Type 2M von Willebrand Disease: F606I and I662F Mutations in the Glycoprotein Ib Binding Domain Selectively Impair Ristocetin- but not Botrocetin-Mediated Binding of von Willebrand Factor to Platelets


VON WILLEBRAND DISEASE (vWD) is a common, autosomal inheritance bleeding disorder caused by a quantitative and/or qualitative deficiency of von Willebrand factor (vWF). We describe two families with a variant form of vWD where affected members of both families have borderline or low vWF antigen levels, normal vWF multimer patterns, disproportionately low ristocetin cofactor activity, and significant bleeding symptoms. Whereas ristocetin-induced binding of plasma vWF from affected members of both families to fixed platelets was reduced, botrocetin-induced platelet binding was normal. The sequencing of genomic DNA identified unique missense mutations in each family in the vWF exon 28. In Family A, a missense mutation at nucleotide 4105T→C resulted in a Phe606Ile amino acid substitution (F606I) and in Family B, a missense mutation at nucleotide 4273A→T resulted in an Ile662Phe amino acid substitution (I662F). Both mutations are within the large disulfide loop between Cys509 and Cys695 in the A1 domain that mediates vWF interaction with platelet glycoprotein Ib. Expression of recombinant vWF containing either F606I or I662F mutations resulted in mutant recombinant vWF with decreased ristocetin-induced platelet binding, but normal multimer structure, botrocetin-induced platelet binding, collagen binding, and binding to the conformation-sensitive monoclonal antibody, AvW-3. Both mutations are phenotypically distinct from the previously reported variant type 2M Milwaukee-1 because of the presence of normal botrocetin-induced platelet binding, collagen binding, and AvW-3 binding, as well as the greater frequency and intensity of clinical bleeding. When the reported type 2M mutations are mapped on the predicted three-dimensional structure of the A1 loop of vWF, the mutations cluster in one region that is distinct from the region in which the type 2B mutations cluster.

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multimer patterns, disproportionately low vWF:RCO activity, and decreased ristocetin-induced platelet binding. Two new missense mutations were identified within the A1 loop of vWF. The vWF defects from affected individuals in the families described in the current report have normal botrocetin-induced platelet binding of vWF, normal collagen binding of vWF, and a stronger history of clinical bleeding and are thus phenotypically distinct from our previous report of type 2M*Missouke-i vWD.13

MATERIALS AND METHODS

Patients. Two unrelated families with abnormal bleeding histories were identified with low vWF:Ag, disproportionately low vWF:RCO activity, and normal multimer structure in the affected members. Available members of three generations of each family were seen in the Pediatric Clinical Research Center at Children’s Hospital of Wisconsin. Plasma was evaluated by the Hemostasis Reference Laboratory at The Blood Center of Southeastern Wisconsin, Milwaukee, WI. Plasma vWF:RCO activity was determined by ristocetin-induced agglutination of formalin-fixed platelets as previously described.15 vWF:Ag levels of the same samples were measured by quantitative Laurell rocket immunoelectrophoresis.16 Plasma vWF multimers were analyzed by electrophoresis on a 0.65% sodium dodecyl sulfate (SDS)/agarose gel using a discontinuous buffer system and detection with 125I-anti-vWF antibody (Ab) as described by Ruggeri and Zimmerman.17,18

Polymerase chain reaction (PCR) amplification of genomic DNA. After obtaining informed consent, blood samples were collected from individuals in both families. Genomic DNA was prepared from peripheral white blood cells from patients AII-1 and AIII-1 in Family A and patients BII-1, BIII-1 and BIII-2 in Family B as previously described.19 The vWF DNA sequence is numbered starting from the initiating Met codon of exon 2.20 Amino acid numbering starts with the mature vWF sequence. For selected Family A patients, vWF exon 28 was amplified from genomic DNA by PCR with Amplitaq Taq polymerase (Perkin-Elmer Cetus, Norwalk, CT) using sense primer Vs273-4, ExoR (GAGGaaATCTGGAATATGGAAGTCTGAG) located in intron 27 and antisense primer Va28-6, BamHI (GAGGataCTTGTGGCAGATGTCATTGAG) located in intron 28 of the vWF gene.21 These primers were chosen for selective amplification of vWF gene sequence without interference from the vWF pseudogene.22 Lower case letters indicate where nucleotides (nt) differ from the vWF gene sequence for the purpose of introducing restriction enzyme sites into the final product. For selected Family B patients, DNA from exon 28 of the vWF gene was amplified by PCR using sense primer Vs273-3 (CCACAGGTTCTCTGCGAACC) located in intron 27 and antisense primer a5040-5020 located in exon 28 of the vWF gene. This was followed by a second amplification using nested sense primer V3673-3697:Nsi I (atgCATGTGTTAGTGTTCCACCTCA) and antisense primer a4488-4462. After PCR amplification, the amplified DNA products were subjected to first round PCR with sense primer VsI27-3 and antisense primer a5040-5020 as described above. After a second PCR amplification with nested primers V3.3673-3697:Nsi I and a4488-4462, a 815-bp product is amplified from vWF genomic DNA. When this PCR product is digested with BclI, only the normal allele is cut into two fragments of 598 and 217 bp, respectively.

Plasmid constructs and expression of recombinant vWF. The Asp I/Nco I restriction fragments (nt 3832-4481 of vWF) of the subcloned PCR products amplified from genomic DNA from patients AII-1 and AIII-1 in Family A and patients BII-1 and BIII-2 in Family B were subcloned into P18vW1, an intermediate vector that was constructed by the insertion of the BamHI/Kpn I restriction fragment (nt 2717-4752 of mature vWF) from the full length vWF cDNA expression plasmid pW198.1 (provided by Dennis Lynch, Dana Farber Cancer Center, Boston, MA) into the plasmid vector pUC-18 (United States Biochemical).19 The BamHI/Kpn I restriction fragment of the resulting construct containing the vWD mutant sequence was ligated into the corresponding BamHI and Kpn I sites of the full-length vWF expression plasmid pW198.1. The pW198.1 and mutant expression plasmids were used to transfect COS-7 cells in the presence of Lipofectamine (GIBCO-BRL, Gaithersburg, MD) using the protocol of Felgner et al.23 After 48 hours, conditioned media were harvested, cleared by low speed centrifugation, and stored at −80°C. vWF:Ag levels in conditioned media were assayed by antigen-capture enzyme-linked immunosorbent assay (ELISA) using monoclonal antibody (MoAb) AvW-124 and detected by anti-vWF rabbit polyclonal Ab followed by biotin conjugated goat antirabbit IgG (Pierce, Rockford, IL). Immune complexes were detected using avidin-horseradish peroxidase, and o-phenylenediamine substrate (Sigma, St Louis, MO).

Multimer analysis of recombinant vWF. Recombinant vWF was immunoprecipitated with vWF MoAb AvW-1 coupled to Sepharose–4B (Pharmacia, Piscatawy, NJ). Immunoprecipitated vWF (14 ng) was analyzed on a 1.5% resolving gel as described by Raines et al.,25 with the following modifications. After adding the samples to the wells, electrophoresis was performed at 150 V (constant) for 7 to 8 hours in a Bio-Rad Model 1415 electrophoresis chamber (Bio-Rad Laboratories, Richmond, CA) cooled to 15°C. After electrophoretic transfer to nitrocellulose, recombinant vWF multimers were detected using anti-vWF rabbit polyclonal Ab followed by horseradish peroxidase-conjugated goat antirabbit IgG (Pierce) and visualized by chemiluminescence using the ECL Western blot detection system (Amersham Corp, Arlington Heights, IL).

Platelet binding assay. The binding of vWF to fixed platelets was measured using a modification of a procedure previously published.19,26 Briefly, AvW-1, a vWF MoAb that does not interfere with vWF binding to either GP Ib or GPIIb/IIIa,24,27 was labeled with 125I (DuPont NEN, Boston, MA) using Iodo-Beads (Pierce).125I-AvW-1 was incubated with either plasma (3 parts plasma: 1 part 125I-AvW-1, 6,000 cpm/µL) or conditioned medium (60 parts conditioned medium: 1 part 125I-AvW-1, 2,000 cpm/µL) for 30 to 60 minutes at 22°C. For recombinant vWF experiments, conditioned medium from transfected COS-7 cells or normal pooled human plasma were diluted in Tris-saline (20 mmol/L Tris pH 7.4, 150 mmol/L NaCl) such that equal amounts of vWF (determined by ELISA as described above) were used within a single platelet binding experiment (range, 50 to 100 ng/mL). Labeled plasma (35 µL, 50,000 cpm) or conditioned media (300 µL, 10,000 cpm) was incubated with formalin-fixed platelets (200 µL of 2 × 10^10/mL for plasma, or 40 µL of 4 × 10^10/mL for recombinant vWF experiments, BioData, Hatboro, PA) in the presence of ristocetin (Helena, Beaumont, TX), botrocetin, or control buffer and gently rocked for 30 to 60 minutes at 22°C. Botrocetin was purified as described by Andrews et al.28 After pelleting platelets and platelet-bound vWF (12,000g, 10 minutes), the upper half of the supernatant was transferred to a clean tube, The amount of radioactivity in the pellet half (a) and the supernatant half (b) fractions was determined using a gamma counter. The percent of vWF
bound to the platelets was calculated using the formula: \( (a - b)/(a + b) \times 100 \).

**Collagen and AvW-3 binding assay.** Type III collagen (6 µg/mL, Southern Biotechnology Associates, Birmingham, AL), vWF MoAb AvW-1 (5 µg/mL), or MoAb AvW-3 (5 µg/mL), a vWF MoAb that binds vWF and inhibits its interaction with GPIb,\(^27\),\(^29\) in a carbonate buffer was plated on microtiter wells (50 µL/well) at 4°C overnight. After blocking (0.05% Tween-20 in Tris-saline, 2 to 3 hours, 22°C) and washing, 50 µL of conditioned medium from transfected COS-7 cells, diluted to approximate concentrations of both 100 ng/mL and 50 ng/mL of recombinant vWF in blocking buffer, was added to wells in triplicate and incubated at 22°C for 60 minutes. After washing the wells, bound recombinant vWF was detected by ELISA using rabbit anti-vWF polyclonal Ab followed by horseradish peroxidase-conjugated goat antirabbit IgG (Pierce). Immune complexes were detected using o-phenylenediamine (Zymed, San Francisco, CA). Bound vWF was quantitated by comparing the resultant optical density (above background) with a standard curve of pooled normal plasma vWF binding that was performed in parallel in each of these studies. The amount of vWF added to the wells was quantitated by binding to AvW-1–coated wells in parallel experiments. The amount of recombinant vWF bound to collagen or AvW-3 was expressed as a ratio of the amount of vWF bound to collagen or AvW-3 divided by the amount of vWF added to the well.

**vWF A1 domain molecular model.** The coordinates for the A domains of integrins \( \alpha_M \) (Mac-1)\(^30\) and \( \alpha_L \) (LFA-1)\(^31\) were generously provided by Robert C. Liddington (University of Leicester, Leicester, UK) and Daniel J. Leahy (Johns Hopkins University, Baltimore, MD), respectively. The sequence for human vWF domain A1 (residues Cys509-Cys695) was aligned with the sequences of the homologous A domains of \( \alpha_M \) and \( \alpha_L \) using the three-dimensional profile method of Bowie et al.\(^32\) Amino acids CSR and LC were added to the amino-terminus and carboxy-terminus, respectively, of the \( \alpha_M \) structure. The two cysteine residues were joined, and the new segment was subjected to molecular dynamics annealing using the program TINKER.\(^33\) Residues in \( \alpha_M \) were replaced by the corresponding aligned residues in vWF domain A1. Improper contacts were removed and the resulting structures were refined using the program WHAT IF.\(^34\),\(^35\) Small insertions or deletions in surface loops were modeled by adding or deleting residues, followed by local energy minimization with the program TINKER (steepest descent conjugate gradient or preconditioned truncated Newton method).\(^33\) The model was evaluated for improper contacts and bond angles; where appropriate, segments with bad conformations underwent molecular dynamics annealing. The entire model was energy-minimized to RMS gradient <0.01 kcal/mol (preconditioned truncated Newton method). The packing quality of the final model was –1.195 sigma.\(^36\)

**RESULTS**

**Description of two family pedigrees.** Figure 1 shows the pedigrees of Family A and Family B (Figs 1A and, B). Members from three generations of each family were available for study. The index case in Family A (AIII-1) presented in childhood with a lifelong history of increased bruising and moderately severe epistaxis; she required 1-desamino-8-D-arginine vasopressin (DDAVP) or vWF replacement therapy on multiple occasions. She also experienced bleeding 2 days posttonsillectomy despite perioperative vWF replacement therapy. The other affected members of Family A (AI-1 and AII-1) have an extensive history of increased bruising. The index case in Family B...
Type 2M von Willebrand Disease

The data for the individuals with type 1 vWD and type 2M Milwaukee-1 vWD (X with low vWF:Ag and normal vWF multimers (Type 1 vWD, type 2M vWD. Depicted are the vWF:RCo/vWF:Ag ratios of individuals vWD has been previously reported.13 As the ratio of these assays in 681 individuals with low vWF:Ag shows a normal distribution pattern of multimers in affected members of both families (Fig IC and D).

The ratio of the clinical assays for vWF:RCo and vWF:Ag of individuals from Family A and Family B were compared with the ratio of these assays in 681 individuals with low vWF:Ag and normal vWF multimers that were previously reported.13 As shown in Fig 2 and Table 1, the affected individuals from Family A and Family B, as well as the previously reported patients with type 2MMilwaukee-1, show vWF:RCo/vWF:Ag ratios that are more than 2 SD below the mean. While the vWF:RCo, vWF:Ag, and the vWF:RCo/vWF:Ag ratios all increase after treatment with DDAVP, the disproportionate ratio of vWF:RCo to vWF:Ag remains more than 2 SD below the normal range (Table 1 and Fig 2). The moderate increase in the vWF:RCo/vWF:Ag ratio after DDAVP is similar to that seen in patients with type 1 vWD after DDAVP therapy (data not shown). In unaffected family members, the vWF:RCo/vWF:Ag ratio is normal (Fig 2). In contrast to the marked reduction in the vWF:RCo/vWF:Ag ratio, there was minimal reduction in the ristocetin-induced platelet binding of plasma vWF from affected individuals of both families (Table 2). Plasma vWF from affected members of both families had normal botrocetin-induced binding to fixed platelets (Table 2).

Identification of unique missense mutations in the vWF A1 binding domain for both families. The site of vWF interaction with platelet GPIb receptor has been localized to the A1 domain of the mature vWF glycoprotein37-39 that is encoded by exon 28 of the vWF gene.8 Therefore, vWF exon 28 was amplified by PCR from genomic DNA from two patients in each family and subcloned into plasmids for DNA sequencing. In Family A, a single T → A missense mutation at nt 4105 was detected for both patient AII-1 and patient AIII-1, resulting in the substitution of Ile for Phe606 (F606I, Fig 3A). The normal allele was

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<th>Table 1. Family A and Family B vWF:RCo and vWF:Ag Levels</th>
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The vWF:RCo (U/dL), vWF:Ag (U/dL), vWF:RCo/vWF:Ag ratio and factor VIII:C activity (U/dL) of three generations of Family A and Family B were determined from testing in a clinical laboratory. The identity of each individual is defined in the pedigree in Fig 1. For selected affected family members only, the vWF:RCo and vWF:Ag levels were also measured 1 hour after treatment with DDAVP.

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<th>Table 2. Ristocetin- and Botrocetin-Induced Binding of Plasma vWF to Platelets</th>
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Plasma labeled with 125I-AvW-1 vWF MoAb was incubated with formalin-fixed platelets in the presence of ristocetin (1.2 mg/mL, Ristocetin), botrocetin (2 μg/mL, Botrocetin) or control buffer (Control) as described in Materials and Methods. After pelleting platelets and associated bound vWF, bound and unbound 125I-AvW-1 were detected by gamma counting, and percent bound 125I-AvW-1 calculated as described in Materials and Methods. The percent 125I-AvW-1 bound from normal pooled plasma (NP), affected Family A member AII-1 plasma (F606I), or affected Family B member BI-1 plasma (I662F) are shown as the mean (± SD) of two separate experiments.
also identified in both of these patients. These patients were also heterozygous for a commonly occurring A/G polymorphism at nt 4141 and a T/C polymorphism at nt 4641 with the 4141A and the 4641T polymorphisms occurring in association with the missense mutation (data not shown). In Family B, an A → T missense mutation at nt 4273 was detected in both patient BII-1 and patient BIII-2, resulting in the substitution of Phe for Ile662 (I662F, Fig 3B). Again, both normal and mutant alleles were identified, showing that the affected individuals were heterozygous for the missense mutation. The patients were also heterozygous for a commonly occurring G/A polymorphism at nt 4196, with the G polymorphism occurring in association with the missense mutation (data not shown).

Detection of vWF missense mutation in affected family members by restriction digestion of PCR products. A Bcl I restriction site unique to the mutation in Family A was created using additional base changes in a PCR primer. Figure 3C illustrates the selective cutting of PCR primer by Bcl I only in the mutant allele. The PCR products were amplified from genomic DNA from a normal control (N) or Family A patients (All.I and All.I). When the PCR product (336 bp) is digested with Bcl I, only the mutant vWF allele is cut into two fragments, 302 and 34 bp long. (D) In Family B, a native restriction site for BstY I is lost in the mutant allele. Genomic DNA from a normal control (N) or Family B patients (BII.I, BIII.I, and BIII.2) is amplified by PCR with primers VsI27-3 and Va5040-5020 followed by a second round of PCR with primers Vs3673:Nsi I and Va4488-4462:Nco I. When PCR product (815 bp) is digested with BstY I, only the normal allele is cut into two fragments, 598 and 217 bp long. Molecular weight standards (bp) are shown on the side.
the affected Family A members, AIII-1 and AII-1, but not in a normal individual. Both Family A members were heterozygous for the full-length (336 bp) and cut (302 bp) PCR products after Bcl I digestion, showing that the affected individual had both normal and mutant alleles. In Family B, the new mutation 4273A → T results in the loss of a restriction site for BsrY I. Figure 3D illustrates the selective retention of full-length PCR product after BsrY I digestion only in the affected Family B members, BII-1, BIII-1, and BIII-2, but not in the normal individual. Again, both full-length and cut PCR products were observed in affected family members, providing further evidence that affected members of Family B are heterozygous for the missense mutation.

Expression and structural characterization of recombinant mutant F606I and I662F vWF. To determine the effect of the missense mutation on vWF structure and function, restriction fragments containing the 4105T → A mutation from Family A and the 4273A → T mutation from Family B were inserted into the full-length expression vector pvW198.1. Wild-type (wt) and both mutant vWFs were transiently expressed in COS-7 cells. As shown in Fig 4, both recombinant F606I and I662F mutant vWFs and wt vWF formed multimer patterns containing similar amounts and proportions of all multimer sizes.

Ristocetin- and botrocetin-induced binding of recombinant mutant vWF to platelets. To test the functional characteristics of the missense mutations, platelet binding assays were performed with the recombinant mutant and wt vWF expressed by the transfected COS-7 cells.19,26 The binding of vWF to platelets was quantitated by a radiolabeled, noninhibitory, vWF MoAb AvW-1 that bound to vWF and was cosedimented with platelets through platelet-associated vWF. As shown in Fig 5, normal plasma vWF and wt recombinant vWF bound to fixed platelets in the presence of ristocetin (1.2 mg/mL, Ristocetin, shaded bars), botrocetin (2 μg/mL, Botrocetin, hatched bars), or control buffer (Control, open bars) as described in Materials and Methods. After pelleting platelets and associated bound vWF, bound and unbound 125I-AvW-1 was detected by gamma counting, and percent bound 125I-AvW-1 calculated as described in Materials and Methods. The percent 125I-AvW-1 bound from normal pooled plasma (NP, n = 3), wt recombinant vWF (WT, n = 4), recombinant vWF containing the mutation from Family A (F606I, n = 5), Family B (I662F, n = 5), type 2M Milwaukee-1 vWD (Δ629-639, n = 2) or conditioned medium from mock-transfected cells (Mock, n = 4) are plotted as the mean ± SD.
MoAb, AP-1,24 and the blocking A1 domain vWF MoAb, AvW-3,27,29 inhibited recombinant mutant vWF binding to platelets in the presence of either ristocetin or botrocetin indicating that the variant vWF binds to platelets via GPIb, similar to wt vWF (data not shown).

Binding of recombinant vWF to collagen. While the A3 domain of vWF is the putative major binding site for collagen,43 the A1 domain of vWF may also contribute to vWF interactions with collagen.42 Recombinant F606I and I662F vWF both bound to collagen under static conditions to levels similar to wt vWF (Fig 6A). In contrast, the type 2M<sub>Milwaukee-1</sub> variant, ΔR629-Q639,13 had reduced levels of binding to collagen (Fig 6A). This suggests that the A1 domain of vWF can significantly contribute to the interaction between vWF and collagen.

Binding of recombinant vWF to AvW-3. Because vWF MoAb AvW-3 inhibits the binding of vWF to GPIb,27,29 the binding of this MoAb to wt, F606I, I662F, and ΔR629-Q639 vWF was determined. As shown in Fig 6B, AvW-3 bound the wt, F606I, and I662F vWF to similar levels, while AvW-3 binding to ΔR629-Q639 vWF was absent. In addition, AvW-3 immunoprecipitates the wt, F606I, and I662F mutant vWF, but does not immunoprecipitate the type 2M<sub>Milwaukee-1</sub> mutant vWF, ΔR629-Q639 (data not shown).

DISCUSSION

Two lines of evidence support the conclusion that the vWF missense mutations F606I in Family A and I662F in Family B are responsible for the functional characteristics of the type 2M vWD. First, both missense mutations and the low vWF:RCo/vWF:Ag ratio are coinherit in an autosomal dominant manner through two or three generations of the affected families. Second, similar to patient plasma vWF, recombinant vWF containing each missense mutation is deficient in ristocetin-mediated vWF binding to platelets, yet retains normal multimer structure, botrocetin-mediated platelet binding, collagen binding, and AvW-3 binding. We speculate that the more severe ristocetin-mediated binding defect observed for mutant recombinant vWF compared with the same patient’s plasma vWF is due to expression of only the defective vWF in transfected cells versus heterozygous expression of mutant and normal vWF in the plasma of the affected patients. Interestingly, the missense mutation in Family A (4105T → A) is also present in the vWF pseudogene.22 However, no other pseudogene specific sequences were present in exon 28 of Family A. Additionally, Meyer et al47 recently reported a French family with a type 2M phenotype that also had the I662F mutation; the genomic nt mutation was not reported and the mutation has not been expressed. However, this preliminary data further supports our conclusion that the I662F mutation is responsible for the type 2M phenotype described in this report.

Both of these two new type 2M vWD variants are characterized by normal multimer structure, low vWF:RCo/vWF:Ag ratio, but normal botrocetin-mediated platelet binding, collagen binding, and AvW-3 binding. Only two other type 2M mutations have been described and confirmed by recombinant expression of mutant vWF: subtype B vWD14 and type 2M<sub>Milwaukee-1</sub> vWD (ΔR629-Q639).13 Subtype B vWD is due to the missense mutation Gly561Ser (G561S) that causes decreased ristocetin-mediated platelet binding, but normal botrocetin-mediated platelet binding and a normal multimer pattern.14 The two new variant forms of vWD described in this study are similar to G561S vWF and type 2M<sub>Milwaukee-1</sub> vWF with regard to the normal multimer structure, decreased ristocetin-induced platelet binding and amino acid modifications localized within the Cys509-Cys695 loop. However, in contrast to the type 2M<sub>Milwaukee-1</sub> vWD that has reduced botrocetin-mediated platelet binding and minimal clinical bleeding symptoms, the G561S vWF and the two new missense mutations described in this study have normal botrocetin-induced platelet binding, yet significant clinical bleeding symptoms. These data suggest a lack of clinical correlation between botrocetin-induced vWF reactivity and in vivo function of vWF. Furthermore, the difference in type III collagen binding between type 2M<sub>Milwaukee-1</sub> vWF (decreased collagen binding) and the clinically symptomatic type 2M F606I and I662F vWF variants described in this study (normal collagen binding) suggest that the effect of these mutations on collagen binding as measured in these static assays do not significantly alter the interaction of vWF with collagen or other subendothelial components during primary hemostasis. However, this data does suggest that the A1 domain can specifically

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![Fig 6](https://www.bloodjournal.org/) Recombinant vWF binds type III collagen and vWF MoAb AvW-3. Conditioned media from transfected COS-7 cells containing recombinant vWF from wt vWF (WT), Family A mutation (F606I), Family B mutation (I662F), or type 2M<sub>Milwaukee-1</sub> vWD (ΔR629-Q639) were incubated in microtiter wells coated with type III collagen (0.3 μg/well) or AvW-3 (0.25 μg/well). Bound vWF was detected by ELISA as described in Materials and Methods. The amount of vWF bound to collagen (A) or AvW-3 (B) is shown as the ratio of vWF bound (ng/mL) divided by the amount of vWF added to the well (ng/mL). The graph depicts the mean ± SD of two experiments.
affect the interaction of vWF with type III collagen. In contrast to the profound reduction in the vWF:RCo/vWF:Ag ratio in affected individuals from the type 2M vWD families described in this study, there was only minimal reduction in the ristocetin-induced binding of plasma vWF to fixed platelets. This suggests that the vWF:RCo/vWF:Ag ratio is more sensitive than the direct platelet-binding assay in the detection of clinically important deficiencies in the interaction of vWF with platelet GPIb.

To facilitate understanding of vWF A1 domain structure-function relationships, a molecular model of the domain was constructed based on the crystallographic structures of the homologous domains of αM (Mac-1) and αL (LFA-1) (Fig 7). These domains consist of a central 5-stranded parallel β-sheet with a short sixth antiparallel strand on one edge. This core is surrounded by amphipathic α-helices in a typical open α/β sheet or dinucleotide-binding fold. Amino acid residues that coordinate metal ions at the top of the αM and αL domains are not conserved in vWF, and this is consistent with the observation that binding functions of the vWF A1 domain do not require divalent cations. The organization of this domain predicted by computer modeling appears to agree with that determined directly by x-ray crystallography, as described in a recent preliminary report, and the model provides a useful framework for understanding the structure-function relationships of the vWF A1 domain.

The locations of residues known to be mutated in patients with type 2M vWD and type 2B vWD are shown within the modeled A1 loop of vWF in Fig 7. Type 2B vWF mutations, which result in enhanced affinity of vWF for the platelet GPIb/IX complex, cluster between Met540 and Arg578 within the amino-terminal half of the disulfide loop. In the molecular model, all the vWD type 2B gain-of function mutations map to a patch of 30x20 Å near the “base” of the globular A1 domain. In contrast, the vWD type 2M mutations, G561S, ΔR629-Q639, I662F, and F606I, appear to cluster at the top of the domain.
despite their significant separation based on the linear sequence location. While the type 2M mutation G561S is sequentially near the region where most of the type 2B gain-of-function mutations are clustered, the three-dimensional molecular model clearly places the Gly561 spatially closer to the other 2M mutations and distant from the 2B mutations.

Although the type 2M mutations I662F and G561S are located near proposed sites for botrocetin binding to vWF as identified by peptide inhibition studies (Asp539-Val553, Lys569-Gln583, and Arg629-Lys643) or scanning mutagenesis (Arg663-Lys667), neither residue is directly within the proposed botrocetin binding segments. Additionally, while a recently proposed model for the regulation of vWF binding to GPIb based on studies of mutants generated by scanning alanine mutagenesis places F606I within one of several discontinuous segments that likely contribute to the interaction of vWF with platelet GPIb, the results of this study show that F606 is not a critical residue for GPIb binding within this segment. Consequently, the type 2M mutations G561S, F606I, and I662F that retain normal botrocetin-induced binding to platelet GPIb do not appear to directly affect residues identified as essential for binding to botrocetin or GPIb.

The type 2M mutations G561S, F606I, and I662F have decreased vWF:RCo yet retain normal botrocetin-induced binding to platelet GPIb. In addition, charged-to-alanine mutations at Glu626 and Asp520-Lys534 and at Lys534, Lys569, and Lys642-Lys645 of vWF resulted in reduced ristocetin-induced binding, but not botrocetin-induced binding of vWF to platelets. These results are consistent with ristocetin and botrocetin each having independent structural requirements or mechanisms for mediating the interaction of vWF with the platelet GPIb/IX complex. The ability of these mutant vWF proteins to bind platelet GPIb in the presence of botrocetin support the conclusion that the GPIb binding site remains intact, while the ristocetin-mediated allosteric regulation of vWF binding is disrupted. Furthermore, these results suggest that discontinuous regions and/or a major portion of the 509-695 disulfide loop are involved in modulation of ristocetin-mediated binding of vWF to platelet GPIb.

These two new type 2M families provide further support for the hypothesis that defects for other vWD variants with a low vWF:RCo/vWF:Ag ratio in the setting of normotilin structure may be localized to exon 28 of the vWF gene. The clustering of mutations for other similar vWD variants within exon 28 will facilitate the rapid genetic diagnosis of these variants. In addition, identification and characterization of genetic defects for these families with variant vWD will provide further insight into the role of the Cys509-Cys695 loop in the interaction of vWF with the platelet GPIb/IX complex and a better understanding of the structural and functional characteristics of vWF in general. Furthermore, an improved classification of vWD, based on known structural and functional characteristics and genetic mutations of vWF, may result for this heterogeneous disorder. An improved classification for vWD based on genotypic analysis should better predict phenotypic expression and therefore aid in diagnosis and management of this disease.

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