Legendre P et al.

Mutations in the A3 domain of von Willebrand factor inducing combined qualitative and quantitative defects in the protein

Supplementary methods and figures

Phenotypic analysis

FVIII activity was measured using a one-stage clotting assay based on the APTT using FVIII-deficient plasma (Diagnostica Stago, Asnières, France) on the STA®-R automate (Diagnostica Stago). VWF antigen (VWF:Ag) was measured using the STA-Liatest® VWF:Ag reagent on the STA®-R automate. VWF ristocetin cofactor activity (VWF:RCo) was assayed by aggregometry using a commercial kit from Behring (Marburg, Germany), consisting of lyophilized platelets and ristocetin A. VWF collagen binding activity (VWF:CB) was performed with a home-made method using equine tendon collagen (Horm collagen; Nycomed Arzneimitte, München, Germany) at 1mg/ml immobilized on microtiter wells. Bound VWF was detected using a polyclonal rabbit anti-human VWF horseradish peroxidase (HRP) conjugate (DakoCytomation, Trappes, France). Results are the mean of two determinations and expressed as IU/dl of plasma. The XIth International Reference Preparation for Factor VIII-related activities (National Institute for Biological Standards and Control, London, UK) was used as a standard.

Multimer analysis was performed by 2% sodium dodecyl sulfate (SDS)-agarose gel electrophoresis followed by immunoblotting and staining with rabbit anti-VWF-HRP. Multimers were visualized using 3,3’diaminobenzidine tetrachloride as substrate.
**Genetic analysis**

Genomic DNAs were obtained from whole blood using Nucleon™ BACC2 Genomic DNA Extraction kit (Amersham Life Science, GE Healthcare). We have screened patient’s DNA for mutations by direct sequencing of the VWF gene (NM_000552.3), using Big Dye V3.1 and a 3130XL capillary sequencer (Applied Biosystems). The sequence data were analyzed with Seqscape v2.5 (Applied Biosystems).

**Cell transfections**

Stable cell lines were made in baby kidney hamster (BHK) cells by transfection of pNUT-VWF, wt or mutant, using jetPEI reagent (Polyplus-transfection SA, Illkirch, France). COS-7 cells (14×10⁶ cells/ml in a 0.7 ml volume) were transfected by electroporation (255 Volts, 1500 µF, ∞ Ohm) using 10 µg of a pNut expression vector containing VWF cDNA wt or mutated. In co-expression experiments, 5 µg of wt-plasmid and 5µg of the mutated plasmid were used. Serum-free conditioned cell medium was collected 72 h after transfection. In all experiments, wt-rVWF was included as positive control for the procedure. The supernatants were concentrated 10-40 fold with aquacid-II (Calbiochem, La Jolla, CA, USA) and dialysed against 25mM Tris-HCl containing 150mM NaCl, pH 7.4 (TBS). Cell lysate was obtained by treatment of cells with lysis buffer (10mM Tris-HCl, 150mM NaCl, 5mM EDTA, 1% Nonidet, pH 8). Amount of rVWF expressed in both cell lines was measured by ELISA.

**Binding of rVWF to monoclonal antibodies (Mabs)**
In house Mabs directed against different domains of VWF were tested. Mab200 (epitope 1688-1868) and Mab505 (epitope 1690-1868) recognize the A3 domain and both inhibit binding of VWF to collagen.\textsuperscript{5,6} Mab418 (epitope 765-816) inhibits VWF binding to FVIII.\textsuperscript{7} Mab9 (epitope 2466-2509) interferes with VWF binding to GPIIbIIIa.\textsuperscript{5} Mab487 is a non-inhibitory antibody recognizing the D4-CK region.

Three Mabs directed against the A1 domain were used: Mab701 inhibits binding to GPIb in presence of ristocetin, botrocetin and bitiscetin;\textsuperscript{6} Mab724 inhibits binding to GPIb in presence of botrocetin and bitiscetin;\textsuperscript{8} and Mab318 inhibits binding to GPIb in presence of ristocetin and bitiscetin.\textsuperscript{9} For binding to rVWF, microtiter plates (cat# 675 161; Greiner bio-one, Les Ulis, France) were coated with the various antibodies (5µg/ml) diluted in 50mM carbonate buffer at pH 9.6 overnight at 4°C. Serial dilutions of rVWF in culture medium was added and incubated 2h at 37°C. Finally, anti-VWF-HRP polyclonal antibodies were added. Bound antibodies were detected via peroxidase hydrolysis of Color Fast O-phenylenediamine (OPD) substrate (Sigma-Aldrich). Absorbance was measured at 492 nm using a plate reader (Power Wave 340, BioTek Instruments, Colmar, France).

**Binding of rVWF to collagen**

Binding to collagen was done as follows: Equine collagen I (Collagen Horm, Kordia, Leiden, The Netherlands) diluted in water at 25µg/ml or human collagen III (Sigma-Aldrich, La Verpillière, France) dissolved in 0.1M acetic acid and diluted in 50mM carbonate buffer at pH 9.6 at 10µg/ml were immobilized on microtiter plates (cat# 675 161; Greiner bio-one, Les Ulis, France) overnight at 4°C. After coating, the plates were washed thrice with phosphate-buffered saline (PBS) 0.1% Tween-20 (PBS/T) and blocked with 3% bovine serum albumin (BSA) (Sigma-Aldrich) in PBS/T. Serial
dilutions of rVWF in culture medium were added to the wells (0-2 µg/ml for collagen I and 0-1 µg/ml for collagen III) and incubated 2h at 37°C. After washing, rabbit anti-VWF-HRP polyclonal antibodies were added for 1h at 37°C. Bound antibody was detected with 3,3',5,5'-tetramethylbenzidine (TMB; Tebu-bio, Le Perray-en-Yvelines, France) for collagen I and with OPD for collagen III. As negative control, rVWF lacking the A3 domain (VWF-delta A3) was used. This deletion variant lacks residues 1673-1876 (p.E1673-p.V1876del; c.G5017-T5627del; overlapping exons 28-33).

**Binding of rVWF to Factor VIII**

Rabbit anti-VWF (DakoCytomation) was immobilized on 96 wells plate at 5µg/ml in 50mM carbonate buffer pH 9.6 overnight at 4°C. After washing thrice with TBS 0.1% Tween-20 (TBS-T), the wells were saturated with 3% BSA in TBS-T. Then serial dilutions of rVWF were added to the wells (0-1 µg/ml) and incubated overnight at 4°C. After washing in TBS-T, CaCl$_2$ (0.35M) was added to the wells, twice for 10 min, followed by 6 washes. Then rFVIII (Kogenate-FS, Bayer Healthcare) diluted at 60mU/well was added. After 2h at 37°C and 3 washes, anti-FVIII-HRP (Kordia) was added to the wells. A two-step measure was performed. First, amount of FVIII/well was measured by detecting bound FVIII antibody using OPD substrate. Plate was rinsed and the amount of VWF immobilized on the wells was subsequently measured by adding rabbit anti-VWF-HRP for 1h at 37°C and bound antibody was detected with OPD. Both VWF and FVIII were quantified using standard curves obtained with normal pooled plasma. For each recombinant, the amount of FVIII bound to rVWF was calculated as a function of immobilized VWF. Results are expressed as the ratio of the slope of the binding curve of FVIII to mutated VWF over the slope of binding to wt-VWF. As negative control, plasma from a compound heterozygous VWD-type
2N patient was used (candidate mutation p.R768Q, c.2303G>A, exon 18 and p.R854Q, c.2561G>A; exon 20).

**Binding of rVWF to GPIIbIIIa**

In house Mab against GPIIbIIIa was immobilized on half-well plates in 50mM carbonate buffer pH 9.6 overnight at 4°C. After washing in 50mM Tris-HCl, 100mM NaCl, 1mM CaCl₂, 1mM MgCl₂, 0.0035% Triton X100, 1% BSA (buffer A), wells were blocked with buffer A containing 3% BSA. Human GPIIbIIIa (Kordia) diluted at 10µg/ml in buffer A was then added for 2h at 37°C. After washing 3 times, rabbit anti-VWF-HRP polyclonal antibodies were added for 1h at 37°C and bound antibody was detected with TMB. As negative control, VWF mutant D2509G (p.D2509G; c.7526A>G & c.7527C>A; exon 44) was used, in which the RGD-motif has been mutated.¹⁰

**Binding of rVWF to GPIb**

Binding to GPIb was done as described by Vanhoorelbeke et al.¹¹ Briefly, a home-made Mab anti GPIb was coated overnight at 4°C in half-well microtiter plates in carbonate buffer. Wells were blocked with TBS-T containing 3% BSA for 1h. rGPIb, wt or p.D235Y/M239V (GPIb 2M; c.751G>T & c.763A>G; exon 1), produced in BHK cells, was added to the wells (5 or 2µg/ml respectively) in TBS and BSA 3 % for 3h at 37°C. Then, serial dilutions of rVWF mutants diluted in TBS BSA 0.1% for GPIb 2M or complemented with 1µg/ml botrocetin for GPIb wt, was added to the wells and incubated 3h at 37°C. Botrocetin was purified in our laboratory.¹² Bound VWF was probed with rabbit anti-VWF-HRP and detected via peroxidase hydrolysis of TMB. Between each step, the plate was washed thrice with TBS-T.
Supplementary references


**Supplementary Table S1: Summary of the results obtained in the expression studies of the two recombinant VWF carrying mutations in the A3 domain**

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<th>Homozygous mutants</th>
<th>Heterozygous mutants</th>
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<tr>
<td></td>
<td>Expression (COS-7 cells)</td>
<td>Binding</td>
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<tr>
<td></td>
<td>SN</td>
<td>LY</td>
</tr>
<tr>
<td>p.L1696R</td>
<td>N↓</td>
<td>N↓</td>
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<tr>
<td>p.P1824H</td>
<td>N↓</td>
<td>A</td>
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SN: supernatant; LY: lysate;
A:- indicates absence of expression or of binding; N indicates similar to wt-VWF; ↓ indicates decreased binding and ↓↓ indicates binding or expression that is not absent but very strongly decreased.
Supplementary Figure S1

Supplementary Figure S1: Schematic representation of the crystal structure of VWF A3 domain indicating the position of the investigated point mutations

The representation uses coordinates from PDB accession 1ATZ. A ribbon represents the backbone structure of the A3 domain. Residues L1696 and P1824 are in red ball-and-stick representation, located in \( \beta \)-strands \( \beta1 \) and \( \beta5 \), respectively, within the central beta-sheet that contains 6 strands and is surrounded by seven \( \alpha \)-helices. Residues previously described as mutated in other VWD patients are in orange ball-and-stick representation (S1731, W1745) while residues previously identified by in vitro approaches as crucial for VWF-collagen interaction are in purple ball-and-stick representation (I1738, T1740, D1742, V1761, E1764). Residues both orange and purple in ball-and-stick representation were described both in patients and in in vitro studies (S1783, H1786).
Supplementary Figure S2: Ratio binding of heterozygous VWF mutants to antibodies Mab200 and Mab418.

To test whether the VWF molecules produced upon co-expression of mutants p.L1696R-rVWF or p.P1824H-rVWF with wt-rVWF, an assay was performed based on the fact that both mutants do not interact with Mab200, whereas binding to Mab418 is unaffected. As such the ratio Mab200/Mab418 is related to the heterozygosity of the protein: it is 1 for wt-rVWF, 0 for mutant VWF and somewhere between 0 and 1 for the heterozygous protein. In the figure, the ratios for wt-rVWF alone and the combinations p.L1696R-rVWF/ut-rVWF and p.P1824H-rVWF/ut-rVWF are provided. Two types of samples were analyzed: supernatants of COS-7 cells and plasma of mice after hydrodynamic gene transfer. In all samples, the ratios vary between 0.4 and 0.6, suggesting that 40-60% of the subunits of the secreted VWF proteins are mutated.