Identification of a New Type 2M von Willebrand Disease Mutation also at Position 1324 of von Willebrand Factor

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Summary
Type 2M von Willebrand disease (VWD) refers to variants with decreased platelet-dependent function that is not associated with the loss of high molecular weight (HMW) von Willebrand factor (VWF) multimers. This category includes the so-called “phenotype B” responsible for inexistent ristocetin-induced but normal botrocetin-induced binding of VWF to platelet glycoprotein Ib. The missense mutation G1324S was identified in the first patient reported to display “phenotype B”. We report here on the identification in four members of a French family of a missense mutation also affecting this glycine residue but changing it into an alanine residue. These individuals are heterozygous for this mutation and two of them display an additional quantitative VWF deficiency resulting from a stop codon at position 2470. After transient transfection in Cos-7 cells, the mutated recombinant protein harbouring the G1324A substitution was shown to exhibit normal multimers and inexistent ristocetin-induced but normal botrocetin-induced binding to GPIb, confirming the classification of this new mutation as a type 2M VWD mutation.

Introduction
Von Willebrand factor (VWF) is a highly multimerised plasma glycoprotein (GP) that has a dual role in haemostasis: VWF is the carrier for factor VIII (FVIII) in the circulation and is involved in platelet adhesion to the subendothelium of injured vessels (1, 2). This function depends upon the interaction of VWF with the platelet membrane GP Ib and with components of the vessel wall. The binding site for platelet GP Ib has been localised between aa residues V1212 and K1491 of VWF (3). In vitro, the binding of VWF to GP Ib can be induced by agonists such as the antibiotic ristocetin (4) or the snake venom protein botrocetin (5). Although ristocetin binds to platelets and induces binding to GPIb, confirming the classification of this new mutation and two of them display an additional quantitative VWF deficiency resulting from a stop codon at position 2470. After transient transfection in Cos-7 cells, we have shown that the G1324A substitution was responsible for the patient’s phenotype: normal multimers and decreased ristocetin-induced but normal botrocetin-induced binding to GPIb, confirming the classification of this new mutation as a type 2M VWD mutation.

Materials and Methods

Patients
The propositus (II-1) is a 26-year old woman who was diagnosed with VWD at 8 years of age (see Fig. 1 for the family tree). She displayed severe bleeding symptoms including severe epistaxis in childhood and menorrhagia. She received cryoprecipitate and VWF concentrate (FACTEUR WILLEBRAND-LFB®, France) before appendectomy and delivery, respectively. II-2, who was also reported to have a severe bleeding tendency with frequent and very severe epistaxis and menorrhagia, was treated with cryoprecipitate for bleeding after tympanoplasty and received FACTEUR WILLEBRAND-LFB® and biological sealant (Biocolle, LFB) for dental extractions. I-1 and III-1 were qualified as type 2M VWD patients (14, database) but expression studies must still be performed to confirm the relationship between the patients’ phenotype and molecular abnormality. In addition to these mutations found in the A1 domain of VWF, a missense mutation (R1205H) has been identified in the D3 domain in a particular subgroup of 2M VWD, named “Vicenza” (15), characterised by the presence of “supernormal” multimers (16).

We report here on the identification in four related French patients of a new mutation affecting, like the first 2M mutation identified (9), the glycine residue at position 1324 of pre-proVWF (aa 561 in mature VWF). A G→C transversion in exon 28 changing glycine residue 1324 into alanine and a C→T transition in exon 43 changing glutamine 2470 (aa 1707 in mature VWF) into a stop codon were identified in the propositus. Using site-directed mutagenesis and transient expression in Cos-7 cells, we have shown that the G1324A substitution was responsible for the patient’s phenotype: normal multimers and decreased ristocetin-induced but normal botrocetin-induced binding of VWF to platelets.

Blood Sample Collection and Routine Coagulation Studies
Ivy’s template bleeding times were determined using a disposable device and closure times were determined using a PFA-100 system (17). Blood samples were collected with 13 mM sodium citrate and stored at ~80° C. The
ristocetin-induced platelet agglutination assay (RIPA) was performed on platelet-rich-plasma (PRP) samples by measuring the extent of platelet agglutination after addition of different concentrations of ristocetin. FVIII coagulant activity (FVIII:C), VWF antigen (VWF:Ag) levels and ristocetin cofactor (VWF:RCo) activity were assayed on citrated platelet-poor plasma (PPP) samples stored at –80°C (18).

Response to DDAVP was evaluated in some patients by measuring bleeding time, VWF:Ag, VWF:RCo and FVIII:C levels before and 1 h after intravenous infusion of 0.3 g/kg Minirin® (Ferring Laboratory).

Identification of Patients’ Molecular Abnormalities

Genomic DNA was extracted from peripheral blood leucocytes according to a method already described (19). The 5’ part of exon 28 of the propositus’s VWF gene was amplified by polymerase-chain reaction (PCR) and sequenced using a dideoxy-sequencing procedure (18). SSCP was also performed on the propositus’s DNA using a triplex strategy and the 3 exons involved in the abnormal SSCP profile were then sequenced. Subsequently, the corresponding exons in the propositus’s relatives were evaluated by direct sequencing to search for the two molecular abnormalities identified in the propositus.

Expression Vector Construction

Plasmid A1324pSVVWFA harbouring the G3971C nucleotide substitution derives from pSVVWFA which contains the full-length cDNA for normal human VWF (20). Nt of the VWF cDNA are numbered from the initiator ATG as +1. A1324pSVVWFA was created by site-directed mutagenesis using the Transformer site-directed mutagenesis kit (Clontech Laboratories, Palo Alto, CA, USA), a mutagenesis primer [nt 3961-3981: (5’) TAC CAC GAC GCC TCC CAC GCC (3’) and a selection primer which mutates the unique restriction site HindIII of the plasmid [nt 2225-2245: (5’) GA GTC CCC GGG AGC TTG CTG C (3’)]. The underlined letters in the sequences indicate the nt that differ from the corresponding normal VWF sequence. The clones containing the desired mutation were selected by allele-specific PCR as previously described (20) with an allele-specific primer [nt 3954-3971: (5’) G GTG GAG TAC CAC GAC GC]. Three different clones containing the mutated DNA were selected and the corresponding mutated plasmids were purified using the Plasmid Midi kit (Qiagen, Chatsworth, CA). Then, to ensure that no additional undesired nt substitution was present, these three plasmids were digested with ApII and NheI and the corresponding digested fragments were cloned into pSVVWFA digested in parallel with the two enzymes. Finally, the three resulting subcloned plasmids were purified using the Plasmid Maxi Kit (Qiagen, Chatsworth, CA) and used for transfection.

Transfection of Cells

Cos-7 cells growing in complete culture medium were transfected using the diethylaminoethyl dextran method previously described (18) with slight modifications. In brief, 7 × 10^4 cells were seeded in 80 cm^2 flasks and grown overnight. After washing the cells twice with Tris-Saline buffer (140 mM NaCl, 3 mM KCl, 1 mM CaCl_2, 0.5 mM MgCl_2, 0.9 mM Na_2PO_4, 25 mM Tris-HCl pH 7.4), the cells were incubated for 1 h in 3.8 ml of Tris-Saline buffer containing 500 μg/ml DEAE dextran and 25 μg of normal (pSVVWFA) or

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**Table 1 Routine laboratory data**

<table>
<thead>
<tr>
<th>Family member</th>
<th>Blood group</th>
<th>Closure time (sec)</th>
<th>ADP</th>
<th>Bleeding time (min)</th>
<th>Platelets 10^3/μl</th>
<th>FVIII:C IU/dl</th>
<th>VWF:Ag IU/dl</th>
<th>VWF:RCo IU/dl</th>
<th>RIPA 1.2 mg/ml</th>
<th>Molecular abnormality</th>
</tr>
</thead>
<tbody>
<tr>
<td>Father I-1</td>
<td>O</td>
<td>ND</td>
<td>ND</td>
<td>5</td>
<td>281</td>
<td>95</td>
<td>57</td>
<td>25</td>
<td>+</td>
<td>G1324A</td>
</tr>
<tr>
<td>Mother I-2</td>
<td>O</td>
<td>&gt;300</td>
<td>260</td>
<td>9</td>
<td>217</td>
<td>97</td>
<td>42</td>
<td>33</td>
<td>+</td>
<td>Q2470X</td>
</tr>
<tr>
<td>Propositus II-1</td>
<td>O</td>
<td>&gt;300</td>
<td>&gt;300</td>
<td>&gt;20</td>
<td>222</td>
<td>107</td>
<td>34</td>
<td>&lt;3</td>
<td>&lt;3</td>
<td>0</td>
</tr>
<tr>
<td>Sister II-2</td>
<td>O</td>
<td>&gt;300</td>
<td>&gt;300</td>
<td>20</td>
<td>150</td>
<td>94</td>
<td>50</td>
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<td>&lt;3</td>
<td>0</td>
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<tr>
<td>Son III-1</td>
<td>ND</td>
<td>282</td>
<td>154</td>
<td>ND</td>
<td>323</td>
<td>98</td>
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<td>50-150</td>
<td>50-150</td>
<td>50-150</td>
<td>+</td>
<td>-</td>
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</tbody>
</table>

ND: not done

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mutated (A1324pSVVFWA) plasmid DNA. In order to mimic the hybrid protein present in the plasma of the patients heterozygous for this mutation, cells were also transfected with 12.5 μg of both the normal and mutated plasmids. After removal of the DNA/DEAE dextran solution, 8 ml of complete culture medium containing 100 μM chloroquine was added and the cells were incubated for an additional 4 h at 37° C. The cells were then washed twice with Tris-Saline buffer and left to rest for 40 h in 10 ml of complete culture medium before addition of 8 ml of expression medium (complete or serum-free culture medium). After 72 h, the medium was collected in the presence of 10 mM benzamidine and 1 mM phenylmethanesulfonylfluorid and centrifuged before analysis of rVWF.

The VWF:Ag present in the culture medium was measured by ELISA as previously described (21). The normal recombinant protein is referred to as WTIVWF; the mutated protein as A1324rVWF and the hybrid protein as A/G1324rVWF.

**Electrophoretic Characterisation of VWF**

The multimeric composition of VWF was analysed in 1.5% agarose gels and the VWF multimers were visualised with alkaline phosphatase-conjugated immunopurified anti-VWF polyclonal antibodies as previously described (18).

**Binding Assays**

The ristocetin- and botrocetin-dependent platelet binding assays were carried out on plasma VWF and rVWF samples as previously described using radiolabelled neutral anti-VWF polyclonal antibodies and formaldehyde-fixed platelets (FFP) (22). The binding is expressed as the percentage of radioactivity bound to platelets.

**Results**

**Phenotype Analysis**

The biologic data of the different family members are reported in Table 1. All the family members tested have normal FVIII:C levels and prolonged closure times. Members I-2, II-1 and II-2 displayed moderately decreased VWF:Ag levels whereas members I-1 and III-1 displayed normal levels. However, with the exception of I-2, they all displayed decreased VWF activity and therefore show a discrepancy between VWF:Ag and VWF:RCo levels characteristic of type 2 VWD. This discrepancy was more pronounced in II-1 and II-2. After a trial infusion with desmopressin, bleeding time was initially very prolonged and VWF:RCo remained unchanged in II-1 and II-2 whereas VWF:Ag and FVIII:C were moderately increased (1.5-times). In I-2, bleeding time was normalised, VWF:Ag and VWF:RCo levels were doubled and FVIII:C was increased threefold. RIPA was nil at 1.2 mg/ml ristocetin for II-1 and II-2 but was normal for I-1 and I-2 (Table 1). As shown in Figure 2A, plasma VWF from the different members contained the full range of multimers. II-1 and II-2 exhibited no ristocetin-induced binding to platelets. A1324rVWF was characterised by the absence of binding to GPIb regardless of the dose of ristocetin added (Fig. 3B). Hybrid A/G1324rVWF showed intermediate ristocetin-induced binding to platelets compared to that of WTIVWF and A1324rVWF (Fig. 3B). In the presence of botrocetin, all the wild-type, mutated and hybrid rVWF bound to platelets in a similar manner and this binding was dependent on the dose of botrocetin added (Fig. 3C).

**Discussion**

Type 2M VWD refers to qualitative variants with decreased platelet-dependent function that is not caused by the loss of HMW multimers of VWF. This category includes the former “phenotype B” characterised by inexistent ristocetin-induced and normal botrocetin-induced binding of plasma VWF to GPIb (23). In the first patient displaying this phenotype, a G1324S mutation was identified within the GPIb-binding domain of VWF (9). We report here on the identification in four related French family members of a second mutation at the same position but changing the glycine residue into an alanine residue. The identification of two different substitutions at the same aa position in patients sharing a similar phenotype and the conservation of this aa (glycine 1324) in 28 different mammalian species studied (24) indicate that this residue is crucial for the normal functional properties of VWF.

After transient expression in Cos-7 cells, mutated A1324rVWF displayed the functional characteristics of “phenotype B” plasma VWF. Furthermore, hybrid A/G1324rVWF assumed to reflect the VWF protein present in patient plasma displayed ristocetin-induced binding to GPIb that was intermediate between that of normal rVWF and mutated rVWF. It is noteworthy that, in contrast, the hybrid S/G1324rVWF was reported to display normal ristocetin-induced binding (9). This suggests that the substitution of the glycine residue 1324 by alanine may have a more deleterious effect than the substitution by serine.

As the ristocetin-induced binding of the propositus’s plasma VWF was nil though the mutation was at heterozygous state, an additional quantitative alteration of the VWF gene was suspected. This hypothesis was supported by the low level of VWF:Ag observed in this patient. After triplex-SSCP and further sequencing of exon 43 of the propositus VWF gene, a stop codon at position 2470 of the pre-proVWF (aa 1707 in mature VWF) was also detected (C7408T). The presence of the two molecular abnormalities was then sought in the propositus’s relatives by direct sequencing of the exons in question. The G1324A abnormality was identified in I-1, II-2 and III-1 whereas the stop codon was detected in I-2 and II-2 (Table 1).

**Expression and Characterisation of rVWF**

Cos-7 cells were transiently transfected either with wild-type or mutated plasmids (3 different clones). Mutated A1324rVWF was present in the culture medium at the same level as WTIVWF and displayed a normal multimeric pattern with the full range of multimers (Fig. 3A). While WTIVWF showed ristocetin dose-dependent binding to platelets, A1324rVWF was characterised by the absence of binding to GPIb regardless of the dose of ristocetin added (Fig. 3B). Hybrid A/G1324rVWF showed intermediate ristocetin-induced binding to platelets compared to that of WTIVWF and A1324rVWF (Fig. 3B).

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**Mutation Detection**

Direct analysis of the PCR-amplified 5’ part of exon 28 from II-1 showed the presence of a cystosine in addition to the normal guanine at position 3971 in the VWF gene. This nt sequence indicates that II-1 is heterozygous for the nt change that substitutes an alanine residue for a glycine in position 1324 of the pre-proVWF (aa 561 in mature VWF). In addition, after triplex-SSCP and further sequencing of exon 43 of the propositus VWF gene, a stop codon at position 2470 of the pre-proVWF (aa 1707 in mature VWF) was also detected (C7408T). The presence of the two molecular abnormalities was then sought in the propositus’s relatives by direct sequencing of the exons in question. The G1324A abnormality was identified in I-1, II-2 and III-1 whereas the stop codon was detected in I-2 and II-2 (Table 1).

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The absence of spontaneous bleeding in I-1 and III-1 and the diagnosis of type 2 VWD in II-1 and II-2 who had haemorrhages requiring substitutive treatment suggest that the G1324A mutation is clinically recessive. However, members harbouring only the G1324A mutation displayed a low VWF:RCo/VWF:Ag ratio. It is noteworthy that the patient with the G1324S mutation also had severe haemorrhages requiring several transfusions but no family history of bleeding. Nevertheless, as her relatives were not available, the fact that the other allele was not expressed could be only assumed but not confirmed (9).

A1324rVWF was unable to induce any measurable shear-induced platelet aggregation (10) and a rVWF fragment comprising residues 1208-1496 of pre-pro VWF with the G1324S mutation showed significantly lower platelet adhesion at different shear rates (25). These data suggest that both mutations are responsible for a modified VWF conformation insensitive to high shear rates and ristocetin. Gly1324 lies on the carboxyl edge of the β-sheet at the beginning of the βC strand and is part of the tight βB-βC turn at the upper front edge of the A1 domain (24, 26). Emsley et al. (26) suggest that the mutant side chain can point out into solution without severe distortion of the main chain and that the ability of botrocetin to overcome the binding deficiency in the mutants may arise from the stabilising effect of its tight binding to an adjacent surface of the A1 domain.

Besides the “phenotype B” and “2B” VWD mutations, missense mutations inducing also a loss-of-function have been reported in the A1 domain but their classification in a definite VWD subtype remains difficult in some cases. For instance, the R1315C mutation which was identified in a Spanish patient as a type 2M VWD mutation (27) was reported to induce a 2A-like phenotype in 10 unrelated French patients (22). The R1374C mutation has been referred to as a type 1 mutation in four families from Sweden (28), a type 2M mutation in an Italian family (29) or a type 2 unclassified mutation in a French family (20). These two examples clearly indicate that differentiation between types 1, 2M and 2A critically depends on the quality of multimer analysis. Furthermore, patients originally diagnosed as type 1 VWD were shown to be misclassified as they displayed discrepancies between the...
VWF:RCo and VWF:Ag measurements (14). In agreement with the patients’ phenotype, the identified mutations (V1279F, Y1321D, R1399C and I1416T) correspond, in the VWF-A1 crystal structure, to potential type 2M defects. Conversely, the “Vicenza” patients who are classified as type 2M VWD patients could be considered as type 1 VWD as the corresponding rVWF does not present abnormal binding to platelets (30). These studies underline the difficulties of correct diagnosis of type 2M VWD and the importance of both in vitro analysis using rVWF and molecular modelling analysis.

In conclusion, we report here on the identification and expression of a new type 2M VWD mutation (G1324A) at the same position where the mutation (G1324S) in the princeps “phenotype B” patient was identified confirming that glycine 1324 plays a major role in the mechanism of VWF/GPIb interaction.

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References


