Type 2M: Milwaukee-1 von Willebrand Disease: An In-frame Deletion in the Cys509-Cys695 Loop of the von Willebrand Factor A1 Domain Causes Deficient Binding of von Willebrand Factor to Platelets


This report examines the genetic basis of a variant form of moderately severe von Willebrand disease (vWD) characterized by low plasma von Willebrand factor antigen (vWF:Ag) levels and normal multimerization, typical of type 1 vWD, but disproportionately low agonist-mediated platelet-binding activity. We identified an in-frame deletion in vWF exon 28 in three generations of affected family members, who are heterozygous for this mutation. The deletion of nucleotides 4,173-4,205 results in the loss of amino acids Arg629-Gln639 in the Cys509-Cys695 loop of the A1 domain in mature vWF.

The secreted mutant vWF showed a normal multimeric profile but did not bind to platelets in the presence of optimal concentrations of either ristocetin or botrocetin. The mutant vWF also failed to interact with heparin, and with vWF monoclonal antibody 2W3, which blocks the binding of vWF to GPIb. In addition, mutant vWF showed reduced secretion from transfected cells concomitant with increased intracellular levels. These results confirm that the deletion is the genetic defect responsible for the reduced interaction of vWF with platelets. We have designated this new variant type 2M: Milwaukee-1 vWD. Our analysis suggests that the potential frequency of this phenotype in individuals diagnosed with type 1 vWD is about 0.5%.

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VON WILLEBRAND disease (vWD) is the most common inherited bleeding disorder in humans with an estimated prevalence as high as 1%.1 vWD results from defects in the function or synthesis of von Willebrand factor (vWF), which is a multimeric plasma protein that is also found in platelets and endothelial cells. vWF functions in hemostasis by serving both as the carrier of coagulation factor VIII, and as the ligand for the primary adhesion of platelets to sites of vascular damage. The heterogeneous nature of vWD has led to a classification scheme broadly-based on quantitative or qualitative defects in vWF.2 Type 1 vWD includes those patients that show a partial quantitative deficiency of vWF and a normal multimer distribution. Type 2 vWD includes those patients whose vWF shows functional defects, and type 3 vWD includes those patients with a virtually complete deficiency of vWF.

Type 2 vWD is divided further into four subtypes to accommodate the complex phenotypes associated with vWD.2 vWD types 2A, 2B, and 2M, are defined by the dysfunctional interaction of vWF with the platelet glycoprotein Ib/IX (GPIb/IX) complex, and type 2N vWD refers to all variants with a markedly-decreased affinity for coagulation factor VIII. Type 2B vWD refers to those variants with increased affinity for the GPIb/IX complex, whereas vWD types 2A or 2M are defined by decreased platelet-dependent function. Classification of vWD as type 2A or 2M depends on whether the functional defect is associated with the absence (type 2A vWD) or presence (type 2M vWD) of high molecular weight (HMW) multimers of vWF. Many mutations responsible for types 2A and 2B vWD have been identified and they are located exclusively within exon 28 of the vWF gene.3-10 Type 2A mutations are clustered in vWF domain A2, and lead to the reduced interaction of vWF with platelets by causing the loss of HMW vWF multimers either by increasing the sensitivity of plasma vWF to proteolysis, or by inhibiting secretion of high molecular weight multimers.11 Type 2B vWD mutations are located in the A1 domain,4-9 which is consistent with the identification of this region as the site of interaction between vWF and the GPIb/IX complex.12-14 Only one type 2M mutation has been identified, and it also is located in the A1 domain.15

In this paper we report on a family with vWD in which inheritance is dominant, vWF multimerization is normal and plasma vWF antigen (vWF:Ag) levels are low, which is characteristic of type 1 vWD. However, in the affected individuals vWF ristocetin cofactor (vWF R:Co) activity levels are two to eightfold less than the vWF:Ag levels. We identified an in-frame deletion within vWF exon 28 that is linked with the variant vWD phenotype and results in the deletion of amino acids Arg629-Gln639 in the A1 domain of mature vWF. Functional analysis of recombinant vWF containing the deletion supports the conclusion that the deletion is the genetic defect responsible for the disproportionately-low vWF R:Co activity characteristic of this new variant, which we classify as 2M: Milwaukee-1 vWD.

MATERIALS AND METHODS

Patients. Members from three generations of a family with a history of bleeding were evaluated by the Hemostasis Reference Laboratory at The Blood Center of Southeastern Wisconsin (Milwaukee). Plasma vWF multimers were analyzed by electrophoresis on a 0.65% sodium dodecyl sulfate (SDS)/agarose gel using a discontinuous buffer system16,17 and detected with 125I-labeled vWF affinity-purified polyclonal antibodies. vWF:Ag levels were determined by quantitative Laurell rocket immunoelectrophoresis.18 The
vWF R:Co activity of the same samples was measured as described by Abildgaard et al.19

Isolation and analysis of patient RNA and genomic DNA. Blood was drawn from patients with their informed consent following Institutional guidelines. RNA was prepared from platelets and genomic DNA was prepared from white blood cells isolated from 50 ml of peripheral blood. vWF cDNA was amplified by polymerase chain reaction (PCR) using AmpliTaq Taq polymerase (Perkin-Elmer Cetus, Norwalk, CT). In brief, 1 μg of total platelet RNA was used as a template for reverse transcription with primer a4,542-4,530, followed by two rounds of amplification (30 cycles each) with nested primers (first round primers, s3,544-3,573 and a4,506-4,480; second round primers, s3,598-3,627 and a4,488-4,462). The final product was 891 bp and contained nucleotides 3,598-4,488 of the vWF cDNA sequence. The vWF cDNA is numbered using adenosine of the initiating Met codon as the first nucleotide. 21

vWF exon 28 was amplified from genomic DNA using sense primer Vs-27-1 (TCTGTGGGAATCAGGAATGCTATG) located in intron 27 and antisense primer Va-28-6 (GAGATCTCTTGGCAG-ATGCATGTag) located in intron 28 of the vWF gene. 22 Primer Vs-27-5 has the same sequence as primer Va-226, which was previously used for the selective amplification of the vWF gene without interference from the vWF pseudogene. 21 Va28-6 was also designed to allow selective amplification of the vWF gene sequence. Lower case letters in the primer sequences identify positions that differ from the vWF gene sequence 23 for the purpose of introducing EcoRI and BamHI restriction sites into the final product. PCR products were subcloned into plasmids pGEM-SZf(+) or pGEM-7Zf(+) (Promega Corp, Madison, WI). The sequence of the cDNA inserts was determined into plasmids pGEM-SZf(+) or pGEM-7Zf(+) (Promega Corp, Madison, WI). The sequence of the cDNA inserts was determined using Sequenase (US Biochemical, Cleveland, OH).

Primers Vs-4003 (AAGCGACCGTCAGAG) and Va-4370 (AGGATGCTTACAGATGCATGTag) which flank the deletion, were used for detection of the type 2M:Milwaukee-1 vWD deletion by PCR. These primers produce a 367 bp product from normal genomic DNA and a 334 bp product from genomic DNA containing the 33 nucleotide deletion. For PCR amplification, reactions containing 200 ng of genomic DNA and the appropriate pair of primers were pre-sonicated at 99°C for 5 minutes without AmpliTaq. After addition of SaullNcoI 4,752) in the plasmid vector PGEM-SZf(+) the DNA was amplified for 30 cycles (1 minute at 94°C, 1 minute at 51°C, and 2 minutes at 72°C) with a final extension for 7 minutes at 72°C. PCR products were separated by electrophoresis through 1% agarose, stained with ethidium bromide, and visualized using UV light.

Expression of recombinant vWF Type 2M:Milwaukee-1 vWF. A SaulNcoI restriction fragment (nucleotides 3,621-4,481) of the subcloned vWF cDNA PCR product amplified from the platelet RNA of patient II-1 was subcloned into pg7wV1, which contained a BamHI/KpnI restriction fragment of vWF cDNA (residues 2,717-4,752) in the plasmid vector pGEM-7Zf(+) . 23 The BamHI/KpnI restriction fragment of the construct containing the type 2M:Milwaukee-1 vWF sequence was inserted into the full-length vWF expression plasmid pW198 (generously provided by Dennis Lynch, Dana-Farber Cancer Institute, Boston, MA).

pW198 and type 2M:Milwaukee-1 vWF expression plasmids were used to transfect HEK 293T cells26 in the presence of Lipofectamine (GIBCO-BRL, Gaithersburg, MD) as described by Kroner et al. 21 Conditioned media were obtained 48 to 60 hours after transfection, cleared by low speed centrifugation, and stored at -80°C. vWF levels in conditioned media were assayed by antigen-capture enzyme-linked immunosorbent assay (ELISA) using anti-vWF monoclonal antibody (MoAb) AVW-1 27 as the capture antibody and an anti-vWF rabbit polyclonal antibody as the primary detection antibody. The anti-vWF polyclonal antibody was detected using a goat anti-rabbit IgG polyclonal antibody coupled to horseradish per-oxidase and the VECTASTAIN ABC kit (Vector Laboratories, Burlingame, CA). Absorbance values were obtained using a THERMOMax microplate reader (Molecular Devices Corp, Menlo Park, CA) and analyzed using SOFTmax software. Absorbance values were converted to ng/mL vWF using a standard curve generated from dilutions of pooled normal plasma assigned a vWF concentration of 10 μg/mL. The reactivity of vWF with MoAb AVW3 27 was performed as previously described using AVW3 as the capture antibody.

vWF monomer and multimer analysis. vWF was immunoprecipitated from conditioned media or pooled normal plasma using vWF MoAb AVW1 coupled to Sepharose 4B (Pharmacia Biotech, Piscataway, NJ). Monomeric vWF was analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions. vWF was visualized after transfer to nitrocellulose using an anti-vWF rabbit polyclonal antibody and horseradish peroxidase-conjugated goat anti-rabbit antibody (Pierce Chemical Corp, Rockford, IL) and the ECL Detection System (Amersham Corp, Arlington Heights, IL). Recombinant vWF multimers were analyzed on a 1.5% resolving gel as described by Raines et al. 23 After electrophoresis vWF multimers were transferred to nitrocellulose and detected as previously described.

Platelet-binding assay. The binding of vWF to platelets was determined using a modification of a procedure described by Scott and Montgomery 24 and Kroner et al. 21 In brief, vWF was indirectly-labeled using 125I-labeled vWF MoAb AVW1, and incubated for 30 minutes with formalin-fixed platelets (BioData Corp, Hatboro, PA) in the presence of various concentrations of ristocetin or botrocetin. Plasma or conditioned media were diluted so that equal amounts of vWF (100 ng/mL) were used in the platelet binding assay. After pelleting the platelets by centrifugation, the upper one-half of the supernatant was transferred to a new tube and was designated the supernatant (b) fraction. The remaining one-half of the supernatant containing the platelet pellet was designated the pellet (a) fraction. The radioactivity in the pellet and supernatant fractions was determined using a gamma counter. The amount of radioactivity bound to platelets was determined using the formula ((a-b)/a+b)×100. Botrocetin was prepared from Bothrops jararaca venom (Sigma Chemical Co, St Louis, MO). 23 Ristocetin sulfate was a gift of H. Lundbeck (Copenhagen, Denmark).

Heparin-binding assay. The binding of vWF to heparin was performed using heparin-Sepharose CL-4B (Pharmacia Biotech, Piscataway, NJ) following a procedure similar to the platelet-binding assay. Each reaction mixture contained 160 μL of vWF (100 ng/mL) indirectly-labeled with 125I-labeled MoAb AVW1, 50 μL of heparin-Sepharose (50% vol/vol), and 20 μL of either Tris-Saline or soluble heparin (100 μg/mL in ddHzO, sodium salt, grade I-A from porcine intestinal mucosa, Sigma Chemical Co). After incubation at room temperature for 30 minutes, the heparin-Sepharose was pelleted by centrifugation and the amount of radioactivity bound to heparin was determined as previously described.

Statistical analysis of Type 1 vWD patient data. The vWF R:Co and vWF:Ag values of 692 type 1 vWD patients evaluated at The Blood Center between 1988 to 1995 were analyzed to identify abnormal vWF R:Co/vWF:Ag ratios. The vWF R:Co/vWF:Ag ratios of patients with normal vWF multimers and vWF:Ag less than 50% were analyzed and plotted using the SigmaPlot Scientific Graphing System, Version 4.10 (Jandel Corp, Corte Madera, CA).

RESULTS

Clinical diagnosis. Fig 1A shows the pedigree of a family with a variant form of vWD. Affected family members have lifelong histories of mild mucocutaneous bleeding characterized by easy bruising and epistaxis. Patients I-2 and I-
Fig 1. Clinical evaluation of a family with type 2M:Milwaukee-1 vWD. (A) Family pedigree. Three generations are illustrated showing affected (shaded symbol) and unaffected (open symbol) family members. vWF:Ag/vWF R:Co activities determined from clinical analysis are shown below selected symbols. vWF:Ag levels and vWF R:Co activities measured 1 hour post DDAVP treatment are indicated in parenthesis. (B) Autoradiographic pattern of plasma vWF multimers analyzed by SDS/agarose gel electrophoresis (0.65% agarose) and detected with 125I-labeled anti-vWF antibody. Lanes 2, 4, 5, and 7 contain normal plasma. Lanes 1, 3, 6, and 8 contain plasma from patients 1-2, 11-2, 11-3, and 11-1, respectively.

I reported prolonged bleeding after tooth extractions and patient III-1 bled excessively following tonsillectomy. Analysis of plasma vWF from affected patients I-2, II-1, II-2, and II-3 showed vWF:Ag levels ranging from 14 to 35 U/dL and a normal vWF multimer profile (Fig 1B). However, the vWF R:Co levels in these patients ranged from 3 to 8 U/dL, which results in vWF R:Co/vWF:Ag ratios of 11% (patient I-2) to 33% (patient II-1). This disproportionality persists following treatment with 1-deamino-8-D-arginine-vasopressin (Fig 1A). The disproportionality between vWF:Ag and vWF R:Co levels was not observed in unaffected family members I-1 and II-4 (Fig 1A). It should be noted that although the vWF:Ag level of unaffected family member II-4 is low, it is within the blood type-matched normal range (type A, 48-233 U/dL). Affected individuals thus have low vWF:Ag levels and normal plasma multimers.
typical of type 1 vWD, combined with a functional defect that reduces the ristocetin-induced binding of vWF to platelets, characteristic of type 2 vWD. We propose that the bleeding disorder in this family be classified as type 2M:Milwaukee-I vWD, which is consistent with the revised classification scheme for vWD.2

**Identification of a mutation in affected family members.**

vWF mutations associated with increased or decreased reactivity of vWF with the platelet GPIb/IX complex are localized to exon 28 of the vWF gene.3 We therefore examined the sequence of vWF exon 28 from affected family members to determine if a mutation in this region was responsible for the reduced vWF R:Co activity of their plasma vWF. Reverse transcription and PCR were used to amplify exon 28 sequence from either platelet RNA or genomic DNA of patients I-2 and I-1. The amplified products were subcloned and the sequence of individual clones was determined. A 33 nucleotide deletion was detected in 3 of 5 clones derived from patient I-2 RNA, 4 of 4 clones from patient II-1 RNA, and 2 of 4 clones from patient II-1 DNA. Representative

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**Fig 2. Identification and detection of the type 2M:Milwaukee-1 deletion mutation.** (A) DNA sequence of normal and variant vWF alleles. vWF exon 28 was amplified from genomic DNA, subcloned, and then sequenced. Representative sequence of clones containing the normal allele and variant allele are shown with the brackets indicating the region of the normal allele that is deleted in the mutant allele (nucleotides 4,173-4,205). As indicated in the translated sequence, the in-frame deletion of nucleotides 4,173-4,205 results in a loss of 11 amino acids, Arg629-Gln639. Moreover, the deletion does not result in an amino acid substitution because the codon for Gln628 is conserved at the deletion junction. (B) Detection of the 4,173-4,205 deletion in vWD patient platelet RNA and genomic DNA. PCR using primers which flank the deletion was used to amplify products of 367 bp and 334 bp from normal and mutant alleles, respectively. The presence of the 334 bp product indicates the presence of the deletion in exon 28. PCR products on the gel on the left were amplified from platelet RNA of patients II-1 and I-2, platelet RNA of a normal control (N), and a no RNA control (Blk). Products amplified from genomic DNA of patients II-3, II-2, II-4, and I-1 are shown on the right. The amplification of vWF pseudogene sequence, in addition to vWF gene sequence, results in the greater intensity of the 367 bp product in the gel on the right. Molecular weight standards are shown in the center.
The deletion corresponds to nucleotides 4,173-4,205 of the vWF coding sequence and encodes amino acids Arg629-Gln639 of the mature protein (Fig 2A). The deletion does not change the reading frame of the vWF protein or result in an amino acid substitution (Fig 2A). According to the sequence analysis, patients I-2 and II-1 are heterozygous for the deletion, as both normal and mutant alleles were identified. Patients are also heterozygous for a commonly occurring G/A polymorphism at nucleotide 4,141,21 with G occurring in association with the deletion.

Members of the affected family were examined for the vWF gene deletion by PCR using primers that span the deleted sequence (Fig 2B). Amplification of the native vWF sequence results in a 367 bp fragment, and amplification of the mutant sequence results in a 334 bp fragment. Analysis of patients II-1 and I-2 using platelet RNA as the primary template revealed the presence of both 334 bp and 367 bp fragments, which is consistent with the sequence data and confirms that these patients are heterozygous for the deletion. This analysis also showed that affected family members II-2 and II-3 are also heterozygous for this deletion, and that the deletion is not present in unaffected family members I-1 and II-4. The specificity of this assay is shown by the amplification of only the 367 bp product from the platelet RNA isolated from an unrelated unaffected individual, and the absence of product in the no RNA or no DNA controls. Occasionally, an artificial higher molecular weight band (~0.5 kbp) was amplified from platelet RNA as shown in lane N of Fig 2B. A similar PCR analysis using genomic DNA demonstrated that patient III-1 is also heterozygous for the deletion of the vWF gene sequence encoding amino acids Arg629-Gln639 of mature vWF (data not shown).

Expression of recombinant mutant vWF. To determine the effect of the Arg629-Gln639 deletion on the synthesis and function of vWF, a restriction fragment containing the deletion was inserted into the full-length vWF expression vector pW198, and both wild type (wt) and mutant vWF (vWF-Δ(629-639)) were transiently expressed in mammalian cells. Although total synthesis vWF-Δ(629-639) was only slightly less that wt vWF (4.38 ± 0.36 μg v 4.89 ± 0.28 μg, respectively), comparison of intracellular and secreted (conditioned media) vWF showed that deletion of amino acids Arg629-Gln639 caused a moderate defect in secretion (Fig 3). Compared to wt vWF, the concentration of vWF-Δ(629-639) in conditioned media was reduced by 33%. In contrast, the intracellular concentration of vWF-Δ(629-639) was twofold higher than wt vWF. The presence of the deletion did not affect the structure of the secreted vWF-Δ(629-639), which showed a monomer size (Fig 4A) and multimeric profile (Fig 4B) that was similar to wt vWF.

Interaction of recombinant vWF with platelets. To determine if the Arg629-Gln639 deletion is responsible for the disproportionately-low vWF R:Co activity in the affected family, platelet-binding assays were performed with normal plasma and expressed wt vWF and vWF-Δ(629-639) using both ristocetin (Fig 5A) and botrocetin (Fig 5B) as agonists. Compared to normal plasma vWF, wt vWF showed reduced binding to platelets in the presence of reduced concentrations of ristocetin, which most likely reflects, in contrast to botrocetin, the preferential interaction of ristocetin with HMW vWF multimers.22 In our expression system the proportion of secreted vWF that consists of HMW multimers is less than the proportion of HMW multimers in plasma vWF. The binding of wt vWF to platelets in the presence of botrocetin is similar to normal plasma vWF, and shows that wt vWF is fully functional. In contrast to wt vWF, vWF-Δ(629-639) does not show significant binding to platelets at any concentration of ristocetin or botrocetin. This supports the conclusion that the deletion is responsible for the reduced platelet-binding activity of the plasma vWF in the affected family.

Interaction of recombinant vWF with heparin. The vWF A1 domain contains a binding site for heparin,13,15 and recent evidence suggests that the heparin-binding domain may participate in the regulation of the vWF/GPIb interaction.24 To determine whether deletion of amino acids Arg629-Gln639 affects the interaction of vWF with heparin, we performed binding assays with wt vWF and vWF-Δ(629-639) using heparin-Sepharose (Fig 6). Although wt vWF showed binding to heparin that is comparable to plasma vWF, vWF-Δ(629-639) lacked heparin-binding activity. The specificity of the interaction of vWF with heparin in this assay was shown by the lack of binding of wt vWF and plasma vWF to heparin-Sepharose in the presence of excess soluble heparin.

Prevalence of the type 2M vWD phenotype among type 1 vWD patients. Because type 2M:Milwaukee-1 vWD has features in common with type 1 vWD, we analyzed the records of type 1 vWD patients to identify additional patients with type 2M:Milwaukee vWD. The analysis included 692
type 1 vWD patients evaluated between 1988 and 1995 by
the Hemostasis Laboratory of The Blood Center of South-
eastern Wisconsin and included patients with normal vWF
multimers and vWF:Ag levels less than 50 U/dL. Patients
with known preexisting conditions or on prescription drug
treatments which could potentially affect plasma vWF anti-
gen levels were excluded from analysis. The vWF R:Co
activities and vWF:Ag levels were determined using the
same pooled normal plasma standard. The vWF R:Co/
vWF:Ag ratios for these patients is shown in Fig 7. For all
patients the mean vWF R:Co/vWF:Ag ratio was 0.99 ±
0.27 (±2 SD). The vWF R:Co/vWF:Ag ratios for affected
members of the type 2M:Milwaukee-I vWD family con-
taining the Arg629-Gln639 deletion are included in Fig 7
and are identified according to Fig 1A. In addition to the
family members that are the focus of this study, three other
individuals who are probands from three different families
show a vWF R:Co/vWF:Ag ratio of less than 0.4. If the
affected family members from this study other than the pro-
band are excluded from this analysis, then the frequency of
patients with the type 2M:Milwaukee vWD phenotype in
this patient population is 0.5%.

**DISCUSSION**

The phenotype of the vWD patients in this study is charac-
terized by normal multimers, low plasma vWF:Ag, and
disproportionately low vWF R:Co activity. Due to the unique
phenotype of these patients, and to the identification and
confirmation of deletion of Arg629-Gln639 in mature vWF
as the cause of the reduced interaction of the patients’ plasma
vWF with platelets, we propose that this vWD variant be
classified as type 2M:Milwaukee-I. Two lines of evidence
support the conclusion that a 33 nucleotide deletion encoding
vWF amino acids Arg629-Gln639 is responsible for the dis-
proportionately low vWF R:Co activity of the affected family
members. First, the 33 nucleotide deletion and low vWF
R:Co activity are co-inherited through three generations of
the affected family. The amplification of both the normal
and mutant alleles from the platelet RNA of the affected
individuals shows they are heterozygous for the defect, and
that their plasma multimers consist of both normal and mu-
tant vWF. Second, vWF-Δ(629-639), like patient plasma
vWF, is deficient in both ristocetin- and botrocetin-mediated
binding of vWF to platelets, vWF-Δ(629-639) has a more
severe platelet-binding defect than plasma vWF, which is
most likely caused by the homozygous expression of the
defect in transfected cells, as compared to the heterozygous
expression of normal and mutant vWF in the patients’
plasma.

The type 2M:Milwaukee-I vWD deletion resides within the
A1 domain of mature vWF, which contains the binding
site for the platelet GPIb/IX complex.12-14 An important fea-
ture of this region is a 185-amino acid loop defined by disul-
fide bonds between Cys509 and Cys695. Several studies
identified discrete functional domains both within and adja-
cent to the loop that may regulate the vWF/GPIb interaction
or form the GPIb-binding site in vWF. For example, Cys474-
Pro488 and Leu694-Pro708 are flanking sequences that may
be involved in the ristocetin-mediated interaction of vWF
with GPIb.55 Functional domains that have been identified
within the Cys509-Cys695 loop include Asp514-Glu542,
which may form part of the GPIb-binding site,56 and three
discontinuous segments, Asp539-Val553, Lys569-Gln583,
and Arg629-Lys643, which may participate in the interaction
of vWF with botrocetin.57 In the type 2M:Milwaukee-I vWD
variant, the deletion of 11 of the 15 amino acids in the
proposed Arg629-Lys643 botrocetin binding domain may
result in the inability of botrocetin to bind to vWF, thus
resulting in the observed absence of botrocetin-mediated
binding of vWF to platelets. This is consistent with alanine
mutagenesis studies of vWF, which showed that Arg636 is
required specifically for botrocetin binding.58 However, as
discussed later it seems more likely that the deletion of vWF
amino acids Arg629-Gln639 results in an overall conforma-
tional change that inhibits either the interaction of vWF with
ristocetin and botrocetin, or the binding of the agonist-vWF
complex to the GPIb/IX complex.

An additional functional defect in vWF-Δ(629-639) is the
loss of heparin-binding activity. The physiological signifi-
cance of the interaction of vWF with heparin is not clear,
but it was recently proposed that the heparin-binding domain in vWF may overlap with the GPIb-binding site, and may play a role in regulating the vWF/GPIb interaction.\textsuperscript{24} Heparin-binding sites in vWF have been proposed both in the amino-terminus of mature vWF\textsuperscript{29} and in the A1 domain.\textsuperscript{33,33} However, the loss of heparin-binding activity after deletion of the A1 domain suggests that the heparin-binding domain in the amino terminus may not be functional in multimeric vWF.\textsuperscript{30} Sobel et al\textsuperscript{31} further localized the heparin-binding domain of vWF in the A1 domain to residues Tyr565-Ala587. Thus it was surprising that vWF-\(\Delta\)(629-639) lacks heparin-binding activity. Our results suggest that either a second heparin-binding site is present in the A1 domain, or that the heparin-binding site is formed from discontinuous segments of the A1 domain. Alternatively, the deletion of Arg629-Gln639 may cause conformational changes in the A1 domain that disrupt the heparin-binding site. Interestingly, Sobel et al\textsuperscript{31} also reported that a secondary consensus heparin-binding sequence is present in the vWF A1 domain between residues Asn633-Val648. The loss of heparin-binding activity by vWF-\(\Delta\)(629-639) suggests that this region may form a heparin-binding site in vWF. Although this is consistent with the results of a mutagenesis study of the A1 domain which showed that substitution of amino acids 642-645 with alanine resulted in reduced heparin-binding activity by full-length multimeric vWF (Kroner et al, submitted), we suspect that, as discussed later, the loss of heparin-binding activity by vWF-\(\Delta\)(629-639) may also be due to conformational changes in the A1 domain that result from the deletion of amino acids Arg629-Gln639.

Several lines of evidence suggest that the multiple defects associated with the deletion of Arg629-Gln639 are caused by a conformational change in the A1 domain. First, although vWF-\(\Delta\)(629-639) retains normal binding to AvW1, which is a MoAb with an epitope in the carboxy terminus of vWF, vWF-\(\Delta\)(629-639) fails to bind to AvW3, which is a conformation-dependent antibody that blocks the binding of vWF to GPIb (data not shown). Second, ristocetin and botrocetin induce the binding of vWF to the GPIb/IX complex by distinct mechanisms,\textsuperscript{37,42} and the Arg629-Gln639 deletion re-
Results in the loss of both ristocetin- and botrocetin-induced binding of vWF to platelets. Third, vWF-Δ(629-639) fails to bind to heparin, and it has been suggested that heparin-binding domains may have both charge and conformational restraints. Fourth, the phenotype of vWF-Δ(629-639) is similar to phenotypes of nine vWF A1 domain deletion mutants all of which show defects in secretion, fail to bind vWF MoAb AvW3, and fail to bind to platelets in the presence of ristocetin or botrocetin (Kroner et al, submitted). Similarly, four to 20 amino acid deletion mutations in the Cys509-Cys695 loop of an A1 domain fragment expressed in Escherichia coli also result in the loss of GPIb-binding activity. The Arg629-Gln639 deletion may be affecting discrete functional domains in vWF, but these observations suggest that the biosynthetic and functional defects in vWF-Δ(629-639) are caused by conformational changes in the A1 domain resulting from the deletion of Arg629-Gln639.

It is not clear if the mild secretory defect caused by the deletion of Arg629-Gln639 is entirely responsible for the low vWF:Ag levels in the type 2M:Milwaukee-1 family. If the major form of vWF in the endoplasmic reticulum is a heterodimer composed of mutant and native vWF monomers, or if the selection for intracellular retention occurs after formation of multimers including both mutant and native vWF monomers, then the selective retention of the mutant vWF could result in the reduced secretion of native vWF as well. Such a result was seen in cotransfection experiments performed with wt vWF and mutant vWF in which Arg611 was changed to either Cys or His. The deletion associated with vWD type 2M:Milwaukee-1 is unusual among the many type 2 vWD mutations that have been identified thus far in that it results in a loss of function (defective agonist-mediated binding to platelets) without affecting multimerization. The Arg629-Gln639 deletion contrasts with type 2B vWD mutations which cause enhanced reactivity of vWF to GPIb. In addition, while most type 2B vWD mutations are clustered in the amino-terminal half of the Cys509-Cys695 loop between residues 540 and 578, the Arg629-Gln639 deletion is located in the carboxy-terminal half of the loop. Other mutations in the vWF Cys509-Cys695 loop that result in the reduced binding of vWF to GPIb have been reported. These mutations include Gly561Ser, and Arg611Cys and Arg611His. Unlike the type 2M:Milwaukee-1 deletion, the Gly561Ser mutation does not affect the secretion of vWF, and abolishes ristocetin-induced, but not botrocetin-induced binding of vWF to the platelet GPIb/IX complex. The phenotypes of recombinant vWF containing either the Arg611Cys or Arg611His substitutions are more similar to the recombinant type 2M:Milwaukee-1 vWF. These mutant proteins show secretory defects and markedly-reduced platelet-binding activity. However, these two mutants differ from type 2M:Milwaukee-1 vWF in that the secretory defect includes the loss of high molecular weight multimers. In addition, the Arg611Cys and Arg611His proteins lacked ristocetin-induced platelet-binding activity, but they retained a low, but significant, ability to bind to platelets in the presence of botrocetin. The identity of “loss-of-function” mutations in the carboxy-terminal half of the Cys509-Cys695 loop supports evidence obtained from mutagenesis that this region contains important regulatory and/or binding domains.

Our analysis suggests that a small subset of type 1 vWD patients have vWF R:Co/vWF:Ag ratios characteristic of type 2M:Milwaukee-1 vWD. We are currently studying these individuals and their families to determine the genetic defect responsible for their bleeding disorder. Our hypothesis is that mutations associated with this phenotype, if different than the 33 nucleotide deletion found in the family reported here, will cluster in the carboxy-terminal half of the Cys509-Cys695 loop near the Arg629-Gln639 deletion. The identification of mutations responsible for type 2M:Milwaukee vWD will facilitate the rapid clinical diagnosis of this vWD variant. In addition, the identification and characterization of the genetic defects responsible for the decreased platelet-dependent function of vWF will provide further insight into the role of the Cys509-Cys695 loop in the interaction of vWF with the platelet GPIb/IX complex, and may define domains important for the physiological regulation of vWF/GPIb interaction.

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