<td>β1-β2 loop</td>
<td>Introduce +ve in region of -ve charge, highly deleterious</td>
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<td></td>
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<td>Loss of conserved +ve in putative a3 binding region</td>
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<td>H817Q</td>
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<td>Loss of conserved +ve in putative a3 binding region</td>
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<tr>
<td>E'</td>
<td>R854W</td>
<td>+42.1</td>
<td>β4-β5 loop</td>
<td>Loss +ve, polar-to-hydrophobic</td>
</tr>
<tr>
<td></td>
<td>R854Q</td>
<td>-40.7</td>
<td>β4-β5 loop</td>
<td>Introduction of SH</td>
</tr>
<tr>
<td></td>
<td>C858F</td>
<td>+97.4</td>
<td>β5</td>
<td>Introduction of SH</td>
</tr>
<tr>
<td></td>
<td>C858S</td>
<td>-0.4</td>
<td>β5</td>
<td>Introduction of SH</td>
</tr>
</tbody>
</table>

*Changes in residue volumes are based on Pontius et al.*

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Solution structure of the major factor VIII binding region on von Willebrand factor