Hum Genet (1995) 95:681–686

ORIGINAL INVESTIGATION

Reinhard Schneppenheim · Kathy B. Thomas
Sonja Krey · Ulrich Budde · Ursula Jessat
Anton H. Sutor · Barbara Zieger

Identification of a candidate missense mutation in a family with von Willebrand disease type IIC

Received: 29 September 1994

Abstract A screening project to identify candidate molecular defects causing von Willebrand disease type IIC (VWD IIC) in a German family was carried out using polymerase chain reaction (PCR) amplification of all 52 exons of the von Willebrand factor (VWF) gene, subsequent electrophoresis of single and double stranded DNA and direct sequencing of PCR products with aberrant electrophoretic patterns. Only one candidate mutation, G550R, caused by a G→A transition, was detected in exon 14 of the pro-VWF gene sequence. This mutation was not found on 200 chromosomes of normal individuals. The propositus was homozygous for the mutation and for an extended intragenic haplotype, composed of eight polymorphic markers. Further family members were heterozygous for the mutation and were phenotypically normal or only mildly affected, in accordance with the recessive pattern of inheritance for VWD type IIC. The mutation could influence one of the presumed active centers for the suspected multimerizing enzymatic activity of pro-VWF localized in the D1 and D2 domain, which corresponds to exon 5 and exon 14 of the VWF gene.

Introduction

Von Willebrand disease (VWD), an inherited disorder of primary hemostasis is characterized by quantitative or qualitative defects of the von Willebrand factor (VWF). Quantitative defects are responsible for the manifestation of VWD type I and the most severe form, type III. Qualitative defects are classified as VWD type II, which is further divided into subtypes IIA through IIH, together with the recently described VWD type Normandy (Ruggeri 1987; Sadler 1994). Most of the known mutations causing VWD type II are missense mutations, and particular defects can be correlated with specific phenotypes that can be recognized by specific aberrant VWF multimer patterns following sodium dodecyl sulfate (SDS) electrophoresis in agarose gels (Ruggeri 1987). To date, a number of different mutations have been identified for VWD type IIA, IIB, type Normandy (Ginsburg and Sadler 1993), and type I New York/Malmö (Holmberg et al. 1993). All these defects are localized in exon 28 and exon 18 (type Normandy) of the sequence coding for the mature VWF, and the detection of these mutations offers the opportunity to test concepts concerning the complex structure-function relationships of the mature protein. Our preliminary attempts to detect mutations causing other subtypes have also been targeted to exon 28, but with no positive results so far (R. Schneppenheim, unpublished).

The primary product of VWF biosynthesis is pre-pro-VWF. The mature VWF lacks the signal (pre-) peptide and the pro-polypeptide of 760 amino acid residues refered to as the D1 and D2 domain of pro-VWF. Complex posttranslational modifications include dimerization, polymerization, glycosylation, and cleavage of pro-VWF (Wagner 1990). It has been shown that the pro-polypeptide is necessary for the multimerization of VWF dimers by disulfide bonding, a process essential for the biological activity of VWF (Verweij et al. 1987; Wise et al. 1988; Mayadas and Wagner 1989). Thus, besides the intrinsic defects within the mature VWF, an abnormal multimerization process could also be caused by a defect in the pro-polypeptide. The multimerizing capacity of pro-VWF has tentatively been localized to the amino acid sequence "Cys-Gly-Leu-Cys" in each of the two D domains (D1 aa 159 – aa 162; D2 aa 521 – aa 524) because of the homology of this sequence with the active site of disulfide isomerases that catalyze disulfide bonding (Mayadas and

R. Schneppenheim (✉) · S. Krey
Universitäts-Kinderklinik Kiel, Schwanenweg 20,
D-24105 Kiel, Germany

K. B. Thomas · U. Jessat · A. H. Sutor
Universitäts-Kinderklinik Freiburg, Freiburg, Germany

U. Budde
Allg. Krankenhaus Harburg, Hamburg, Germany

B. Zieger
Scripps Clinic and Research Foundation, La Jolla,
California, USA

Wagner 1992). These amino acids correspond to exon 5 and exon 14 of the VWF gene, respectively, and recently, an attempt to identify the molecular defect of a new VWD type IIC variant has been concentrated on that gene region, but with no success (Ledford et al. 1993).

Our search for candidate mutations causing VWD type II, other than IIA, IIB and type Normandy, comprised the complete gene sequence coding for the mature VWF and for the pro-polypeptide sequence. Of particular interest for us was VWD type IIC, which differs from other type II subtypes by its recessive mode of inheritance and an apparent variability of clinical symptoms, hemostasis parameters, and electrophoretic multimer patterns (Armitage and Rizza 1979; Ruggeri et al. 1982; Mannucci et al. 1983; Batlle et al. 1986; Mazurier et al. 1986). Identification of the responsible molecular defects should allow a more reliable classification of this subtype.

Materials and methods

Patients

The propositus, who was diagnosed as having VWD type IIC, was the only severely affected member in a three-generation family. Reported bleeding symptoms were frequent epistaxis, easy bruising, and heavy menorrhagia that finally required hysterectomy. The patient's mother also had a history of frequent epistaxis in her childhood and some secondary bleeding after abdominal surgery. Both of the patient's children were phenotypically normal. Informed consent was obtained from all investigated individuals.

Hemostasis parameters

Citrated plasma was used for the determination of factor VIII procoagulant activity (FVIII:C) by means of a coagulometric assay (Behring, Marburg, Germany). Von Willebrand factor antigen (VWF:AG) was determined by an enzyme-linked immunosorbent assay (ELISA) (Mazurier et al. 1977), and VWF biological activity was measured as ristocetin cofactor (VWF:RiCof) activity, by means of formalin-fixed platelets (Macfarlane et al. 1975), and as collagen-binding activity (VWF:CBA) by ELISA, with equine collagen type I (Hormon Chemie, Munich, Germany) coating the microtiter plate (Brown and Bosack 1986; Thomas et al. 1994). A normal plasma pool, prepared from 19 normal donors, was used to construct the standard curves for the VWF parameters and also for the multimer analysis. The VWF parameters were expressed in arbitrary units/ml (IU/ml), where one unit was the amount of that parameter present in 1 ml normal pooled plasma. Multimeric analysis was carried out by SDS-agarose gel electrophoresis in 2.2% HGT(P)-agarose (Sea-Kem, Bio-zym, Hameln, Germany; Ruggeri and Zimmermann 1981) with modifications (Raines et al. 1990). The diagnosis of VWD type IIC was confirmed in two other laboratories (U. Budde, Hamburg-Harburg, Germany; C. Mazurier, Lille, France). In addition to visual qualitative evaluation, multimers were also scanned by video-densitometry (Bio-Rad, model 620).

Mutation screening

Preparation of high molecular weight genomic DNA from leukocytes was according to published protocols (Maniatis et al. 1982). Primer sequences for amplification by the polymerase chain reaction (PCR; Saiki et al. 1988) of exons 1 through 22 and exons 35 through 52 were chosen from the published VWF gene sequence (Mancuso et al. 1989) using a computer program (Lowe et al. 1990); primer sequences for PCR amplification of exons 23 through 34, which correspond to a pseudogenic region on chromosome 22, were taken from the available literature (Mancuso et al. 1991). In addition, the amplified sequences, each comprising single exons, were flanked by sufficient intronic DNA to allow the detection of splice site mutations. Only the primer sequences for exons 5 and 14, which are of particular interest concerning the multimerization process of VWF (Mayadas and Wagner 1992), are given (primer sequences for the amplification of other exons can be provided on request). The primers for exon 5 also included exon 4 and intron 4: exon 4–5 sense primer 5′-CTG CTG AGA AAA GGT TAC G-3′; antisense primer 5′-GCA AAG AGA TAA GGT TGG C-3′; exon 14-sense primer 5′-CCA GAG TGG CCT GGT CTC TC-3′, antisense primer 5′-GAT CGC TGT GCT GCT TCT GC-3′.

Annealing temperatures were 55° C for exon 4–5, and 63° C for exon 14. PCR was carried out by means of the Thermal Reactor (Perkin Elmer Cetus, Hamburg, Germany) using reagents and *Taq* polymerase from the same company. Double stranded or single stranded PCR products were analyzed by non-denaturing polyacrylamide gel electrophoresis (PAGE; Budowle et al. 1991; Orita et al. 1989); the separated bands were visualized by silver staining (Budowle et al. 1991). Samples with aberrant electrophoretic patterns were further analyzed by direct sequencing of single stranded DNA obtained by asymmetric PCR. The results were confirmed by sequencing the complementary strand. The Sequenase 2.0 kit (U.S.B., Bad Homburg, Germany) was used for the sequencing reactions that were primed by the respective PCR-limiting sense or antisense oligonucleotides.

Haplotype analysis

Haplotypes were constructed to establish the genetic background of VWD type IIC alleles. Eight intragenic polymorphisms were studied: PCR *Mae*II, exon 12 (Bowen et al. 1991), PCR *Acc*I, exon 14 (Kunkel et al. 1991), length polymorphism, intron 15, unclassified (R. Schneppenheim, unpublished), PCR *Rsa*I, exon 18 (Kunkel et al. 1990), PCR *Msp*I, exon 19 (Mercier et al. 1990), PCR *Hph*I, exon 28 (Donner et al. 1991), variable number of tandem repeats (VNTR) I, intron 40 (Peake et al. 1990), and VNTR II, intron 40 (Ploos van Amstel and Reitsma 1990). The PCR products containing restriction fragment length polymorphisms (RFLPs) were digested by the appropriate restriction enzymes (Boehringer Mannheim, Germany). They were subsequently analyzed by PAGE and visualized by silver staining (Budowle et al. 1991).

Results

Hemostasis studies

Hemostasis parameters, essential to the diagnosis of VWD in the investigated family, are shown in Table 1. The platelet count was normal in all members and the skin bleeding time was prolonged only in the propositus. In contrast to other patients with VWD type IIC, VWF:Ag in our patient was normal to elevated; however, the functional parameters, measured as the VWF:RiCof and VWF:CBA were pathologically decreased. In all other investigated family members, the VWF:Ag values were normal to elevated; the functional parameters were within or just below the normal range, but were disproportionally lower than the corresponding VWF:Ag. The multimeric patterns of all family members are shown in Fig. 1. In the propositus, the multimer analysis of the VWF showed an unusual banding pattern, characterized by a reduction of the high molecular weight multimer species, the presence of a pronounced protomer, and markedly reduced abnormal satel-

Table 1 Hemostasis parameters for the patient and family members, (*ID* identification number referring to the pedigree in Fig. 3, *BT* bleeding time, *VWF:Ag* von Willebrand factor antigen, *RiCof* ristocetin-cofactor, *VWF:CBA* collagen binding activity of VWF, *F VIII:C* factor VIII procoagulant activity)

ID	BT [min]	VWF: Ag [IU/ml]	RiCof [IU/ml]	VWF: CBA [IU/ml]	F VIII:C [IU/ml]
I 1	3.5	2.09	1.45	1.44	> 1.00
II 1	10.0	1.59	0.25	0.29	> 1.00
III 1	2.0	1.34	0.80	0.79	> 1.00
III 2	6.0	0.86	0.66	0.57	> 1.00
Normal range	< 6.0	0.6–1.6	0.6–1.6	0.6–1.6	0.7–1.4

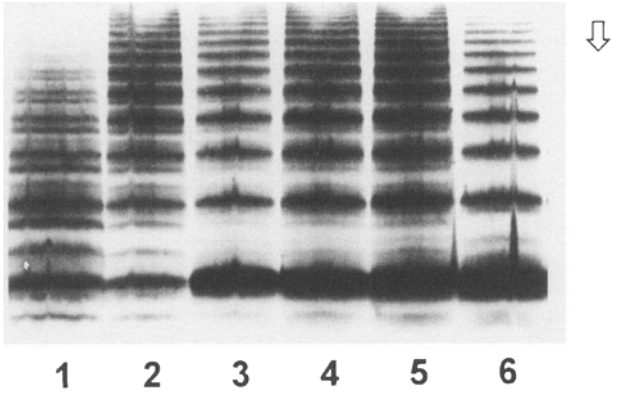

Fig. 1 SDS agarose gel electrophoresis of VWF multimers, visualized by enzyme immunostaining after capillary transfer onto PVDF membranes. Note the loss of some high molecular weight multimers, the lack of satellite bands, and the presence of a pronounced protomer in the propositus *(lane 6)*. The electrophoretic phenotypes of the other family members appeared normal, except for the presence of a pronounced protomer and a lower intensity of satellite bands. *Lane 1* VWD type IIB, *lane 2* normal control, *lane 3* I 1, *lane 4* III 1, *lane 5* III 2 (referring to the pedigree in Fig. 3)

lite bands, confirming the diagnosis of VWD type IIC. In the other family members, all multimers were seen, although the pronounced protomer remained. The multimer banding pattern in these family members contained a mixture of intervening satellite bands that corresponded to those of the normal plasma pool and to those seen in the propositus. Densitometric evaluation confirmed these findings (Fig. 2).

Mutation screening

The search for the molecular defect causing VWD type IIC in our patient comprised the complete coding sequence of the VWF gene and yielded only one candidate mutation in exon 14. The mutation is a G→A transition at nucleotide 1898 (nucleotide "+ 1" = transcription cap site, 250 nucleotides before the initiation codon; Sadler 1994) and results in the amino acid substitution G550R (numbering starting at the initiation codon). The patient was

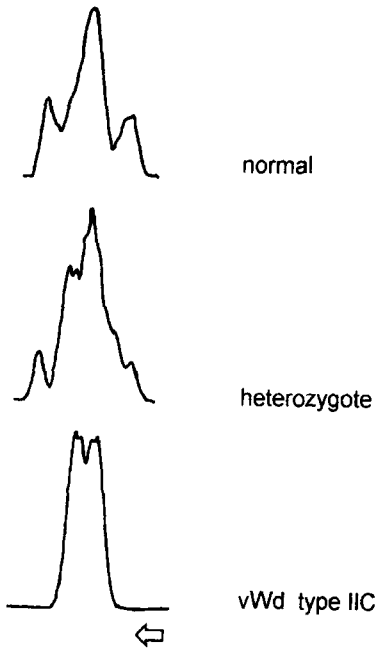

Fig. 2 Densitometric evaluation of the second multimer and its corresponding satellite bands in a normal control (lane 2 in Fig. 1), in a heterozygous individual for the VWD IIC defect (lane 3 in Fig. 1), and the patient, being homozygous for the VWD type IIC mutation (lane 6 in Fig. 1). The VWD type IIC multimer lacks satellite bands and has a symmetrically split main band; the heterozygous multimer species show a decrease of satellite bands, compared with normal plasma, and an asymmetrically split main band with a pronounced second sub-band. The *arrow* indicates the direction of electrophoretic migration

homozygous for this mutation, whereas the patient's mother and both of the patient's children were heterozygous (Fig. 3). In all other 51 exons of the complete VWF gene sequence, no additional deviant sequences were detected, except those known as polymorphisms. The candidate mutation was not found on 200 chromosomes in a population study of unrelated anonymous control individuals.

Haplotype analysis

Five out of eight investigated intragenic polymorphisms were informative in the family of our patient, who was homozygous for both the mutation and the extended haplotype. A common genetic background for the maternally and the paternally derived allele was thus suggested by our results (Fig. 3, electropherograms not shown). The propositus' father was not available for study. No information regarding the family's history was given and thus the degree of relatedness between father and mother could not be established.

Discussion

The primary translation product of VWF is a pre-pro-polypeptide. After the removal of the signal (pre-) peptide,

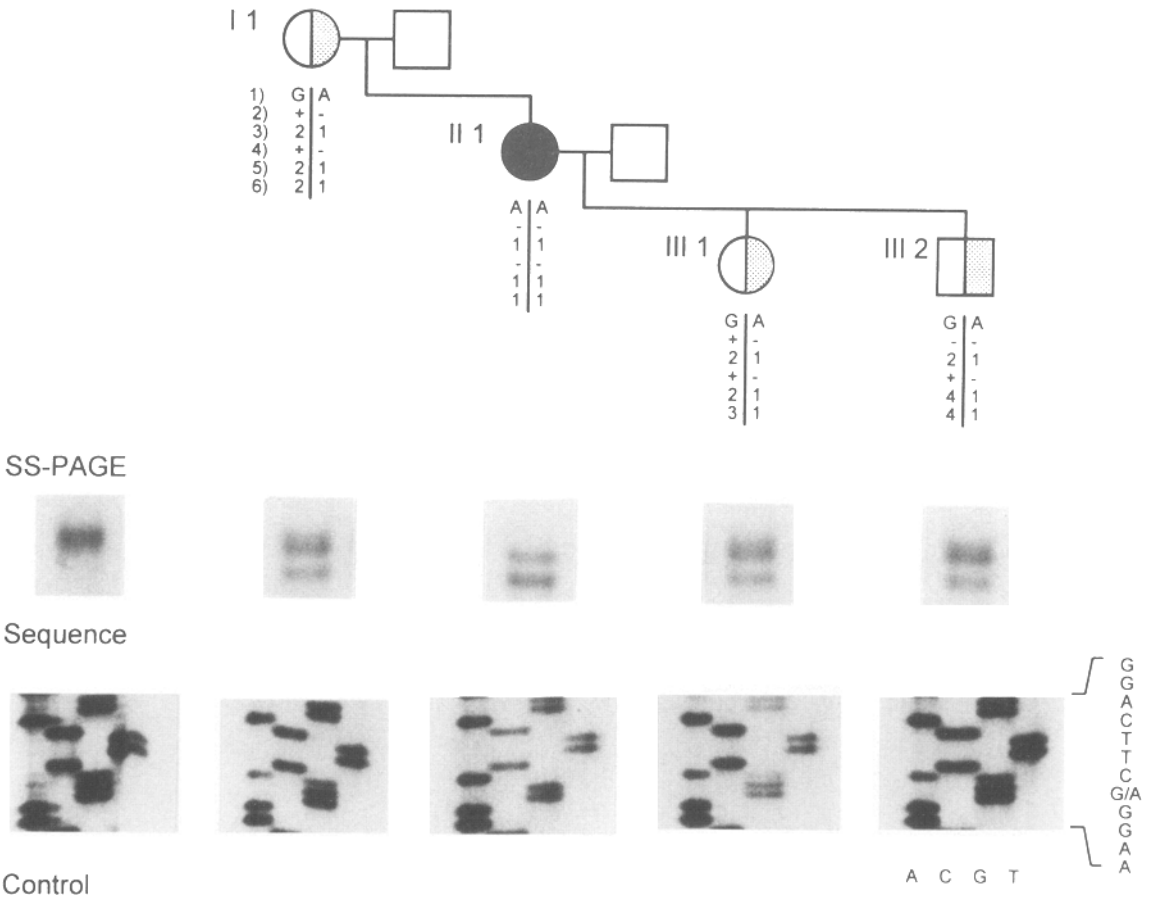

Fig. 3 Homozygous candidate missense mutation (G550R) caused by a G/A transition located in exon 14 of the VWF gene in the propositus (II 1) with VWD type IIC. The mutation was detected by polyacrylamide gel electrophoresis of single stranded PCR products *(SS-PAGE)* and was identified by direct sequencing of the sense and antisense strand. All other investigated family members (I 1, III 1, III 2) were heterozygous for the mutation. The patient's father and her husband were not available for analysis. *Half-filled shaded symbols* Midly affected or unaffected heterozygous carrier of the mutation. Haplotypes, constructed from five informative intragenic marker polymorphisms, are shown *below* the pedigree symbols: *1) G* wildtype, *A* candidate mutation; *2)* PCR *Mae*II, exon 12; *3)* diallelic length polymorphism, intron 15; *4)* PCR *Rsa*I, exon 18; *5)* VNTR I, intron 40; *6)* VNTR II, intron 40

the pro-VWF forms dimers by means of interchain disulfide bonding at the carboxyl terminals (Fowler et al. 1985). These pro-VWF dimers presumably multimerize at the N-terminals by the formation of interchain disulfide bonds (Fowler et al. 1985); in vitro, the propeptide plays an essential role in the multimerization process (Verweij et al. 1987; Wise et al. 1988; Mayadas and Wagner 1989). The detection of consensus sequences in the D1 and D2 domain of pro-VWF, corresponding to the active center of disulfide isomerases, supports this hypothetical function (Mayadas and Wagner 1992). Vicinal cysteines are part of the consensus sequence; they are separated by two amino acids (Cys-Xaa-Xaa-Cys) and form unstable intrachain disulfide bonds that cycle between the disulfide and the dithiol state, and that thus function as a redox system (Bulleid and Freedman 1988; Edman et al. 1985). This redox activity catalyzes thiol protein disulfide interchange, and it has been suggested that this function is responsible for the multimerization of VWF dimers by interchain disulfide bonding (Mayadas and Wagner 1992). This hypothesis has offered a rationale for screening for mutations, even in those regions of the VWF gene that do not code for the mature VWF primary amino acid sequence but that could nevertheless cause an aberrant multimeric structure and defective biological activity of VWF. The detection of a homozygous candidate mutation in exon 14 of the pro-VWF gene sequence (D2 domain) in our patient with VWD type IIC is in accordance with these considerations. The mutation, G1898A, corresponds to a C→T transition on the antisense strand in a CpG dinucleotide that is considered to be a hot spot of mutation (Bird 1980). It results in an exchange of a glycine to an arginine, a neutral versus an alkaline amino acid. Although this mutation is located only in the neighborhood of the consensus sequence "521Cys-Gly-Leu-Cys524" that corresponds to the catalytic site of different protein disulfide isomerases (Mayadas and Wagner 1992), it could alter the hypothesized protein disulfide isomerase enzymatic activity of this region.

It has been suggested that the aberrant multimer subunit composition seen in type IIC VWD results from reduced susceptibility to the normally occurring proteolytic cleavage of the VWF after its release into the blood stream

(Zimmerman et al. 1986). Such reduced proteolytic processing may be caused by an abnormal multimer formation after the initial dimerization process. One can only speculate about the nature of this alteration, since the exact location of the sulfhydryl bonding in the cysteine-rich N-terminal region of VWF is still unknown. However, an altered specificity of the potential catalytic site in pro-VWF toward disulfide bonding of alternative cysteine residues is a possible explanation, such that a change in the location of the disulfide bonds at the N-terminals of VWF subunits could reduce the susceptibility of VWF to proteolysis. This is supported by the observation of an altered proteolytic cleavage pattern of the N-terminal portion of IIC VWF after treatment with V8 protease (Takahashi et al. 1988). An altered site for disulfide bonding could also explain the lack of high molecular weight multimers, the presence of the pronounced protomer, and the increased prevalence of smaller multimers in IIC VWF attributable to a less efficient multimerization process (Fig.).

The significance of G550R in exon 14 as a candidate mutation was substantiated by the negative results of our mutation screening in any ot the other 51 exons of the complete VWF gene sequence. Nevertheless, the applied methods cannot be regarded as 100% sensitive, and other mutations could have been missed. Additional evidence is however provided by the observed homozygosity for the mutation in our patient and by the negative results of a population screening of 100 unrelated individuals. Although the synopsis of our results speak in favor of G550R in exon 14 as the molecular defect causing VWD type IIC in our patient, screening for this mutation in additional VWD IIC patients and expression studies concerning the mutation are required. Some of these requirements have been fulfilled recently. During preparation of this manuscript, Gaucher et al. (1994) reported two different candidate mutations in two unrelated patients with VWD type IIC, both being located in exon 15 of the VWF propeptide DNA sequence, which is in close proximity to the mutation detected by us (exon 14). These mutations, a GCG insertion that results in an additional glycine at position 625 and a missense mutation (C2119G; numbering starting at the transcription cap site) that results in the substitution of tryptophan for cysteine at position 623 (C623W), are even more distant from the coding sequence for the presumed catalytic site (521Cys-Gly-Leu-Cys524) than the mutation detected by us (G1898A/G550R). The identification of these three mutations within a limited region of the VWF propeptide provides strong evidence that these defects cause VWD type IIC in our patient and the patients of Gaucher et al. (1994), respectively.

To date, VWD type IIC is the only structurally abnormal well-established subtype showing recessive inheritance, whereas all other common type II subtypes with abnormal electrophoretic multimer patterns are inherited in a dominant fashion. The observed homozygosity for G550R in our patient is in accordance with a recessive trait. Nevertheless, the patient's mother who is heterozygous for the candidate mutation had mild bleeding symptoms, whereas both of the patient's children carry the same heterozygous mutation but are unaffected. All of them could be diagnosed as heterozygotes by their abnormal function-to-antigen ratio of VWF and by the multimer analysis. This suggests the codominant inheritance of the VWD type IIC defect itself. Like the severe VWD type III, however, the characteristic phenotype of VWD type IIC, is most probably inherited in a recessive manner.

Acknowledgements The authors wish to thank A. Grohmann for technical assistance, and C. Mazurier for confirming our multimer patterns and for constructive comments and suggestions. We are also grateful to J.E. Sadler for information regarding the PCR amplification of exons 14 and 15, and H. Niederhoff for supplying us with part of the clinical data. This work was supported by grant Schn 325/2–1 of the Deutsche Forschungsgemeinschaft.

References

Armitage H, Rizza CR (1979) Two populations of factor VIII-related antigen in a family with von Willebrand's disease. Br J Haematol 41:279–289

Batlle J, Lopez Fernandez MF, Lasierra J, Fernandez Villamor A, Lopez Berges C, Lopez Borrasca A, Ruggeri ZM, Zimmerman TS (1986) Von Willebrand disease type IIC with different abnormalities of von Willebrand factor in the same sibship. Am J Hematol 21:177–188

Bird AP (1980) DNA methylation and the frequency of CpG in animal DNA. Nucleic Acids Res 8:1499–1504

Bowen DJ, Webb CE, Peake IR, Bloom AL (1991) *Aat*II polymorphism in von Willebrand factor gene at codon 471. Nucleic Acids Res 19:3159

Brown JE, Bosak JO (1986) An ELISA test for the binding of von Willebrand antigen to collagen. Thromb Res 43:303–311

Budowle B, Chakraborty R, Giusti AM, Eisenberg AJ, Allen RC (1991) Analysis of the VNTR locus DIS80 by the PCR followed by high resolution PAGE. Am J Hum Genet 48:137–144

Bulleid NJ, Freedman RB (1988) Defective cotranslational formation of disulphide bonds in protein disulphide-isomerase-deficient microsomes. Nature 335:649–651

Donner M, Holmberg L, Kristoffersson AC, Nilsson IM (1991) An *Hph*I-polymorphism in exon 28 of the von Willebrand factor gene, and its frequency among patients with various forms of von Willebrand's disease. Br J Haematol 78:403–407

Edman JC, Ellis L, Blacher RW, Roth RA, Rutter WJ (1985) Sequence of protein disulphide isomerase and implications of its relationship to thioredoxin. Nature 317:267–270

Fowler WE, Fretto LJ, Hamilton KK, Erickson HP, McKee PA (1985) Substructure of human von Willebrand factor. J Clin Invest 76:1491–1500

Gaucher C, Diéval J, Mazurier C (1994) Characterization of von Willebrand factor gene defects in two unrelated patients with type IIC von Willebrand disease. Blood 84:1024–1030

Ginsburg D, Sadler JE (1993) Von Willebrand disease: a database of point mutations, insertions, and deletions. For the Consortium on von Willebrand Factor Mutations and Polymorphisms, and the Subcommittee on von Willebrand Factor of the Scientific and Standardization Committee of the International Society on Thrombosis and Haemostasis. Thromb Haemost 69:177–184

Holmberg L, Dent JA, Schneppenheim R, Budde U, Ware J, Ruggeri ZM (1993) Von Willebrand factor mutation enhancing interaction with platelets in patients with normal multimeric structure. J Clin Invest 91:2169–2177

Kunkel GR, Graham JB, Fowlkes DM, Lord ST (1990) *Rsa*I polymorphism in von Willebrand factor (VWF) at codon 789. Nucleic Acids Res 18:4961

Kunkel GR, Graham JB, Fowlkes DM, Lord ST (1991) *Acc*I polymorphism in von Willebrand factor (F8VWF) at codon 516. Nucleic Acids Res 19:1729

Ledford MR, Rabinowitz I, Sadler JE, Kent JW, Civantos F (1993) New variant of von Willebrand disease type II with markedly increased levels of von Willebrand factor antigen and dominant mode of inheritance: von Willebrand disease type IIC Miami. Blood 82:169–175

Lowe T, Sharefkin J, Yang SQ, Dieffenbach CW (1990) A computer program for selection of oligonucleotide primers for polymerase chain reactions. Nucleic Acids Res 18:1757–1761

Macfarlane DE, Stibbe J, Kirby EP, Zucker MB, Grant RA, McPherson J (1975) Letter: a method for assaying von Willebrand factor (ristocetin cofactor). Thromb Diath Haemorrh 34: 306–308

Mancuso DJ, Tuley EA, Westfield LA, Worrall NK, Shelton Inloes BB, Sorace JM, Alevy YG, Sadler JE (1989) Structure of the gene for human von Willebrand factor. J Biol Chem 264: 19514–19527

Mancuso DJ, Tuley EA, Westfield LA, Lester Mancuso TL, Le Beau MM, Sorace JM, Sadler JE (1991) Human von Willebrand factor gene and pseudogene: structural analysis and differentiation by polymerase chain reaction. Biochemistry 30: 253–269

Maniatis T, Fritsch EF, Sambrook J (1982) Molecular cloning: a laboratory manual, Cold Spring Harbor Laboratory Cold Spring Harbor, NY

Mannucci PM, Lombardi R, Pareti FI, Solinas S, Mazzucconi MG, Mariani G (1983) A variant of von Willebrand's disease characterized by recessive inheritance and missing triplet structure of von Willebrand factor multimers. Blood 62:1000–1005

Mayadas TN, Wagner DD (1989) In vitro multimerization of von Willebrand factor is triggered by low pH. Importance of the propolypeptide and free sulfhydryls. J Biol Chem 264:13497–13503

Mayadas TN, Wagner DD (1992) Vicinal cysteines in the prosequence play a role in von Willebrand factor multimer assembly. Proc Natl Acad Sci USA 89:3531–3535

Mazurier C, Parquet Gernez A, Goudemand M (1977) Enzyme-linked immunoabsorbent assay of factor VIII-related antigen. Interest in study of von Willebrand's disease. Pathol Biol (Paris) 25:18–24

Mazurier C, Mannucci PM, Parquet Gernez A, Goudemand M, Meyer D (1986) Investigation of a case of subtype IIC von Willebrand disease: characterization of the variability of this subtype. Am J Hematol 22:301–311

Mercier B, Gaucher C, Mazurier C (1990) An *Msp*I polymorphism in the von Willebrand factor gene. Nucleic Acids Res 18:7467

Orita M, Iwahana H, Kanazawa H, Hayashi K, Sekiya T (1989) Detection of polymorphisms of human DNA by gel electrophoresis as single-strand conformation polymorphisms. Proc Natl Acad Sci USA 86:2766–2770

Peake IR, Bowen D, Bignell P, Liddell MB, Sadler JE, Standen G, Bloom AL (1990) Family studies and prenatal diagnosis in severe von Willebrand disease by polymerase chain reaction amplification of a variable number tandem repeat region of the von Willebrand factor gene. Blood 76:555–561

Ploos van Amstel HK, Reitsma PH (1990) Tetranucleotide repeat polymorphism in the VWF gene. Nucleic Acids Res 18:4957

Raines G, Aumann H, Sykes S, Street A (1990) Multimeric analysis of von Willebrand factor by molecular sieving electrophoresis in sodium dodecyl sulphate agarose gel. Thromb Res 60: 201–212

Ruggeri ZM (1987) Classification of von Willebrand disease. In: Verstraete M, Vermylen J, Lijnen R, Arnout J (eds) Thrombosis and haemostasis. Leuven University Press, Leuven pp 419–445

Ruggeri ZM, Zimmerman TS (1981) The complex multimeric composition of factor VIII/von Willebrand factor. Blood 57: 1140–1143

Ruggeri ZM, Nilsson IM, Lombardi R, Holmberg L, Zimmerman TS (1982) Aberrant multimeric structure of von Willebrand factor in a new variant of von Willebrand's disease (type IIC). J Clin Invest 70:1124–1127

Sadler JE (1994) A revised classification of von Willebrand disease. Thromb Haemost 71:520–525

Saiki RK, Gelfand DH, Stoffel S, Scharf SJ, Higuchi R, Horn GT, Mullis KB, Erlich HA (1988) Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. Science 239:487–491

Takahashi Y, Kalafatis M, Girma JP, Meyer D (1988) Abnormality of the N-terminal portion of von Willebrand factor in type IIA and IIC von Willebrand disease. Thromb Haemost 60:498–505

Thomas KB, Sutor AH, Zieger B, Jessat U, Budde U, Grohmann A, Choong SC, Tune EP (1994) Von Willebrand factor – collagen binding activity and ristocetin cofactor activity. A comparison of methods able to differentiate von Willebrand disease type I and II. Hämostaseologie 14:133–139

Verweij CL, Hart M, Pannekoek H (1987) Expression of variant von Willebrand factor (VWF) cDNA in heterologous cells: requirement of the pro-polypeptide in VWF multimer formation. EMBO J 6:2885–2890

Wagner DD (1990) Cell biology of von Willebrand factor. Annu Rev Cell Biol 6:217–246

Wise RJ, Pittman DD, Handin RI, Kaufman RJ, Orkin SH (1988) The propeptide of von Willebrand factor independently mediates the assembly of von Willebrand multimers. Cell 52:229–236

Zimmerman TS, Dent JA, Ruggerie ZM, Nannini LH (1986) Subunit composition of plasma von Willebrand factor. Cleavage is present in normal individuals, increased in IIA and IIB von Willebrand disease, but minimal in variants with aberrant structure of individual oligomers (type IIC, IID and IIE). J Clin Invest 77:947–951