We previously reported a functional defect of von Willebrand factor (vWF) in a new variant of von Willebrand disease (vWD) tentatively named vWD “Normandy.” The present work has attempted to characterize the molecular abnormality of this vWF that fails to bind factor VIII (FVIII). The immunopurified vWF from normal and patient’s plasma were digested by trypsin and the resulting peptides were compared. The electrophoresis of “vWF Normandy” showed a shift in the band corresponding to a polypeptide from amino acid 1 to 272. Consequently, we performed the molecular analysis of the portion of the vWF gene of this patient encoding this amino acid sequence. Exons 18-24 were amplified by the use of polymerase chain reaction and their nucleotide sequences corresponding to 1.8 kb were determined.

**VON WILLEBRAND FACTOR (vWF)** is a large multimeric glycoprotein found in plasma and in platelets that is synthesized by endothelial cells and megakaryocytes. Besides its major role as a mediator of initial platelet adhesion to vascular subendothelium, vWF is also the carrier of factor VIII (FVIII) in plasma.12 The association of vWF with FVIII has been shown in vitro to stabilize the coagulant activity of both human1 and recombinant3 FVIII. Furthermore, clinical observations in patients confirm the importance of normal vWF in prolonging FVIII half-life.6,7

The human vWF gene has been studied in some detail and is located on chromosome 12.5,6 It is 178 kb in length and contains 52 exons, the intron boundaries of which were recently determined, 19% of the gene being sequenced.16 The 9-kb vWF mRNA encodes a 2,813-amino acid (AA) precursor consisting of a 22-AA signal peptide, a 741-AA propeptide, and a 2,050-AA mature subunit. Functional domains involved in binding to platelet membrane glycoproteins, collagen, and heparin have been localized on the vWF subunit.1 More recently, a major FVIII-binding domain of vWF was characterized on the NH2-terminal region of mature vWF. FVIII binds to Sp fragment III’ (AA 1-910), but not to Sp fragments I (AA 911-1,365) or II (AA 1,366-2,050), obtained by digestion of vWF with Staphylococcus aureus V8 protease.5,6 Further, this function is maintained on a tryptic fragment (SpIII-T4) containing the amino-terminal 272 AA of vWF.14 von Willebrand disease (vWD), the most common inherited bleeding disorder, is heterogeneous and originates from either quantitative (vWD types III and I) or qualitative (vWD type II) alterations of vWF. Many phenotypic subtypes of vWD have been described, generally according to the multimeric profile of plasma and platelet vWF. Depending on the disease type or subtype, the inheritance has been found either dominant (type I, subtypes IIA and IIB) or recessive (type III and subtype IIC). The cause of vWD at the level of gene structure is known in only a few families. Total or partial gene deletions for vWD type III1,10 as well as single point mutations for vWD types IIA19 and IIB22 have been reported.

In an earlier article,23 we described a new variant form of vWD, tentatively named vWD “Normandy,” in a female patient with FVIII deficiency but normal primary hemostasis. Her plasma vWF, which did not present any quantitative or multimerization abnormality, was shown to be unable to bind FVIII. This report details the study we have performed to characterize the underlying molecular pathology of this patient.

**PATIENT AND METHODS**

Case report. For a complete data report refer to our earlier publication.23 A 50-year-old French woman with a lifelong history of bleeding was referred to us in August 1988. Investigations confirmed FVIII deficiency (5 to 8 IU/dL) but showed normal levels of vWF antigen and ristocetin cofactor activity and normal vWF multimeric pattern. FVIII-binding assays showed that the patient’s vWF, in contrast to normal vWF, was unable to bind FVIII. Her two children, a son and a daughter, as well as her parents, who were third cousins, were said to have no bleeding history. However, plasma from the patient’s son showed both moderate FVIII deficiency and FVIII-binding values intermediate between the normal values and the patient’s values (data not shown).

**Antibodies.** Monoclonal antibody (MoAb)-239 to human vWF, prepared in collaboration with Immunotech (Marseilles, France), recognizes all the multimeric forms of vWF.24 MoAb-418 was a generous gift of D. Meyer (INSERM U143, Paris, France); it specifically inhibits the binding of FVIII to vWF and reacts with the unreduced N-terminal tryptic (AA 1-272) or plasmic (AA 1-298) fragment of the vWF subunit.15 Its epitope was further localized to the first 106 AA of the mature vWF subunit.25 Two other MoAbs, obtained by immunization of Balb/c mice with SpIII-T4 fragment,12 were also used. MoAb-175-35A8 inhibits the FVIII binding to vWF and recognizes both reduced and unreduced SpIII-T4 fragment; MoAb-175-35A8 inhibits the FVIII binding to vWF and recognizes both reduced and unreduced SpIII-T4 fragment; MoAb-175-35A8 inhibits the FVIII binding to vWF and recognizes both reduced and unreduced SpIII-T4 fragment.

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MoAb-175-31H3 does not inhibit FVIII:vWF interaction and reacts only with unreduced SpIII-T4 fragment.

**vWF purification and analysis of trypsin digest.** vWF was purified from the patient’s and normal plasma, using immunoaffinity chromatography on anti-vWF MoAb-239 as previously reported. Purified vWF was submitted to a trypsin treatment according to a previously published method with some minor modifications: immunopurified vWF diluted in Tris 50 mmol/L, NaCl 150 mmol/L, pH 7.35, was incubated at 37°C for 10 minutes with L-(tosylamido 2-phenyl) ethyl chloro methyl ketone (TPCK)-treated trypsin (Worthington Biomedical Corp, Freehold, NJ) and an enzyme to substrate ratio of 1:25 (wt/wt).

**Polymerase chain reaction (PCR) amplification of genomic DNA.** All PCR amplifications were performed on genomic DNA extracted from peripheral blood mononuclear cells according to the method described by Miller et al. Amplifications were performed using Taq polymerase (Amersham, Buckinghamshire, England) in a reaction mixture containing 0.5 μg genomic DNA, 100 pmol of each primer, 200 μmol/L of each deoxynucleotide triphosphate (dNTP), and the enzyme buffer (10×) provided by the manufacturer. Before addition of the enzyme (2 to 2.5 U/100 μL reaction), the reaction mixture was heated at 94°C for 5 minutes to allow proper genomic DNA denaturation. Amplification cycles were subsequently performed under standard conditions: 94°C for 1 minute, 55°C for 1 minute, and 72°C for 2 minutes. After 30 cycles, the PCR products were purified on Centricon 30 (Amicon Corp, Danvers, MA) to remove unincorporated primers and dNTPs.

Each set of primers was designed to allow amplification of introns sequences adjacent to the exon coding sequences.

**Restriction endonuclease analysis.** PCR-amplified exon 18 fragments were digested with Maui II restriction endonuclease (Boehringer, Mannheim, Germany) according to the manufacturer’s conditions and separated on a 12% polyacrylamide gel. After electrophoresis, the gel was stained with ethidium bromide for direct visualization under UV light.

**PCR products direct sequencing.** Single-stranded DNA suitable for sequencing was obtained by performing a second PCR amplification of 25 to 30 cycles on double-stranded PCR products as a template with only one of the two primers used in the first PCR reaction. Before sequencing, single-stranded products were purified on Centricon 100 or on diethyl aminoethyl (DEAE) cellulose paper during agarose gel electrophoresis to remove double-stranded template, unincorporated primers, and dNTPs. Sequencing was performed with a sequenase kit (USB, Cleveland, OH) using PCR primers and deoxyadenosine 5’-32P thiotriphosphate (α-32P dATP) (Amersham) as a label. The samples were analyzed on a 6% denaturing sequencing gel.

**RESULTS**

**vWF trypic fragments analysis.** Fragments resulting from trypsin digestion of purified vWF were separated with sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Fig 1). They displayed a different pattern in the patient compared with the control fragments. The smaller peptide characterized migrated faster than the corresponding 31-Kd peptide obtained with normal vWF, with an apparent molecular weight of approximately 29 Kd. However, under reducing conditions, no mobility shift was observed, the two peptides migrating with an apparent molecular weight of 34 Kd. Western blot analysis performed on both trypic digests showed that MoAb-418, which recognizes normal unreduced 31-Kd peptide, was unable to recognize the patient’s peptide. On the other hand, both the patient’s unreduced 29-Kd peptide as well as normal 31-Kd peptide, were recognized by MoAbs-175-35A8 and 175-31H3 (data not shown).

**DNA sequencing.** Direct sequencing of PCR-amplified exons 18 to 24, covering the N-terminal region of mature vWF up to AA 311, was performed on both sense and antisense strands. DNA sequence analysis showed a single base mutation C to T in exon 18 at nucleotide 2372. This T for C base replacement changes an ACG codon to an ATG codon and predicts the substitution of Threonine (Thr) by Methionine (Met) at position 28 in the mature vWF. The patient was found to be homozygous for the substitution while her son was found to be heterozygous (Fig 2).

**Restriction endonuclease analysis.** The mutation ACG → ATG destroyed a Maui II restriction site ACGT. PCR-
amplified exon 18 fragments of the patient, her son, and the controls were digested to confirm the sequencing data and to search for this nucleotide substitution in normal individuals. The undigested pattern (one single band of 281 bp) found in the patient confirmed her homozygous state for the mutation. Because her son exhibited both digested (113 + 168 bp) and undigested (281 bp) restriction fragments, he was confirmed as heterozygous. On the other hand, all the 28 control DNAs from normal individuals tested presented the homozygous digested pattern with two bands of 113 and 168 bp (Fig 3).

DISCUSSION

Because the vWF defect of the patient tentatively named "vWD Normandy" resulted from a specific FVIII-binding alteration, as shown in our previous report, we decided to direct our investigations toward the vWF N-terminal region containing a major FVIII-binding domain. More precisely, we focused on the tryptic fragment lying between AA 1 and 272 of mature vWF and able to inhibit FVIII binding to vWF, although another potential FVIII-binding site has been reported in the C-terminal region of the vWF subunit but without precise localization. Comparison of normal and the patient's vWF tryptic digests confirmed that a defect was indeed located in the N-terminal region of the vWF molecule, as shown by the mobility shift observed on the patient's peptide, migrating in SDS-PAGE with an apparent lower molecular weight than normal. This mobility alteration could originate from a small deletion or from a point mutation in the vWF gene. Indeed, a single AA substitution may induce either a charge modification creating a different SDS-peptide stochiometry, the disappearance of a glycosylation site, or a conformational alteration of the vWF N-terminal peptide. However, the identical behavior of reduced normal and patient's tryptic peptides suggests that a conformational change is the most likely explanation for the change in electrophoretic mobility. This structure alteration hypothesis was further supported by our immunoblotting analysis because MoAb-418, a conformational MoAb unable to recognize normal reduced SpIII-T4, failed to detect the patient's unreduced corresponding peptide.

Having verified and more precisely localized the vWF region altered in vWD "Normandy", the molecular analysis of the 7 exons of vWF gene encoding the 311 N-terminal AA of mature vWF was performed. In the 1.8-kb region sequenced, corresponding to exons 18 to 24, we found a single base mutation in exon 18, changing the Thr at position 28 of mature vWF into a Met. This mutation, substituting codon ATG for ACG, may result from a methylation-induced C to T transition at a CpG dinucleotide, well known for being mutational hotspots in mammals. The base substitution, destroying a Mae II ACGT restriction site, was conveniently confirmed by restriction digest analysis of PCR-amplified exon 18. The patient was homozygous for this mutation and her son was heterozygous. In a previous report, we discussed the possible mode of inheritance of this defect, which could be in theory either dominant, with only one gene affected in the patient and not transmitted in this case to her children, or recessive, with either double heterozygosity for one mutated allele and one silent allele, or homozygosity for a defective gene. The present genotypic data for the patient and her son confirm the hypothesis of a recessive gene abnormality, which appeared the most likely in light of the consanguinity of the patient's parents.

The failure to find this mutation among normal individuals indicates that it is not a common (non-pathogenic) amino acid sequence polymorphism and suggests that the C to T transition is probably responsible for the FVIII-
binding defect in this patient. It is worth noting that the altered AA 28 is localized on the 13-Kd polypeptide (AA 1 to 106) containing the epitope of MoAb-418, which totally inhibits FVIII binding to vWF, but not on the nonapeptide sequence (AA 78 to 96) shown to be the epitope of another anti-vWF MoAb, MoAb W5-6A, also inhibiting FVIII/vWF interaction. Whether Thr2* of vWF directly cappeptide sequence of this cystine-rich region. The identification of the gene participates in the FVIII/vWF interaction, or its substitu- Met* alters the conformation of the N-terminal portion of mature vWF, which seems to be crucial for FVIII/vWF interaction, is an open question. It would be particu- in other patients whose vWF fails to bind FVIII-34 will be useful in providing an answer. Nevertheless, to prove that this mutation is responsible for the FVIII-binding defect characterized, the insertion of this mutation in a eukaryotic expression vector of vWF-cDNA is required. Studying the FVIII-binding ability of the recombinant mutant vWF should help us to elucidate the origin of the FVIII-binding deficiency observed in the vWD “Normandy” variants and provide valuable information on the vWF/FVIII interaction.

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