Proc. Natl. Acad. Sci. USA
Vol. 88, pp. 6377–6381, July 1991
Medical Sciences

Expression of von Willebrand factor "Normandy": An autosomal mutation that mimics hemophilia A

(factor VIII/hemostasis)

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Communicated by Philip W. Majerus, April 22, 1991 (received for review March 21, 1991)

ABSTRACT **von Willebrand disease Normandy (vWD Normandy) is a recently described phenotype in which a mutant von Willebrand factor (vWF) appears structurally and functionally normal except that it does not bind to blood coagulation factor VIII. This interaction is required for normal survival of factor VIII in the circulation; consequently, vWD Normandy can present as apparent hemophilia A but with autosomal recessive rather than X chromosome-linked inheritance. A vWF missense mutation, $Thr^{28} \rightarrow$ Met, was identified in the propositus in or near the factor VIII binding site. The corresponding mutant recombinant vWF(T28M) formed normal multimers and had normal ristocetin cofactor activity. However, vWF(T28M) exhibited the same defect in factor VIII binding as natural vWF Normandy, confirming that this mutation causes the vWD Normandy phenotype. The distinction between hemophilia A and vWD Normandy is clinically important and should be considered in families affected by apparent mild hemophilia A that fail to show strict X chromosome-linked inheritance and, particularly, in potential female carriers with low factor VIII levels attributed to extreme lyonization.**

Hemophilia A and von Willebrand disease (vWD) are caused by deficiency or structural defect of blood coagulation factor VIII and von Willebrand factor (vWF), respectively, and these two diseases are the most common inherited bleeding disorders of man. Factor VIII is encoded by a gene on chromosome Xq28, and hemophilia A shows X chromosome-linked recessive inheritance. vWF is encoded by a gene on chromosome 12p, and most forms of vWD show autosomal dominant inheritance. Before the cloning of these genes, the distinction between factor VIII and vWF was obscured by the tendency of these proteins to form a noncovalent factor VIII–vWF complex in blood plasma. Factor VIII constitutes <1% (by weight) of the factor VIII–vWF complex, and plasma factor VIII levels generally parallel vWF levels (1).

The binding of factor VIII to plasma vWF is necessary for the normal survival of factor VIII in the blood circulation. In either normal individuals or patients with hemophilia A, transfused factor VIII is cleared from the circulation with a half-life of ≈12 hr (2–4), and the clearance of factor VIII is indistinguishable from that of either vWF or preformed factor VIII–vWF complex (4). This probably reflects the rapid binding of exogenous factor VIII to vWF and the subsequent slow degradation of factor VIII–vWF complexes. In patients with severe vWD, however, the half-life of transfused factor VIII is only ≈2.4 hr (4). The lack of stabilization of factor VIII by binding to vWF appears to explain the low factor VIII levels that occur in patients with severe deficiency of vWF. In the absence of vWF, endogenous factor VIII is cleared rapidly, causing a secondary deficiency of factor VIII.

Another important role of vWF is to promote platelet adhesion to subendothelium, and this is the initial event of primary hemostasis after vascular injury. Discrete domains that bind to platelet receptor sites and collagen have been localized on vWF, and these domains apparently are not involved in binding to factor VIII (5). These observations suggest that, in principle, a defect in the factor VIII binding site of vWF could result in isolated deficiency of factor VIII without affecting other vWF functions in hemostasis, producing an apparently autosomal form of hemophilia A (1). Recently, three patients from two unrelated families from France were reported with such a defect (6, 7). One patient appeared to have a normal bleeding time, normal plasma vWF level and multimer pattern, normal binding to platelet receptors and to collagen, but complete deficiency of factor VIII–vWF binding. Her variant of vWD was tentatively named vWD Normandy (6).

A candidate missense mutation was identified in the vWF gene of the propositus with vWD Normandy (8), in or near the factor VIII binding site. Expression and characterization of the corresponding mutant recombinant vWF proves that this missense mutation abolishes the binding of factor VIII to vWF, demonstrating that a point mutation in the autosomal gene for vWF can cause a bleeding disorder that clinically resembles X chromosome-linked hemophilia A.

MATERIALS AND METHODS

Plasmid Constructs. Plasmid pSVHVWF1 contains a full-length cDNA insert for human vWF cloned into the expression vector pSV7D (9) and was constructed as described (10). Plasmid pSVHNOR contains a C → T transition causing a Thr → Met substitution at amino acid 28 of the mature vWF subunit and was derived from pSVHVWF1 by mutagenesis using the polymerase chain reaction (11). A fragment containing a unique *Hin*dIII site at the 5′ end and the desired substitution near the 3′ end was amplified using the primers 5′-TCCCCGGAAGCTTGCTGCCTGACGC-3′ (vWF nucleotides 2478–2502) and 5′-CAGGTCATAGTTCTGGCACATTTTGGTACACTCGAGCCCT-3′ (vWF nucleotides 2602–2641 complement, substitution is underlined) using pSVHVWF1 as template. The 163-base-pair product fragment was purified by ultrafiltration (Centricon-30, Amicon)

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Abbreviations: vWD, von Willebrand disease; vWF, von Willebrand factor.

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and employed with the primer 5'-TCAGGGTCACTGGGAT-TCAAGGTGA-3' (vWF nucleotides 3885–3909 complement) using pSVHVWF1 as template to amplify a 1431-base-pair product with a unique *Nae* I site near the 3' end. This fragment was digested with *Hin*dIII and *Nae* I, and the resultant 1373-base-pair fragment was cloned into the *Hin*dIII and *Nae* I sites of pSVHVWF1. Clones containing the desired mutation were identified by specific oligonucleotide hybridization, and the DNA sequence was confirmed (12) for the segment subjected to enzymatic amplification.

Expression of Recombinant vWF. COS-7 cells were transfected with plasmids pSVHVWF1 (normal) and pSVHNOR [vWF(T28M)] using a DEAE-dextran method (13). After 24 hr, medium was replaced with serum-free DME(HG) (GIBCO/BRL, 430-2100) containing sodium bicarbonate at 3.7 g/liter for an additional 72 hr. Conditioned medium was concentrated ≈10-fold by ultrafiltration (Centriprep-30, Amicon) and dialyzed at 4°C against 50 mM Tris Cl, pH 8.0/0.15 M NaCl. CHO-DG44 dhfr$^-$ cells (14) were cotransfected using calcium phosphate (15) with the dihydrofolate reductase plasmid pCVSEII (16) and either pSVHVWF1 or pSVH-NOR, and clones were selected with nucleotide-deficient α minimal essential medium (α-MEM) and dialyzed serum. Cell lines expressing recombinant normal vWF or vWF(T28M) were incubated in serum-free α-MEM for 72 hr, and conditioned medium was concentrated ≈20-fold and dialyzed as above.

Total protein was assayed using a Coomassie blue G-250 dye-binding method (Bio-Rad) with bovine serum albumin as the standard. vWF antigen was assayed by a sandwich ELISA method using rabbit polyclonal anti-human vWF (Dakopatts 082, DAKO, Carpinteria, CA) at a 1:500 dilution for coating and a similar antibody conjugated to horseradish peroxidase (Dakopatts P226) at a 1:5000 dilution for detection with the substrate *o*-phenylenediamine. Product was assayed by absorbance at 490 nm using an EL312 Bio-Kinetics (Bio-Tek Instruments, Winooski, VT) plate reader. vWF was quantitated in plasma antigen units using assayed reference plasma from Helena Laboratories. One unit is approximately equal to 10 μg of vWF (17).

Plasmin Digestion of vWF. Samples of either plasma or concentrated conditioned medium containing 0.4–0.7 unit of vWF antigen were incubated for 2 hr at 4°C with 100 μl of Sephacryl-S1000 beads on which 0.08 mg of anti-vWF monoclonal antibody MAb239 was coupled. After washing, the beads were suspended in 10 μl of 0.02 M Tris Cl, pH 7.4/0.15 M NaCl, heated for 15 min at 60°C, and cooled to room temperature. Plasmin digestion was then performed overnight at 37°C after adding 0.046 unit of plasmin. SDS was added to a final concentration of 2% (wt/vol) and each sample was heated in boiling water for 5 min. Beads were removed by centrifugation and supernatant solutions were analyzed by SDS/polyacrylamide gel electrophoresis (18) on a 3–16% gradient gel under nonreducing or reducing conditions.

Factor VIII Binding. Factor VIII binding to vWF was assayed as described (19). Briefly, increasing amounts of vWF were immobilized by binding to anti-vWF monoclonal antibody-coated microplates and any endogenous factor VIII was removed by washing with 0.4 M $CaCl_2$. Purified plasma factor VIII was allowed to bind to the immobilized vWF, and the bound factor VIII was then detected by adding the reagents for a chromogenic assay of factor VIII-dependent factor X activation (Diagnostica Stago, Asnieres-sur-Seine, France) and measuring the hydrolysis by factor Xa of substrate CBS 48.03 (CH_3OCO-D-Leu-Gly-Arg-*p*-nitroanilide) as the rate of change of optical density at 450 nm. The vWF bound per well was then quantitated by addition of ^{125}I-labeled anti-vWF monoclonal antibody and quantitation of bound radioactivity (cpm). For this assay the background value for immobilized vWF was 110 cpm, and the background value for bound factor VIII was 0.27×10^{-3} unit.

RESULTS

Expression and Characterization of Recombinant vWF. The factor VIII binding site on vWF has been localized to an ≈34-kDa trypsin or plasmin fragment containing amino acid residues 1–272 of the vWF subunit (20, 21), suggesting that vWD Normandy might be caused by a mutation in this region. This segment of the protein is encoded by exons 18–23 of the vWF gene (22). Exons 18–24 were amplified from the propositus and sequenced. A single potential missense mutation was identified in exon 18, consisting of a CG → TG transition

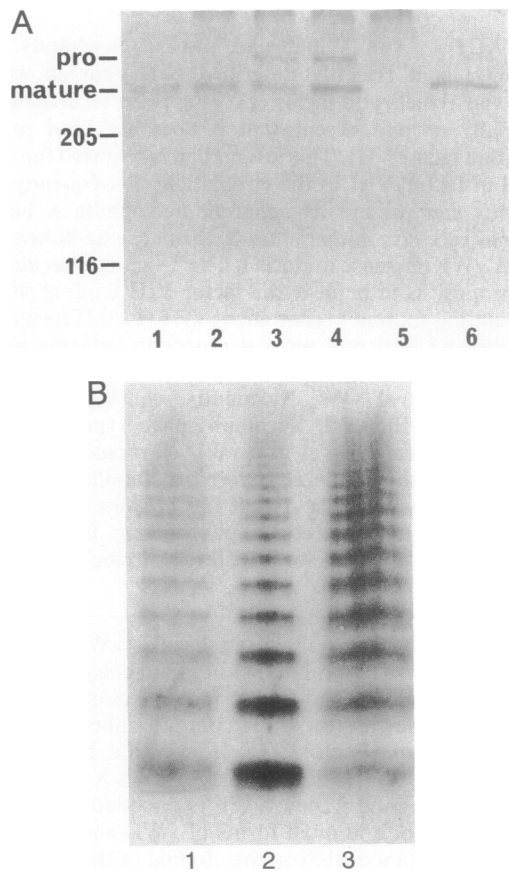

FIG. 1. Expression of recombinant normal vWF and vWF(T28M). (*A*) SDS/polyacrylamide gel electrophoresis under reducing conditions. vWF was immunoprecipitated with rabbit polyclonal anti-human vWF antibody (Dakopatts 082) and protein A-Sepharose from samples of concentrated conditioned medium containing ≈250 ng of vWF protein by ELISA, reduced with 7% (vol/vol) 2-mercaptoethanol, electrophoresed through 5% polyacrylamide gels (18), electroblotted onto nitrocellulose membrane (LKB Novablot), and visualized with anti-human vWF antibody, biotinylated goat anti-rabbit IgG, and Vectastain avidin–biotin complex horseradish peroxidase (Vector Laboratories). The positions of pro-vWF and mature vWF subunits and the apparent mass (kDa) of marker proteins are indicated on the left. Lanes: 1, recombinant normal vWF from COS cells; 2, recombinant vWF(T28M) from COS cells; 3, recombinant normal vWF from CHO cells; 4, recombinant vWF(T28M) from CHO cells; 5, medium from CHO cells transfected with vector only; 6, normal human plasma containing ≈250 ng of vWF. (*B*) SDS/agarose multimer gel electrophoresis. Samples of normal plasma or concentrated COS cell conditioned medium containing ≈25 ng of vWF proteins by ELISA were electrophoresed through a 1.5% agarose gel for analysis of the vWF multimer pattern (24). Lanes: 1, recombinant vWF(T28M); 2, recombinant normal vWF; 3, normal human plasma.

that resulted in the substitution $Thr^{28} \rightarrow Met$. This patient was born of a consanguineous marriage and was shown to be homozygous for this substitution (8). This substitution was incorporated into an expression vector containing a full-length vWF cDNA insert. The normal vWF and mutant vWF(T28M) plasmids were used to prepare the corresponding recombinant proteins in transiently transfected COS cells and stably transfected CHO cell lines.

Control experiments showed that, except for the factor VIII binding domain, recombinant normal vWF and vWF(T28M) were similar in structure and function to plasma vWF. SDS/polyacrylamide gel electrophoresis under reducing conditions showed a major vWF subunit species with an apparent mass of ≈220 kDa and a minor pro-vWF subunit species with an apparent mass of ≈270 kDa for both recombinant normal vWF and vWF(T28M) (Fig. 1*A*). This is consistent with normal partial processing of the ≈270-kDa pro-vWF precursor to the ≈220-kDa mature vWF subunit (23). The extent of processing was similar for both recombinant proteins, although processing was more complete in COS cells than in CHO cells. SDS/agarose gel electrophoresis under nonreducing conditions (Fig. 1*B*) showed multimers ranging from dimers to species of very large size for both normal and mutant proteins. Differences between recombinant normal vWF and vWF(T28M) cannot, therefore, be attributed to differences in proteolytic processing or multimer formation.

Platelets can bind vWF through a specific receptor, the glycoprotein Ib–IX complex (5). This interaction is stimulated by addition of the antibiotic ristocetin, leading to platelet agglutination by polyvalent vWF (25). This property of ristocetin has been exploited to devise clinically useful procedures to assay vWF activity. Recombinant normal vWF and vWF(T28M) supported ristocetin-induced vWF-dependent platelet agglutination (Fig. 2) and had similar collagen binding activity (S.J. and C.M., unpublished observations). This is consistent with the properties of natural plasma-derived vWF Normandy, which exhibits normal ristocetin-dependent binding to platelets and also binds normally to collagen (6).

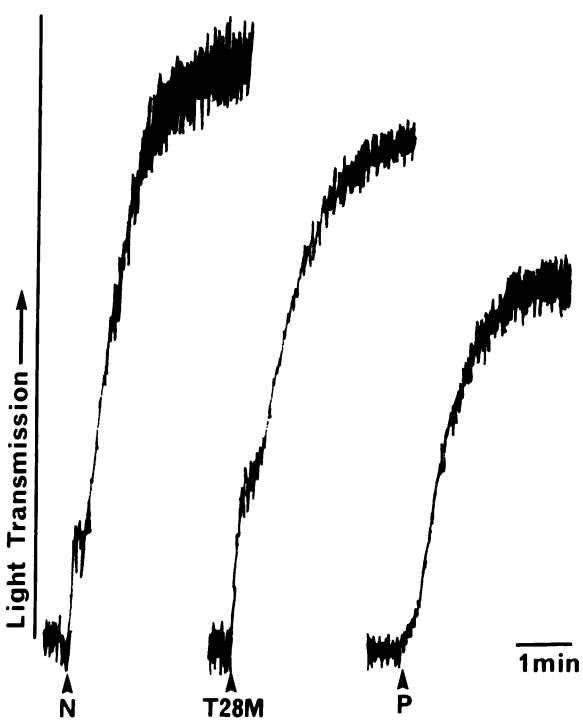

FIG. 2. Ristocetin cofactor activity of recombinant normal vWF and vWF(T28M). Lyophilized platelets were reconstituted to 200,000 platelets per μl in 60 mM Tris Cl, pH 7.5/0.15 M NaCl (Biodata, Hatboro, PA). Ristocetin (15 mg/ml; 50 μl; Sigma) was added to platelet suspension (400 μl) and stirred for 3 min at 37°C. A sample of normal human plasma or of recombinant vWF diluted in 50 μl of buffer was added and light transmission was monitored continuously with a Payton Associates (Buffalo, NY) aggregation module. The horizontal bar represents 1 min. The ordinate is in arbitrary light transmission units. The aggregation traces shown were obtained with the following final concentrations of vWF: N, normal recombinant vWF (0.18 unit/ml); T28M, recombinant vWF(T28M) (0.18 unit/ml); P, normal human plasma vWF (0.12 unit/ml).

Recombinant vWF(T28M) Reproduces the Structural and Functional Defects in the Factor VIII Binding Domain of Plasma-Derived vWF Normandy. Normal plasma vWF and vWF Normandy show a clear structural difference after digestion with plasmin (Fig. 3). Upon SDS/polyacrylamide gel electrophoresis, the amino-terminal plasmin fragment of vWF Normandy has slightly increased mobility (lane 2, ≈29 kDa) compared to the corresponding plasmin fragment (lane 1, ≈31 kDa) of normal vWF, when analyzed without prior reduction. These electrophoretic mobilities are reproduced by the amino-terminal plasmin fragments of recombinant normal vWF and vWF(T28M) (Fig. 3, lanes 4 and 3, respectively). After reduction this difference disappears, and these fragments migrate with an apparent mass of 34 kDa for the plasma-derived (lanes 5 and 6) (ref. 8; C.G., B. Mercier, S.J., D. Oufkir, and C.M., unpublished observations) or recombinant proteins (lanes 7 and 8). Factor VIII binding to amino-terminal fragments of plasma-derived vWF is abolished by reduction (20, 26). These observations suggest that the conformation of the amino-terminal part of vWF is important for factor VIII binding, and this conformation is disrupted either by reduction or by alterations in the secondary structure of plasma vWF Normandy (8) and vWF(T28M) (Fig. 3).

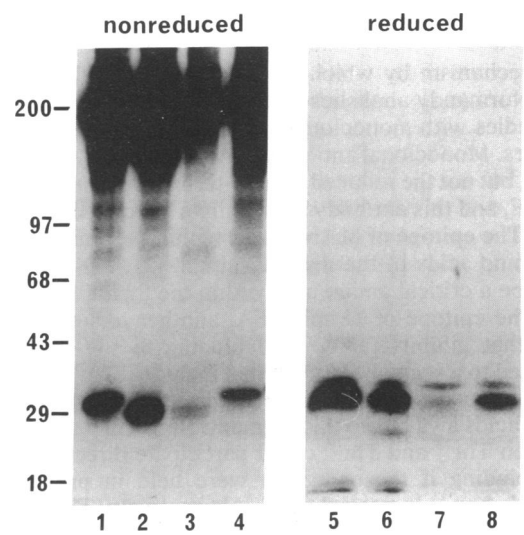

FIG. 3. Electrophoretic mobility of the plasmin fragments of either plasma or recombinant normal vWF and vWF(T28M). The increased electrophoretic mobility of the factor VIII-binding amino-terminal unreduced plasmin fragment of plasma-derived vWF Normandy is reproduced by recombinant vWF(T28M). After electrophoretic transfer to nitrocellulose, the vWF peptide fragments were identified using ^{125}I-labeled antibodies. Rabbit anti-human vWF polyclonal antibodies were used for lanes 1–4. Monoclonal antibody MAb184-14A12, which recognizes both the unreduced and reduced amino-terminal trypsin and plasmin fragments of vWF (C.G., B. Mercier, S.J., D. Oufkir, and C.M., unpublished observations), was used for lanes 5–8. Lanes: 1 and 5, a pool of normal plasmas; 2 and 6, vWF Normandy patient plasma; 3 and 7, recombinant vWF(T28M); 4 and 8, normal recombinant vWF.

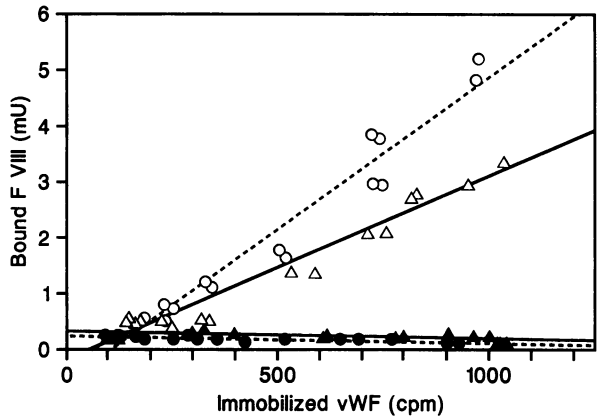

FIG. 4. Factor VIII binding activity of recombinant normal vWF and vWF(T28M). Recombinant normal vWF binds factor VIII in a manner similar to normal plasma vWF, whereas recombinant vWF(T28M) does not bind factor VIII and reproduces the phenotype of plasma vWF Normandy. ○, Normal plasma; △, recombinant normal vWF; ●, vWD Normandy patient plasma; ▲, recombinant vWF(T28M). mU, milliunit(s).

The defect in factor VIII binding of plasma-derived vWF Normandy (6) is reproduced with recombinant vWF(T28M) (Fig. 4). Recombinant normal vWF binds factor VIII in a manner similar to normal plasma vWF, whereas recombinant vWF(T28M) and plasma-derived vWF Normandy have no factor VIII binding activity. The entire coding sequence of the vWF Normandy allele was not examined, so additional amino acid substitutions between residues 311 and 2050 cannot be excluded. However, the defective factor VIII binding of vWF(T28M) indicates that this single amino acid substitution is sufficient to account for the vWD Normandy phenotype (6).

DISCUSSION

The mechanism by which the $Thr^{28} \rightarrow$ Met substitution in vWF Normandy abolishes factor VIII binding is not known, but studies with monoclonal antibodies suggest several possibilities. Monoclonal antibody MAb418 recognizes the unreduced, but not the reduced, amino-terminal plasmin fragment of vWF, and this antibody also inhibits factor VIII binding to vWF. The epitope of MAb418 lies within the amino-terminal 106 amino acids of the mature subunit (21, 27). Thus Thr^{28} could be a critical amino acid within the factor VIII binding site. The epitope of MAbW5-6A, another monoclonal antibody that inhibits factor VIII binding to vWF, has been localized to a segment of vWF that includes Thr^{78}–Thr^{96} (26), and the $Thr^{28} \rightarrow$ Met substitution in vWF Normandy is 50 amino acids away from this segment. Both Thr^{28} and residues between Thr^{78} and Thr^{96} could participate directly in factor VIII binding if these regions were held in proximity by disulfide bonds in normal vWF. Alternatively, Thr^{28} may not be part of the factor VIII binding site, but the $Thr^{28} \rightarrow$ Met substitution may indirectly alter the conformation of this site. Thr^{28} is adjacent to Cys^{29}, and the $Thr^{28} \rightarrow$ Met substitution may prevent the normal disulfide pairing of this residue. Such an effect on secondary structure would be consistent with the mobilities on SDS/polyacrylamide gel electrophoresis of the amino-terminal plasmin fragments of normal vWF and vWF Normandy or vWF(T28M), which are different before reduction but become indistinguishable after reduction (Fig. 3).

Since the first propositus was reported, three additional unrelated families with the vWD Normandy phenotype have been identified (ref. 19; C.G., B. Mercier, S.J., D. Oufkir, and C.M., unpublished observations). In all cases the disorder appears to be recessive, since obligate heterozygotes have intermediate values for factor VIII–vWF binding and are clinically unaffected (6, 19). Aside from the first patient identified there is no evidence for consanguinity of the parents, and these additional patients may be compound heterozygous. Individuals with one vWF Normandy allele and one vWF null allele should exhibit the vWD Normandy phenotype. The distribution of genotypes associated with vWD Normandy will depend on the frequencies of such alleles, which have yet to be determined.

One family affected with the vWD Normandy phenotype was misdiagnosed as affected with mild hemophilia A, and correct diagnosis led to important changes in genetic counseling (19). Such families frequently contain affected females, whose low plasma factor VIII levels were attributed to heterozygosity for hemophilia A with extreme lyonization. Sporadic new mutations account for approximately one-third of all cases of hemophilia A, so that even genetic studies using intragenic factor VIII DNA markers may not reliably identify female carriers. Misdiagnosis of hemophilia A carrier status can delay or prevent recognition of vWD Normandy, and this error can be avoided by direct evaluation of factor VIII–vWF binding.

Recognition of the vWD Normandy phenotype has implications for the treatment of bleeding episodes in patients with factor VIII deficiency. In vWD Normandy, transfused purified factor VIII has a markedly shortened survival, whereas preparations containing some vWF show normal or prolonged factor VIII survival (6, 19). In fact, sustained normalization of plasma factor VIII levels can be achieved by infusion of purified vWF alone (19). As highly purified therapeutic products become more widely used, recognition of this variant may become especially important because some factor VIII concentrates do not contain sufficient vWF to promote normal factor VIII survival. Furthermore, in contrast to patients with reduced factor VIII levels because of mild hemophilia A, treatment of patients having the vWD Normandy phenotype with vasopressin analogues will be ineffective.

Characterization of recombinant vWF(T28M) confirms the genetic basis of vWD Normandy, a congenital factor VIII deficiency unlinked to the factor VIII locus that can mimic hemophilia A. Characterization of mutations in other patients showing the same factor VIII binding defect will extend our understanding of the structure–function relationships and physiological importance of the factor VIII–vWF interaction.

We thank Dr. Anna M. Randi for many helpful discussions and Lisa A. Westfield for the synthesis of oligonucleotides. We also acknowledge Dr. Hervé Broly for help with the preparation of monoclonal antibodies, Bernard Mercier for primer design and PCR analysis, Driss Oufkir for performing the analysis of plasmin fragments, Denis Hoguet for nucleotide sequencing, and Véronique Duretz and Sabine Belmont for technical assistance. This work was supported by the Howard Hughes Medical Institute (E.A.T. and J.E.S.) and by an American Heart Association Medical student research fellowship (N.K.W.).

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