Online Supplementary Methods

Patients

Five patients, diagnosed with VWD (2 patients with type 1, 2 patients with type 2A and 1 with type 3) were included in the study. Bleeding severity was quantified by the bleeding score (BS) calculated on the basis of condensed MCMDM -1 VWD questionnaire.[17] This study was approved by the local ethics committee and informed consent was obtained from all patients (vote 091/09).

Coagulation and multimer analysis

Laboratory investigation of VWF antigen (VWF:Ag), VWF ristocetin cofactor activity (VWF:RCo), FVIII coagulant activity (FVIII:C) and VWF multimers (1.2% [w/v] and 1,6% [w/v] agarose gels) was performed as previously described [16, 18.] VWF multimer profiles were classified as normal or abnormal.

Mutation detection

All mutations were identified by direct sequencing of *VWF* coding region, exon–intron boundaries and 5' and 3' non-coding regions as previously described.[16]

Plasmid constructs

Plasmid pMT2-VWF containing the human full-length wild-type (WT) VWF cDNA was kindly provided by Professor Schneppenheim (Department of Pediatric Hematology and Oncology, University Medical Center Hamburg-Eppendorf, Hamburg, Germany). The mutant constructs were generated using a

QuickChange II XL site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA). The mutagenesis primers are available on request. The sequences of all constructs were verified by DNA sequencing.

Cell culture and transfection

Human embryonic kidney cell lines (HEK 293T; DSMZ, Braunschweig, Germany) were used to evaluate the secretion and intracellular retention of WT and mutants recombinant VWF (rVWF). HEK293 cell lines (DSMZ, Braunschweig, Germany) were used to analyze the intracellular location of VWF by confocal immunofluorescence microscopy. Both cell lines were cultured in Dulbecco's modified Eagel medium (DMEM) containing 4.5g L^{-1} glucose and 2mM L-glutamine supplemented with 10% (v/v) fetal bovine serum, 100IU mL^{-1} penicillin, 100 µg mL^{-1} streptomycin and 0.25 UG/ML Fungizone (all reagents supplied by Life Technologies, CA, USA) at 37°C in 5% CO2 atmosphere.

Cells were transiently transfected with 8µg of WT-VWF or mutated VWF constructs using liposomal transfer (Lipofectamine 2000; Life Technologies, CA, USA) according to the manufacturer's instructions. Co-transfections were performed using an equal amount of WT and mutant vectors (4 µg each of WT and mutant DNA) to reproduce the patients' heterozygous state. Seventy-two hours after transfection of HEK293T cells, supernatants were collected and cells were lysed for analysis of intracellular VWF. VWF secreted in the medium was concentrated on Amicon Centrifugal 50K filter devices (Millipore, USA) to one-forth of the original volume before subsequent analysis. The transfected cells

were lysed by 3 rounds of freezing (-80˚C) and thawing in lysis buffer (0.1M Tris/HCl pH 8.0; 0.6 [v/v] Triton X-100).[19]

Quantitative and qualitative analysis of VWF

The amount of VWF:Ag secreted into the medium and VWF:Ag present in the cellular lysate were determined, and the values were expressed as a percentage of the corresponding WT recombinant VWF. Activity of secreted rVWF was assessed by binding to platelet GPIb, collagen type III (VWF:CB), and VWF:RCo. The VWF activities were expressed as ratios to secreted VWF. VWF:GPIb binding was determined using a particle enhanced assay (Siemens Healthcare, Marburg, Germany) and VWF:CB was assessed by ELISA method (Technoclone, Vienna, Austria) according to the manufacturer's instructions. Comparison between the mean values of the groups was performed with the Student *t*-test. Multimer analysis of concentrated secreted rVWF was performed as described above.

ADAMTS13 assay

To assess the susceptibility of mutants of VWF to proteolysis, full-length WT and mutated rVWF (6μg/ml) were cleaved by 3μg/ml of recombinant ADAMTS13 (R&D systems, USA) in the presence of 1.5M urea at 37˚ C for 2 hours following the manufacturer's instructions. The multimer patterns of mutated and rVWF-WT were visually compared on agarose gel electrophoresis after ADAMTS13 digestion.

Immunofluorescence analysis

HEK293 cells were grown on gelatin pre-coated glass coverslips in 24-well plates. Cells were fixed 48 h after transfection by 4% (w/v) paraformaldehyde in PBS, blocked with 0.1% (v/v) Triton-X 100 in PBS azide supplemented with 10% (v/v) FBS. Cells were incubated with first antibodies for 2 h and then with fluorescence-conjugated secondary antibodies for 1 h. Polyclonal sheep to human VWF (abcam, England) and rabbit anti–human VWF antibodies (Dako, Glostrup, Denmark) were used to visualize VWF. Monoclonal anti-GM130 (BD Bioscience, CA) and polyclonal rabbit anti-human TGN46 (Sigma-Aldrich, USA) were used to stain *cis*- and *trans*-Golgi networks, respectively.[20] To visualize the endoplasmic reticulum (ER), the HEK293 cells were prepared and stained with rabbit anti-human protein-disulfide isomerase (PDI) antibody using SelectFX® Endoplasmic Reticulum Labeling kit (Life Technologies, CA, USA) according to the manufacturer's instructions. Secondary antibodies were conjugated with either AlexaFluor-488 or AlexaFluor-594 (Life Technologies, CA, USA). The coverslips were mounted onto microscope slides with Vectorshield (Vector Labs, Burlingame, CA, USA) and analyzed with the Olympus Fluo View FV1000 or Leica SL confocal microscope.

Structure analysis

Homology modeling of VWF C1, C5, C6 domains

Homology models for the C1, C5, and C6 domains were constructed using various software packages. The domain boundaries for C1, C5, and C6 domains were based on domain annotations from a recently published article.[14] The sequences for the respective domains were from the VWF (Accession number P04275) full length sequence. Templates for each of the domains were searched on the LOMETS server from Zhang.[21] Close packed alignments (using the conserved cysteines as guideposts) were generated between the best LOMETS templates and the original domain sequences using Jalview. The alignments as well as the templates were entered into YASARA version 12.8.6[22] to generate full-length models. The resulting models were further refined by a 500ps MD simulation (AMBER03 force field) in YASARA version 12.8.6. The models with the lowest force field energies were chosen as the final models for the respective domains. The stereochemical quality of these models were checked on the MOLPROBITY server [http://molprobity.biochem.duke.edu/;accessed between 15.07.2012 and 15.09.2012].[23] The monomer models were run on the online server CLUSPRO in dimer mode (http://cluspro.bu.edu/home.php;accessed between 15.08.2012 and 15.10.2012) and the best dimers were chosen based on cluster size for further interface analysis.[24] Impact of mutation on folding was calculated using the FOLDX plugin incorporated in YASARA version 12.8.6.[25]

Molecular models of the VWF mutations

The missense mutations were modeled on the final refined wild-type VWF domain structures. The mutant structure was optimized for the best possible rotamer using the SCRWL library in YASARA version 12.8.6.[26] The variant structures were subjected to rounds of steepest descent and annealing energy minimizations. Subsequently MD simulation was performed for 10 ns (nanosecond) for each mutated structure as well as the wild type. The simulation trajectory for both wild type and mutant proteins were analyzed with respect to each other.

Residue	B (Disulphide Bond)	A.S.A	R.O.G	R.M.S.D	Charge	ΔΔG
p.Cys2283Arg	4CYS-30CYS	2.01/47.02	17.42/20.03	2.24/4.67	+	+12.20
p.Cys2327Trp	-	47.25/83.91	17.42/18.01	2.24/2.32	0	+0.17
p.Cys2619Tyr	26CYS-42CYS	44.40/139.98	16.32/18.15	2.13/3.24	0	+7.65
p.Cys2676Phe	3CYS-30CYS	1.07/3.79	17.58/16.46	2.07/3.52	0	+8.51

Supplementary Table 1. Structural changes observed during MD.

The structural characteristics are represented side by side for wild type/mutant residue. B: Bonds broken/added post mutation, A.S.A: Average surface accessible area for the simulation trajectory (in Angstrom units), R.O.G: Radius of gyration for the simulation trajectory (in Angstrom units), R.M.S.D (root mean square deviation): Average R.M.S.D for the simulation trajectory (in Angstrom units), Charge: Charge added or removed on mutation, ΔΔG: Average change in folding energy for mutation calculated with FOLDX [The stability of the mutated structure was estimated as the difference between the free energy (calculated from folding/unfolding algorithms) of the wild type protein and that of the mutant protein represented as ΔΔG values (stability/free energy changes);reported in kJ/mol; higher values indicate an unstable protein]. The numbering of cysteines mentioned in the second column is according to their linear appearance in the modelled domain structures.

Domain	Disulfide bond	Chi1	Chi2	Chi3	Chi2´	Chi1´	Type of Disulfide bond
C1	4 CYS-30CYS	-165.6	79.7	77.3	130.3	-143.8	-RHSpiral
C5	26CYS-42CYS	-43.5	-101.8	91.3	-86.9	-50.5	-RHStaple
C6	3CYS-30CYS	-66.8	-169.6	-101.3	169.0	59.5	+/-LHHook

Supplementary Table 2. Cysteine Disulfide bonds.

The table lists the torsional angles for disulfide bonds on which mutations have been identified and the classification of these disulfide bonds based on the nature of (+or-) the torsional angles. Classification based on the sign of disulfide bonds (+: grey,-: blank) is annotated from previous literature as well as disulfide bond analysis file (*disulfide_analysis_06_Aug_2012.zip*) accessed on 07/10/2012 (*http://www.med.unsw.edu.au/CRCWeb.nsf/page/Disulfide+Bond+Analysis*).