We report the identification of von Willebrand factor (vWF) gene mutations within exon 28 occurring in three unrelated families with an infrequent form of type 2 von Willebrand disease (vWD). A C → T transition and a G → A transition, both at the codon for arginine 611 of the mature vWF subunit, were found. They result in either a cysteine or an histidine substitution, respectively. Patients were found to be heterozygous for these substitutions and the vWD was transmitted dominantly. These substitutions have been reproduced by in vitro mutagenesis of full-length cDNA of vWF and transiently expressed in Cos-7 cells. The corresponding recombinant vWFs (rvWF) exhibited decreased expression and a significant decrease in the high molecular weight multimeric forms. In addition, ristocetin- and botrocetin-induced binding of mutated rvWFs to platelets were markedly decreased as compared with that for the wild-type rvWFs. Thus, the structural and functional characterization of both mutated rvWFs confirmed that the two nucleotide substitutions identified at position 611 of the mature subunit of vWF are real mutations. Although they are located in the A1 loop containing most of the type 2B mutations inducing increased affinity of vWF for platelet glycoprotein Ib, they are responsible for abnormal vWF with decreased platelet-dependent function.

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Identification of Two Mutations (Arg611Cys and Arg611His) in the A1 Loop of von Willebrand Factor (vWF) Responsible for Type 2 von Willebrand Disease With Decreased Platelet-Dependent Function of vWF

By Lysiane Hilbert, Christine Gaucher, and Claudine Mazurier

VON WILLEBRAND FACTOR (vWF) is a highly multimerized glycoprotein that mediates platelet adhesion to the subendothelium of injured vessels; it is also the carrier for factor VIII (FVIII) in the circulation. It is synthesized in megakaryocytes and endothelial cells, described by a 9-kb mRNA resulting from an ≈180-kb gene including 52 exons and encoding a translation product of 2,813 amino acids (aa). The pro-peptide (763 aa) and the mature subunit (2,050 aa) of vWF are composed of four types of repeating regions designated A through D and arranged in the following order: D1-D2-D'-D3-A1-A2-A3-D4-B1-B2-B3-C1-C2. Before secretion, vWF undergoes an extensive posttranslational processing including cleavage of the propeptide (D1-D2) and multimerization. Secreted vWF consists of a series of multimers that range in size from 500 to over 10,000 kD depending on the number of dimers of the mature subunit (D2 to C2) that are disulfide bonded. Several distinct domains have been identified within the protein, including regions binding to FVIII, to the glycoprotein (GP) platelet receptors GPIb and GPIIb/IIIa and to components of the extracellular matrix, such as collagen. A region important for the interaction of vWF with GPIb has been localized between aa residues 449 and 728 of the mature subunit of vWF. In vitro, binding of vWF to GPIb can be induced by the antibiotic ristocetin or by the snake venom protein botrocetin. Although ristocetin binds to platelets and vWF, botrocetin binds directly to vWF, leading to a conformation change that allows the interaction of vWF with GPIb. The cysteine loop (Cys 509-Cys 695) in the A1 domain of vWF has been identified as an important element in the regulation of binding to GPIb because most of the mutations identified so far in the patients displaying increased reactivity of vWF with GPIb, in the presence of ristocetin, are located between aa residues 540 and 578. von Willebrand disease (vWD) is the most common inherited bleeding disorder that results from either quantitative or qualitative abnormalities of vWF. Quantitative defects are found in the dominant type 1 and the recessive type 3 vWD. Qualitative disorders (type 2 vWD) have recently been divided into four categories and result, in general, from missense mutations or small inframe deletions and insertions in vWF gene. Type 2A vWD refers to variants with decreased platelet-dependent function that is associated with the absence of high molecular weight (HMW) multimers, whereas type 2M refers to variants with decreased platelet-dependent function that is not caused by the absence of HMW vWF multimers. In contrast, type 2B vWD refers to variants with an increased affinity of vWF for platelet GPIb. The most common phenotype in type 2A vWD, previously named type IIA vWD, is characterized by the absence of both HMW and intermediate molecular weight vWF multimers and by the increase in the intensity of the satellite bands of each multimeric unit. The mutations responsible for type IIA vWD have been localized within the A2 domain of vWF, between aa residues 742 and 885, containing the Tyr842-Met843 sequence sensitive to in vivo proteolysis.

We report here the identification of two vWF gene mutations occurring in three unrelated families with type 2 vWD. Sequence analysis of polymerase chain reaction (PCR)-amplified exon 28 of vWF gene in affected patients identified two different nucleotide (nt) substitutions at the codon for arginine 611 of the mature vWF subunit resulting in either a cysteine or a histidine residue. Using site-directed mutagenesis and transient expression in Cos-7 cells, we have shown that these substitutions (Arg611Cys and Arg611His) are the mutations responsible for the quantitative defect, the decrease in HMW multimers, and the decreased platelet-dependent function previously shown in the patients' vWF. Although they are located in the A1 loop, where are clustered most of the mutations found in patients with type 2B vWD.
TWO TYPE 2 vWD MUTATIONS AT POSITION 611

and relatively far from the mutations in the A2 domain found in the patients with IIA phenotype of vWD, they are responsible for a type 2 vWD, with a slight qualitative defect in the multimer distribution, but markedly decreased in platelet-dependent function.

MATERIALS AND METHODS

Patients. This study was performed on the patients of three unrelated families living in northern France. Hemorrhagic symptoms included hemorrhages after tooth extraction, surgery or delivery, menorrhagia, easy bruising, prolonged bleeding after superficial cuts, and epistaxis. The patients or their parents gave informed consent for this study.

Family A. Seven members belonging to three generations were studied. Their family tree is shown in Fig 1A and their biologic data are reported in Table 1. Five of them gave evidence of a vWD phenotype. Patient A11 was diagnosed after severe bleeding after a head injury and his son, AII, because of prolonged bleeding time detected before tonsillectomy. Patient AII showed bleeding symptoms as ecchymosis, hematomas, and gum bleedings and hemorrhages after dental extractions, but no epistaxis. Hematomas and epistaxis episodes were reported in patient AII2 who was transfused when he was 4 years old; he also received cryoprecipitate twice and vWF concentrate (CRTS, Lille, France) once, for surgical procedures. Patient AII5 bled for a long time after bites and superficial cuts and displayed hematomas and gum bleedings. No bleeding disorder was reported in patient AII2. Patient AII4 also received vWF concentrate for tonsillectomy and was infused once with 1-deamino-8-D-Arginine-Vasopressin (dDAVP, Minirin, Ferring, Malmö, Sweden).

Family B. Twelve family members, also belonging to three generations, were studied. Their family tree is shown in Fig 1B and their biologic data are reported in Table 1. All affected family members have been tested on 4 to 5 occasions for vWF and FVIII levels. Among the seven subjects with abnormal data, BII1 is asymptomatic, whereas his father and one of his sisters were reported to have bleeding symptoms. This patient was infused once with dDAVP. The six other patients studied displayed moderate bleeding symptoms including ecchymosis, menorrhagia, and epistaxis. Patient BIII1 once required an infusion of vWF concentrate (CRTS) to stop epistaxis.

Patient C. The biologic data of this 19-year-old patient are reported in Table 1. He has suffered from frequent epistaxis leading to severe anemia. For this, he had to be hospitalized on several occasions and received blood and vWF concentrate (CRTS). As the propositus’s parents were not available, no family study could be performed.

Blood samples collection and routine coagulation studies. Ivy’s template bleeding times were determined using a Simplate 1 device (Organon Technica, Durham, NC). Blood samples were collected with 13 mmol/L sodium citrate and stored frozen at −20°C. Ristocetin-induced platelet agglutination assay (RIPA) was performed on platelet-rich-plasma (PRP) by measuring the extent of agglutination after addition of two concentrations (0.5 and 1 mg/mL) of ristocetin (Diagnostica, Asnières sur Seine, France). FVIII coagulant activity (FVIII:CA), FVIII antigen (FVIII:A) level, vWF antigen (vWF:Ag) level, and ristocetin cofactor activity (vWF:RCo) were determined on platelet-poor from plasma as previously described. For platelet lysate analysis, blood samples were collected into 1/10 volume of 1p100 (wt/vol) EDTA-0.15 mol/L NaCl and centrifuged at 750g for 3 minutes. After different washes, PRP was lysed in the presence of Triton X100 for 1 to 2 minutes. Platelet lysate was frozen at −70°C until used.

Sequencing of PCR-amplified DNA. Fragments of exon 28 were amplified by PCR and sequenced as previously described.

Allele-specific PCR. This specific amplification was performed on genomic DNA extracted from peripheral blood leukocytes from members of family B using primer W9 and primer 611Arg or 611His matching either the normal or the mutated sequence. The reaction mixture was as previously described and amplification was performed in a Perkin Elmer Cetus model 9600 programmable thermal cycler (Applied Biosystem Roche, Roissy Charles de Gaulle, France). After a 1-minute denaturation at 94°C, all samples were subjected to 28 amplification cycles (eight cycles of 20 seconds at 94°C, 20 seconds at 62°C to 58°C—the temperature of annealing was automatically decreased from 0.5°C at each cycle—20 seconds at 72°C and 20 cycles of 20 seconds at 94°C, 20 seconds at 58°C and 20 seconds at 72°C). The amplification was terminated by a final elongation step at 72°C for 5 minutes. Ten microliters of PCR products were run in a 2% agarose gel containing 1 g/mL of ristocetin and visualized under ultraviolet (UV) illumination.

Expression vector construction. Three vectors, pSVvWFCA, pSVvWFb, and pSVvWFA, were used for the construction of vectors containing the Arg611Cys and the Arg611His mutations. pSVvWFA derives from pSVvWF and contains a new unique restric-
tion site, AII f. This AIIf restriction site was created in pSVvWFA using primer 533 AII f (nt 24/402-433) containing a mismatched nt for the creation of the AII f restriction site at position 533 in the mature vWF subunit (5'HCC TGT TGT AAG GCC TTT GTG GT'; the underlined letter in the sequence indicates the nt that differs from the corresponding normal vWF sequence and the bold letters correspond to the desired mutation and to exclude polymerase errors. Wild-type Cys611, His611 vWF and Cys611, His611 vWF. Cotransfection experiments with normal and mutated plasmids (1:1) were also performed.

**Cell culture and transfection.** Cos-7 cells were cultured and transfected using the diethylaminoethyl dextran method, as previously described.18 The recombinant proteins (vWF) produced by plasmids pSVvWFA and pSVvWFA will be called WtrvWF and WtrvWFA. The mutated rVFs will be respectively referred to as Cys611rvWF and His611rvWFA. Cotransfection experiments with normal and mutated plasmids (1:1) were also performed.

**GP Ib binding analysis.** Ristocetin- or botrocetin-dependent platelet binding assays were performed using methods already described.19 Samples were diluted to obtain vWF:Ag levels of 15 IU/dL. Dilutions of plasma samples were performed in the plasma from a severe type 3 vWD patient, while each concentrated culture medium sample was diluted in serum-free culture medium.

**vWF electrophoretic characterization.** A total of 1 mlU vWF:Ag was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (5% to 9% gradient gels) in the presence of 5% β-mercaptoethanol. After electrotransfer of the gel, the samples were visualized by probing the nitrocellulose sheets with rabbit anti-vWF polyclonal antibodies conjugated to peroxidase20 and further revelation by 4-chloro-1-naphtol (Serva Fine Chemicals, Heidelberg, Germany). Myosin (215 kD) and phosphorylase B (105 kD) were included as molecular weight markers.

The multimeric composition of vWF (2.5 mlU) was analyzed by electrophoresis in either 1.5% or 2.5% agarose gels in the presence of 0.1% SDS.21 The vWF multimers were visualized with rabbit anti-vWF polyclonal antibodies22 conjugated to alkaline phosphatase using an immunopure maleimide alkaline phosphatase conjugation kit (Pierce, Rockford IL). The gels were then scanned with a densitometer taking care that the scanning was in the linear range of the multimeric profile. For the pool of normal plasmas, the scanning of 10 multimeric profiles obtained from the same pool of normal plasmas electrophoresed in 10 independent gels allowed us to determine the relative percentages of the protomer (3.8% ± 1.3%) and the multimers ≥5 mers (74.6% ± 5.2%).

**Data analysis.** Data are given as mean ± standard deviation (SD). The Student's t-test was used to compare data. Differences were considered significant when P < .05.

**RESULTS**

**Phenotype analysis.** Phenotypic data of individuals studied in the three families are reported in Table 1. All affected members shared common features including increased bleeding time, normal platelet count, decreased level of vWF with vWF:Ag values higher than vWF:RCo, drastic decrease in RIPA at 1 mg/mL, and a decreased level of FVIII. For the plasma from patients AII2, BII1, BII3, and C, the relative percentage of HMW vWF multimers ≥5 mers was significantly decreased, while there was a significant increase in the relative percentage of the protomer (Table 1). For the

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**Table 1. Routine Laboratory and Electrophoretic Data**

<table>
<thead>
<tr>
<th>Family Member</th>
<th>Bleeding Time (min)</th>
<th>FVIII:C (IU/dL)</th>
<th>vWF:Ag (IU/dL)</th>
<th>vWF:RCo (IU/dL)</th>
<th>RIPA (% of aggregation)</th>
<th>% of vWF Multimers in Plasma</th>
<th>% of vWF Multimers in Platelets</th>
</tr>
</thead>
<tbody>
<tr>
<td>Family A</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I1 (Affected)</td>
<td>&gt;15 (2)</td>
<td>13 ± 4 (4)</td>
<td>10 (2)</td>
<td>&lt;10 (3)</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>I2 (Affected)</td>
<td>&gt;10 (3)</td>
<td>12 ± 0.8 (4)</td>
<td>&lt;20 (3)</td>
<td>&lt;10 (3)</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>I3 (Affected)</td>
<td>10 ± 3</td>
<td>22.6 ± 2.5 (3)</td>
<td>24.4 ± 7 (4)</td>
<td>&lt;10 (4)</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>I4 (Affected)</td>
<td>14 (3)</td>
<td>31 ± 1 (5)</td>
<td>19 ± 2</td>
<td>&lt;10 (2)</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>I5 (Normal)</td>
<td></td>
<td>130 ± 1 (1)</td>
<td>100 ± 1</td>
<td>92 ± 1</td>
<td>1</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>I6 (Normal)</td>
<td>&gt;10 (2)</td>
<td>66 ± 1 (2)</td>
<td>67.5 ± 3.6</td>
<td>&lt;10 (2)</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Family B</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I1 (Affected)</td>
<td>15 ± 3</td>
<td>35 ± 16 (3)</td>
<td>33 ± 7 (4)</td>
<td>&lt;10 (3)</td>
<td>0</td>
<td>0</td>
<td>9.5 ± 3 (3)</td>
</tr>
<tr>
<td>I2 (Normal)</td>
<td>7 (1)</td>
<td>117 ± 1 (1)</td>
<td>100 ± 1</td>
<td>63 ± 1</td>
<td>0</td>
<td>0</td>
<td>5.8 ± 1 (1)</td>
</tr>
<tr>
<td>I3 (Affected)</td>
<td>&gt;20 (1)</td>
<td>32 ± 4 (2)</td>
<td>26 ± 6 (4)</td>
<td>&lt;10 (6)</td>
<td>0</td>
<td>0</td>
<td>9 (1)</td>
</tr>
<tr>
<td>I4 (Affected)</td>
<td>&gt;20 (1)</td>
<td>42 ± 2 (2)</td>
<td>16 ± 2 (2)</td>
<td>&lt;10 (2)</td>
<td>0</td>
<td>0</td>
<td>55 ± 2 (3)</td>
</tr>
<tr>
<td>I5 (Normal)</td>
<td>&gt;10 (1)</td>
<td>65 ± 1 (1)</td>
<td>92 ± 1</td>
<td>90 ± 1</td>
<td>0</td>
<td>0</td>
<td>71 (1)</td>
</tr>
<tr>
<td>I6 (Normal)</td>
<td>&gt;20 (1)</td>
<td>26 ± 4 (5)</td>
<td>14 ± 5 (3)</td>
<td>&lt;10 (4)</td>
<td>0</td>
<td>0</td>
<td>60 (1)</td>
</tr>
<tr>
<td>I7 (Normal)</td>
<td>&gt;10 (2)</td>
<td>100 ± 2</td>
<td>&gt;100 ± 2</td>
<td>95 ± 21 (2)</td>
<td>0</td>
<td>0</td>
<td>60 (2)</td>
</tr>
<tr>
<td>I8 (Affected)</td>
<td>8'30 (1)</td>
<td>27 ± 5 (6)</td>
<td>24 ± 5 (4)</td>
<td>&lt;10 (6)</td>
<td>0</td>
<td>0</td>
<td>45 ± 1</td>
</tr>
<tr>
<td>I9 (Affected)</td>
<td>&gt;20 (1)</td>
<td>11.5 ± 2 (4)</td>
<td>18 ± 2.5 (4)</td>
<td>&lt;10 (6)</td>
<td>0</td>
<td>0</td>
<td>7 ± 1</td>
</tr>
<tr>
<td>I10 (Normal)</td>
<td>2.5 (1)</td>
<td>125 ± 1</td>
<td>140 ± 1</td>
<td>110 ± 1</td>
<td>0</td>
<td>0</td>
<td>10 ± 5</td>
</tr>
<tr>
<td>I11 (Affected)</td>
<td>31.5 ± 4 (5)</td>
<td>17 ± 2 (5)</td>
<td>&lt;10 (5)</td>
<td></td>
<td>0</td>
<td>0</td>
<td>6 ± 1</td>
</tr>
<tr>
<td>I12 (Normal)</td>
<td>24 ± 1</td>
<td>54 ± 1</td>
<td>54 ± 1</td>
<td>&lt;100 ± 1</td>
<td>0</td>
<td>0</td>
<td>7 ± 1</td>
</tr>
<tr>
<td>Family C</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Patient C</td>
<td>10 ± 2</td>
<td>39 ± 10 (5)</td>
<td>25 ± 3 (3)</td>
<td>&lt;10 (3)</td>
<td>0</td>
<td>0</td>
<td>52 ± 2 (3)</td>
</tr>
<tr>
<td>Normal individuals</td>
<td>&gt;8</td>
<td>50 ± 180</td>
<td>50 ± 180</td>
<td>50 ± 180</td>
<td>0</td>
<td>70 ± 80</td>
<td>3.8 ± 1.3 (10)</td>
</tr>
</tbody>
</table>

Results are expressed as the mean ± SD. The number of different determinations is shown in parentheses.
Fig 2. A composite of SDS-agarose gels depicting the multimeric composition of plasma and platelet vWF is shown. Origin is at the top and anode at the bottom. Samples (2.5 mIU vWF:Ag) are probed with anti-vWF polyclonal antibodies. Numbers correspond to the various multimers of the vWF protomer numbered 1, whereas arrows indicate the position of satellite bands. A pool of normal (N) plasmas and normal platelet lysate were used as controls; patients AI2 and BII plasma and platelet lysate were studied, whereas only patient C plasma was available. Plasma of a subtype IIA vWD variant was used as a reference. (A) 1.5% agarose gel electrophoresis of plasma and platelet vWF. Note the absence of HMW vWF multimers in patients BII and C and the presence of a smear in HMW multimers for patient AI2. Intermediate multimers are present in patients' plasma samples, whereas they are lacking in subtype IIA vWD variant. (B) 2.5% agarose gel electrophoresis of plasma vWF. Note that the relative intensity of satellite bands is not increased in the patients' plasma samples, as compared with that for the pool of normal plasmas, whereas it is dramatically enhanced for the subtype IIA vWD patient.

other patients, these percentages of multimers ≥5 mers and protomer were respectively lower and higher than the mean ± 2.2 SD of the corresponding values for the pool of normal plasmas. The typical multimeric pattern in low-resolution gel is exemplified in Fig 2A. In contrast with subtype IIA vWD patients, all the patients tested displayed intermediate multimers. However, a difference could be noted between plasma patterns of family A patients and those in family B or patient C. In family A patients, a smear was observed in the upper part of the gels suggesting that some HMW multimers were present. In high-resolution gels (Fig 2B), satellite bands in each multimeric unit of patients' plasma vWF were detected in a position identical to that for the normal plasma pattern and they did not show the increased intensity characteristic of subtype IIA vWD. Furthermore, the pattern of the proteolytic fragments of plasma vWF obtained in reduced gels was normal in all the patients (data not shown). The analysis of platelet lysate of patients AI2 and BII (Fig 2A) showed that the percentage of multimers ≥5 mers was significantly decreased as compared with that for normal platelet lysate (Table 1). No platelet lysate of patient C was available for study. As exemplified in Fig 3A for patients AI2, BII, and C, binding of plasma vWF to normal platelets was significantly reduced at concentrations of ristocetin ≥0.4 mg/mL as compared with that for the pool of normal plasmas. At 1 mg/mL of ristocetin, vWF binding reached 50% of normal plasma value (Fig 3A). In the presence of botrocetin, binding of patients plasma vWF to plate-
from patient B11 (01, from patient C
leted by centrifugation and the amount of vWF bound was measured
 incubation at room temperature for 30 minutes, platelets were pel-
 by counting the pellet radioactivity. Binding assays were performed
controls the pool of normal plasmas (MI, or the pool of normal plasma
percentages of total radioactivity in duplicate on three occasions for patient AII2 and BII1 and once for patient C. Values are expressed in percentage of total radioactivity in the presence of ristocetin (A) or botrocetin (B) are shown. Plasma from patient AII2
The sequencing profile in this patient indicates that he is heterozygous for this histidine for arginine substitution. The presence of the mutated codon (CAC) was studied by allele-specific PCR in all family B members. This technique allowed us to confirm that the mutated codon was present only in the seven affected family members, whereas the normal codon (CCG) was detected both in the five normal and in the seven affected members showing that the patients are heterozygous for the substitution (data not shown). No other nt substitution, except known polymorphisms, were found in the amplified products and in the remaining exon 28 sequences of the patients studied in the three families.

**Level of expression of recombinant vWFs.** Cos-7 cells were transfected with vWF expression plasmids containing each of the two nt substitutions previously identified. Quantitative analysis of cell media obtained from four different transfection experiments with independent plasmid preparations showed that the amount of vWF present in the cell medium was similar (10 ± 2 IU/dL/72 h) for the two plasmids containing the normal codon (pSVvWF and pSvWFA). It was moderately, but significantly, decreased for His61rvWFA (6.8 ± 1.2 IU/dL/72 h) and markedly reduced for Cys61rvWFA (4.2 ± 0.4 IU/dL/72 h). The secretion levels of His/Arg61rvWFA and Cys/Arg61rvWFA, recombinant vWFs resulting from cotransfections experiments, were also significantly decreased (7.4 ± 0.7 IU/dL/72 h and 6 ± 0.7 IU/dL/72 h, respectively).

**Structural characterization of recombinant vWFs.** Equal amounts of each rWF obtained from four independent transfections were analyzed by electrophoresis in four SDS–1.5% agarose gels as exemplified in Fig 5. The relative percentage of HMW multimers ≥5 mers of WTrvWF (75% ± 4.5%) and WTrvWF (78% ± 1.4%) was not statistically different from that of the pool of normal plasma (74.6% ± 5.2%). For His61rvWFA, the relative percentage of HMW multimers ≥5 mers was significantly decreased (64.5% ± 2%), while the relative percentage of the protomer was slightly increased (5.6% ± 1.5%) as compared with that for WTrvWF (2.5% ± 0.1%) (Fig 5). Hybrid His/Arg61rv-
WFA displayed an intermediate multimeric pattern between that observed for WTrvWF and His61rvWFA with a significant decrease in the relative percentage of HMW multimers ≥5 mers (68.7% ± 4.9%) and a significant increase in the relative percentage of the protomer (4.0% ± 0.9%). For Cys61rvWFA, the relative percentage of the different multimers could not be measured because of the presence of a smear in the area of intermediate and HMW multimers. Nevertheless, it was obvious that the multimeric patterns of both Cys61rvWFA and Cys/Arg61rvWFA were abnormal.

After reduction and SDS-PAGE, normal and mutated rVWFs appeared as a major band with an apparent MW of 275 kD corresponding to mature vWF (data not shown). A faint band of 360 kD corresponding to pro-vWF was observed, but no proteolytic fragments were detected in either rVWF.

**Functional characterization of recombinant vWFs.** Nor-

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**Fig 3.** GPIb binding assays of plasma vWF in the presence of ristocetin (A) or botrocetin (B) are shown. Plasma from patient AII2 (III), from patient BII1 (II), from Patient C (I), or the pool of normal plasma (A) was mixed with neutral radiolabeled anti-vWF monoclonal antibodies and then added to formalin-fixed platelets in the presence of graded concentrations of ristocetin (A) or botrocetin (B). After incubation at room temperature for 30 minutes, platelets were pelleted by centrifugation and the amount of vWF bound was measured by counting the pellet radioactivity. Binding assays were performed in duplicate on three occasions for patient AII2 and BII1 and once for patient C. Values are expressed in percentage of total radioactivity bound to platelets (mean ± SD).

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lets was also significantly reduced at concentrations of botro-
cetin ≥1 μg/mL (Fig 3B). No spontaneous binding was observed in the absence of modulator.

**Mutation detection.** Direct sequencing of PCR-amplified leukocyte DNA from patient AII5 showed the presence of a thymidine residue in addition to the normal cytosine residue at nt 4,120 in exon 28 of the vWF gene (Fig 4A), (the nt position is based on the cDNA sequence of Bonthron et al [22] with residue 1 the first nt of the ATG initiation codon). The sequencing profile in this patient indicates that he is heterozygous for the nt change that substitutes a cysteine residue for an arginine in position 611 of the mature vWF subunit. The presence of this substitution could not be confirmed in the other affected patients, as no blood samples were available. As exemplified in Fig 4B, a G → A transition at nt 4,121 in codon 611 was found in the vWF sequence
TWO TYPE 2 vWD MUTATIONS AT POSITION 611

Fig 4. Identification of the nt substitutions at codon for aa residue 611 of mature vWF in the exon 28 sequences is shown. Sequence of the fragments obtained from patients All5 (A) or B11 (B) is shown. The lettering indicates the base sequence of the coding strands (with respective 3' and 5' ends specified). The asterisk indicates the simultaneous presence of two nucleotides. The C to T transition at nt 4,120 in patient All5 results in the change of Arg → Cys, whereas the G to A transition at nt 4,121 in patients B11 and C results in the change of Arg → His. Affected patients are heterozygous for the mutation as shown by the sequencing profiles.

Fig 5. Multimeric composition of recombinant vWfs is shown. vWfs were analyzed by electrophoresis in 1.5% SDS-agarose gel. Lane 1, Cys611rvWF; lane 2, Cys/Arg611rvWF; lane 3, WTrvWF; lane 4, His611rvWF; lane 5, His/Arg611rvWF; lane 6, WTwWFA; lane 7, pool of normal plasmas. Numbers correspond to the various multimers of the vWF protomer numbered 1.

DISCUSSION

In the present study, we have identified two missense mutations in the vWF gene of several members of three unrelated families in which the vWD diagnosis was first established on the basis of prolonged bleeding time and vWF quantitative deficiency. The classification of these patients was first approached by using a low-resolution electrophoretic technique where each vWF multimer appears as a single band. Electrophoretic patterns showed that the percentage of HMW multimers was decreased in both plasma and platelet vWF indicating that the patients also have a qualitative deficiency of vWF and, thus, belong to type I1 (revised type 2) vWD. The phenotype observed in our patients was then compared with that of the previously reported different subtypes of type I1 vWD. They showed an almost complete lack of RIPA at 1 mg/mL of ristocetin and decreased binding of plasma vWF to platelets in the presence of two agonists, ristocetin and botrocetin. Nevertheless, they do not belong to type IIA vWD variants because their plasma vWF displayed intermediate multimers and satellite bands with normal intensity. Furthermore, the pattern of the proteolytic fragments of plasma vWF obtained in reduced gels was normal in the patients reported here, whereas proteolytic fragments are markedly increased in type IIA vWD variants. Because our patients are characterized by an alteration of the multimeric pattern of both plasma and platelet vWF with only a decrease in the HMW forms, they do not belong to subtypes IIB, IIF, IIG, and II-1 vWD variants. The normal banding pattern of each multimer clearly differentiates our patients from subtypes IIC, IIE, and IIH vWD variants where the satellite bands are absent or reduced in intensity and from subtype IID where these satellite bands migrate in an abnormal position. Therefore, the phenotype reported here likely represents an infrequent form of type 2 vWD.

A C to T transition at nt 4,120 was identified in family A patients, while a G to A transition at nt 4,121 was identified in family B patients and in patient C. These substitutions are located within a CG dinucleotide, which is consistent with the observation that CG dinucleotides are "hotspots"
for mutations. Both these base changes affect the arginine residue at position 611 in the mature subunit of vWF and induce nonconservative aa substitutions. In family A, the arginine residue is changed into cysteine, whereas in family B and in patient C, it is changed into histidine. It is noteworthy that the Arg611Cys substitution has been previously reported in an abstract where the patients were reported to display all multimers sizes, but with a relatively reduced intensity of the larger multimers, in agreement with our observations. The Arg611Cys substitution does introduce a potentially unpaired thiol group in vWF aa sequence and, thus, may disturb the proper tridimensional conformation of vWF.

The identification of two different aa substitutions at the same position in patients sharing similar phenotype provides support that these substitutions may constitute the mutations responsible for the observed phenotypic abnormalities. Direct evidence that these aa substitutions are responsible for the peculiar vWD phenotype characterized was brought by the analysis of expression products from Cos-7 cells transfected with full-length vWF cDNAs containing both nt substitutions. As the majority of vWF molecules in heterogeneous patients is likely composed of a mixture of normal and abnormal subunits, hybrid rvWFs resulting from cotransfections (1:1) with normal and mutated vWF cDNA were prepared to better mimic the simultaneous presence of normal and mutated vWF alleles present in the affected patients. In these conditions, the Arg611Cys mutation resulted in extracellular levels corresponding to ~60% of that obtained for WTvWF, whereas the Arg611His mutation resulted in extracellular levels at approximately 75% of WTvWF. Thus, the secretion of mutated rvWFs is only moderately decreased in cotransfection experiments as compared with the profound decrease in vWF:Ag seen in most of the patients. The differences between hybrid rvWFs and patients’ plasma vWF may reflect variability in the effects of a given nt substitution on the synthesis and secretion of a protein by different cells (ie, human endothelial cells and Cos-7) or on some postsecretion events occurring in circulating blood, but not in culture medium. Both mutated rvWFs also showed a qualitative defect in multimer distribution with the slight decrease in the proportion of HMW vWF forms previously shown in patients’ plasma vWF. In agreement with the marked decrease in vWF:RCo and vWF platelet-dependent function in the patients, the hybrid rvWFs resulting from cotransfections also showed binding values for platelets that were significantly decreased as compared with normal rvWFs. Thus, although small differences could be observed between in vivo and in vitro results, both mutated rvWFs reproduce the quantitative defect, the abnormal multimeric structure, and the decrease in platelet-dependent function of the patients’ vWF.

The two substitutions identified here are located within the Cys509-Cys695 loop of the A1 domain of vWF where most of subtype 2B vWD mutations have already been reported. However, in contrast to 2B patients displaying vWF increased affinity for GPIb, our patients showed a decreased binding of plasma vWF for GPIb. The vWF of a few other patients harboring mutation in the A1 loop are also not associated with an increased reactivity for GPIb. This has already been reported for two patients with phenotype IIA and Cys509Arg and Val551Phe mutation, respectively. However, the mutated rvWF with phenylalanine in position 551 displayed an increased affinity for GPIb, while the function of mutated rvWF with arginine in position 509 has not yet been reported. Furthermore, a point mutation affecting aa 561 (Gly561Ser) has been identified in a patient with type vWD.
B vWD, now classified in type 2M, characterized by no ristocetin-induced but normal botrocetin-induced binding to GPIb. The corresponding Ser561rvWF showed the same platelet-dependent function. Lastly, another patient, reported to have a variant form of type 1 vWD and also displaying decreased ristocetin cofactor activity, was found to have the Phe606Ile mutation very close to the mutations characterized here. However, in this case, no study of corresponding rvWF has yet been reported to confirm that this aa change is the mutation causing the loss of function. In our study, the expression of mutated rvWFs confirms that the two Arg611Cys and Arg611His substitutions are real mutations responsible for type 2 vWD with a slight qualitative defect in multimer distribution, but a profound decrease in vWF binding to platelets in the presence of both ristocetin and botrocetin. In the new classification, both types 2A and 2M refer to qualitative variants with decreased platelet-dependent function. In the former type, this decrease is defined as associated with the absence of HMW vWF multimers, whereas in the last type, it is said to not be caused by the absence of HMW vWF multimers. It has been previously reported that the capacity of normal vWF to interact with platelet GPIb is highly dependent on the size of the multimers and that the loss of vWF function in type IIA patients results from the lack of the large-sized multimers. In our patients, plasma vWF does not present the profound decrease in HMW forms that is observed in type IIA patients. Therefore, the dramatically decreased reactivity for platelet GPIb of plasma vWF and rvWF associated with the mutations reported here may reflect the existence of specific structural alterations affecting the GPIb binding site, in addition to the slight impairment of the multimeric structure. Consequently, these patients are difficult to classify as either type 2A or type 2M and are designated simply as type 2 vWD variants. This apparently particular form of type 2 vWD with decreased platelet-dependent function caused by aa substitutions in the A1 loop may provide important insights for understanding the mechanisms of vWF/GPIb interaction, which is one of the main steps in primary hemostasis.

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Identification of two mutations (Arg611Cys and Arg611His) in the A1 loop of von Willebrand factor (vWF) responsible for type 2 von Willebrand disease with decreased platelet-dependent function of vWF [see comments]

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