Two novel type 2N von Willebrand disease–causing mutations that result in defective factor VIII binding, multimerization, and secretion of von Willebrand factor

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Two novel mutations, a T-to-C transition at nucleotide 2612 and a T-to-G transition at nucleotide 3923 of the von Willebrand factor (vWF) complementary DNA, were detected by analysis of the vWF gene in DNA from members of 2 families with atypical von Willebrand disease. The T2612C transition predicts substitution of cysteine by arginine at amino acid position 786 (C788R), and the T3923G transition predicts substitution of cysteine by glycine at position 1225 (C1225G) of pre-pro-vWF. The patients homozygous for the C788R and C1225G mutations both had a partial vWF deficiency (0.18 IU/mL and 0.07 IU/mL vWF antigen, respectively); vWF in plasma from patients homozygous for either the C788R or the C1225G mutation failed to bind factor VIII and lacked high molecular weight multimers. Recombinant (r) vWF molecules having the C788R or C1225G mutation were expressed in COS-7 cells. Both rVWF C788R and rVWF C1225G exhibited significantly impaired secretion and failed to bind factor VIII. Recombinant vWF C788R in COS-7 culture medium showed a severe reduction in high molecular weight multimers, whereas rVWF C1225G showed a very mild reduction in high molecular weight multimers when compared with wild-type rVWF. (Blood. 2000;95:2000-2007)

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S.A. and A.M.A. contributed equally to this work.

Introduction

Von Willebrand factor (vWF) is a large glycoprotein synthesized by endothelial cells and megakaryocytes that circulates in plasma as disulphide-linked multimers ranging in size from 5 × 10^5 to 20 × 10^5 dalton; vWF has two roles in hemostasis. First, it acts as a carrier for factor VIII, protecting it from proteolytic degradation in plasma. Second, vWF mediates platelet-subendothelium and platelet–platelet interactions at the site of vascular injury.1 The 178-kb gene encoding vWF has been localized to chromosome 12 and contains 52 exons. The 8.7-kb messenger RNA encodes a precursor of 2813 amino acids that includes a signal peptide (22 amino acids), a propeptide (741 amino acids), and the mature subunit (2050 amino acids).2,3 The domains of vWF are in the following order from N- to C-terminus: D1-D2-D’-D3-A1-A2-A3-D4-B1-B2-B3-C1-C2-CK.4 The functions of vWF have been located to specific domains. Regions essential for factor VIII binding reside in the D’ (amino acids 769 to 865 in pre-pro-vWF) and D (amino acids 866 to 1242 in pre-pro-vWF) domains.5,6

Mutations in the vWF gene resulting in quantitative deficiencies or qualitative abnormalities of vWF lead to von Willebrand disease (vWD). This is the most common bleeding disorder in humans. The current classification of vWD recognizes three types.7 Type 1 vWD is the most common form of the disease; it accounts for 70% of cases1,7 and is characterized by a partial quantitative deficiency of vWF and is subdivided into types 2A, 2B, 2M, and 2N.7 Type 2A and 2M variants show decreased platelet binding. In type 2A vWD, but not in type 2M vWD, this is associated with an absence of high molecular weight multimers. Type 2B variants have an increased affinity for platelet glycoprotein Ib. Type 2N vWD refers to variants that have a decreased affinity for factor VIII.8,9

This report describes the molecular defects underlying cases of atypical vWD in 2 different consanguineous families from Turkey. As with previously described cases of type 2N vWD, the vWF in plasma from both affected individuals failed to bind factor VIII. However, unlike the situation in classical type 2N vWD, both individuals had significantly reduced levels of plasma vWF and lacked high molecular weight multimers. Analysis of the vWF gene sequence in DNA from affected members of both families detected a novel mutation in each family, T2612C and T3923G, predicting substitution of cysteine residues by arginine and by glycine at amino acid positions 786 and 1225 in pre-pro-vWF, respectively.

Recombinant (r) vWF molecules having either the C788R or C1225G mutation were expressed in COS-7 cells. Both rVWF C788R and rVWF C1225G exhibited significantly impaired secretion and failed to bind factor VIII. Recombinant vWF C788R in COS-7 culture medium showed a severe reduction in high molecular weight multimers, whereas rVWF C1225G showed a very mild reduction in high molecular weight multimers when compared with wild-type rVWF.
Materials and methods

Materials

Plasma from a patient homozygous for the T791M mutation was kindly supplied by Dr C. Mazurier (Lille, France). Plasmid pSVH vWF1 containing the full-length human vWF complementary DNA (cDNA) was kindly supplied by Dr A. Inbal (Tel Aviv, Israel). Oligonucleotides were synthesized by means of an Applied Biosystems 394 DNA/RNA synthesizer (Foster City, CA). QAEX kits and Mega-plasmid purification kits were purchased from Qiagen (Crawley, UK). Seakem agarose HGT (P) was purchased from FMC BioProducts (Rockland, ME). All antibodies and antibody conjugates were purchased from Dako (Glostrup, Denmark). Nitrocellulose membranes were purchased from Biorad (Hertfordshire, UK). The SuperSignal–enhanced chemiluminescence kit was purchased from Pierce (Rockford, IL). Recombinant factor VIII was purchased from Baxter (Deerfield, IL). Coated factor VIII chromogenic assay kits were purchased from Quadratrace (London, UK), and deoxyxynucleotide triphosphates (dNTPs) were purchased from Pharmacia (Uppsala, Sweden). BACC2 DNA extraction kits were purchased from Nucleon Biosciences (Glasgow, UK). Thermosequenase cycle sequencing kits were purchased from Amersham International (Bucks, UK). The GeneEditor mutagenesis kit and plasmids pSP70 and pSP72 were purchased from Promega (Madison, WI). T4 polymerase and T4 DNA ligase were purchased from New England Biolabs (Hitchin, UK). T4 polynucleotide kinase was purchased from Helena Laboratories (Sunderland, UK). Taq DNA polymerase was purchased from Bioline (London, UK). Tissue-culture reagents were purchased from Gibco Life Technologies (Glasgow, UK). Promix was purchased from ICN (Oxford, UK). All other reagents were purchased from Sigma (Poole, UK).

Methods

Blood samples. Peripheral venous blood was collected from the two propositi, their parents, and their siblings in Turkey following informed consent. Citrated blood was centrifuged at 2500g for 10 minutes to separate plasma, which was stored at –70°C, and plasma was shipped to Aarhus, Denmark, where the laboratory assays for vWF antigen (vWF:Ag), ristocetin cofactor (RcoF), factor VIII:Ag, factor VIII:C and vWF subunit.10 Bleeding times were determined with the use of standard methods.10,11

Factor VIII coagulant assay. The affinity of vWF for factor VIII was assessed as described previously.13 Results were plotted as concentration of vWF:Ag (U/dL) against activity of bound factor VIII expressed as absorbance at 405 nm. Normal plasma pooled from 20 healthy individuals and plasma from an individual homozygous for the T791M mutation in vWF, which showed a complete absence of factor VIII binding,14 were used as control samples.

VIII binding assay. The affinity of vWF for factor VIII was assessed as described previously.13 Results were plotted as concentration of vWF:Ag (U/dL) against activity of bound factor VIII expressed as absorbance at 405 nm. Normal plasma pooled from 20 healthy individuals and plasma from an individual homozygous for the T791M mutation in vWF, which showed a complete absence of factor VIII binding,14 were used as control samples.

Materials

Termiology. We use vWF to refer to the complete pre-pro- vWF protein in relation to the numbering of amino acids. Thus, C788 and C1225 are C25 and C462 of the mature vWF subunit, respectively. This scheme is adopted to allow for numbering of mutations throughout the pre-pro-vWF product in this and subsequent publications from our group. We also use this system to refer to mutations previously reported by other groups and indicate the numbering previously used in these publications for the mature vWF subunit.

Mutation screening. Genomic DNA was extracted from citrated blood with the use of the BACC2 DNA extraction kit and the protocol supplied by the manufacturer. DNA corresponding to the 2.2-kb promoter (nucleotides 1 through 2181)12 and exons 1 through 52, including exon/intron boundaries of the vWF gene, was amplified by means of the polymerase chain reaction (PCR). Apart from the promoter and exons 9, 10, 15, 26, and 28, which were amplified with the use of the primers indicated (Table 1), the fragments were amplified with the use of the oligonucleotide primers previously designed by Zhang et al.15,16 PCRs contained 0.5 μg of genomic DNA diluted in a final volume of 50 μL containing 200 μmol/L dNTPs, 300 ng of each primer, 1 U Taq DNA polymerase, 67 mmol/L Tris/HCl pH 8.8, 16.6 mmol/L (NH₄)₂SO₄, 10 mmol/L β-mercaptoethanol, 100 μg/mL bovine serum albumin (BSA), and 1.5 to 4.0 mmol/L MgCl₂. Samples were heated at 94°C for 1 minute and then subjected to 35 cycles of denaturation at 94°C for 1 minute, annealing at 50°C to 60°C for 30 seconds, and extension at 72°C for 30 seconds.

The variable number tandem repeats, VNTR-1 and VNTR-2, in intron 40 of the vWF gene were amplified and analyzed as described previously.17-19 Following PCR amplification of exons 36 through 52, chemical cleavage mismatch analysis (CCMA) was used to screen fragments for the presence of mutations in the vWF gene.20 CCMA was then replaced by a more rapid screening method: conformation-sensitive gel electrophoresis (CSGE), which does not require a radioactive probe. CSGE was used to analyze exons 1 through 35 and the promoter, as described previously.13

DNA sequencing. Following electrophoresis in 1% agarose, amplified DNA fragments were purified with the use of a QAEX kit and the protocol supplied by the manufacturer. Sequence analysis of amplified DNA fragments and plasmid DNA was performed with the use of a Thermosequenase cycle sequencing kit with 32P end-labeled PCR primers as directed by the manufacturer. Automated sequencing of plasmid DNA was performed with the use of an Applied Biosystems DNA sequencer (model 373).

Plasmid construction. Plasmid pSVH vWF1 contains full-length wild-type human vWF cDNA cloned into the expression vector pSV7D as described previously.22 Plasmid pSVH/WFC788R contained a T-to-C transition at nucleotide 2612 of the vWF cDNA predicted to result in substitution of cysteine by arginine at amino acid 788 in pre-pro-vWF. Plasmid pSVH/WFC788R was constructed by mutagenesis of a shuttle vector pSP70/BglII, which contains a 4.7-kb fragment (nucleotides 2248 through 6936) of the vWF cDNA obtained by BglII digestion of pSVH/WFC1 by sequence analysis. The mutated fragment was subcloned into the BglII sites of pSVH/WFC1 to obtain pSV vWF C788R. The presence of the appropriate mutation in pSV-WFC788R was confirmed again by sequence analysis.

Plasmid pSVvvF/C1225G contained a T-to-G transversion at nucleotide 3923 of the vWF cDNA, predicting the substitution of cysteine by glycine at amino acid 1225 in pre-pro-vWF. We constructed pSVvF/WFC1225G by PCR amplification of the vWF cDNA at the site of mutation using a mutagenic 59mer oligo: 5′-TTGGGCTCAGGAGAAAGAGTGACTACCTGTAATCCAGTGGCTACCCCAAGGTCG-3′ (nucleotides 3865 through 3924 in the vWF cDNA) in combination with the oligonucleotide 2B3: 5′-ACTTCACTCGTGGGACCATGAAGGGCTCGAGCGTACCAAAACGGTGC3′ (reverse primer complementary to nucleotides 3974 through 3993 in the vWF cDNA). The resulting 431-bp PCR product was sequenced to confirm the presence of the T3923G transition, then digested with BsrUI and TbaI11I, and subcloned into shuttle vector pSP72BA, which contains a 2-kb fragment (nucleotides 2817 through 4852) of the vWF cDNA obtained by digestion of psVH...
vWF1 with BamHI and Asp718. The fragment obtained by digestion of the resulting plasmid, pSP72BA-T3923G, with BamHI and Asp718 was then subcloned into the BamHI and Asp718 sites of pSBgII, and finally the BglII fragment was subcloned into the BglII sites of pSVHvWF1 to obtain pSVvWF1C1225G. The presence of the appropriate mutation in pSVvWF1C1225G was confirmed again by sequence analysis.

Expression of wild-type pSVHvWF1, pSVvWF1C788R, and pSVvWF1C872S. COS-7 cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) (Gibco Life Technologies) supplemented with 10% fetal bovine serum (FBS). Cells, at 60% confluence, in 10-cm dishes were washed twice with serum-free DMEM and incubated in the same medium containing 0.4 mg/mL DEAE-dextran and 3 µg/mL of the appropriate plasmid DNA (15 µg plasmid DNA per dish). Where wild-type vWF was coexpressed with mutant vWF, 7.5 µg wild-type and 7.5 µg mutant plasmid DNA were used per dish. The cells were then washed 3 times with DMEM containing 10% FBS and incubated in the same medium containing 100 µmol/L chloroquine for 3 hours. Following 3 further washes with DMEM containing 10% FBS, the cells were incubated in the same medium prior to analysis as described below.

Steady-state analysis of von Willebrand factor secretion. For steady-state analysis of vWF secretion, the COS-7 cells were washed 3 times in serum-free DMEM 24 hours posttransfection and incubated for a further 48 hours in 5 mL of serum-free DMEM supplemented with 0.5% BSA and a 1% insulin/transferrin/selenium supplement. The medium was then removed, and the cells were washed twice with ice-cold PBS before lysis with 0.75 mL of ice-cold Triton lysis buffer on ice for at least 1 hour. Lysates were adjusted to 1 mL with lysis buffer and then centrifuged for 15 minutes at 12 000g to pellet nuclei and cell debris. To each 5 mL medium sample, 0.5 mL of 10% concentrated Triton lysis buffer was added.

Immunoprecipitation of von Willebrand factor. Cell lysates (1 mL) and medium supernatants (5 mL) were precleared with 100 µL and 200 µL of protein-A sepharose (10% wt/vol in PBS), respectively, at 4°C for 1 hour to preclear the samples of protein-A binding components. Precleared samples were then incubated overnight at 4°C with 100 µL (cell lysates) or 200 µL (medium supernatants) of protein-A sepharose and 5 µL (cell lysates) or 20 µL (medium supernatants) of rabbit anti-human vWF polyclonal antibody. The sepharose beads were pelleted, washed 4 times with Triton lysis buffer, and then prepared for analysis by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) as described below.

SDS–polyacrylamide gel electrophoresis. Immunoprecipitation sepharose beads were resuspended in 100 µL of SDS-PAGE sample buffer (0.25 mmol/L Tris-HCl, pH 6.8, containing 2% wt/vol SDS, 20% vol/vol glycerol, 50 mmol/L DTT and 0.004% wt/vol bromphenol blue). The samples were boiled for 5 minutes prior to electrophoresis. Cooled samples were loaded onto 6% SDS-polyacrylamide gels with 3% stacking gels for electrophoresis. Electrophoresis was performed overnight for 18 hours at room temperature at a constant current of 7 mA until the bromphenol blue dye reached the bottom of the gel. Gels were fixed in methanol/acetic acid:water (1:1:9) for 1 hour, then dried under vacuum, and subjected to autoradiography with the use of Kodak Biomax film for 48 hours.

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**Table 1. Primers used for vWF gene amplification**

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<td>55</td>
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</table>

* A, C, F, G, and As are forward primers. B, E, D, H, and Bs are reverse primers.
†Location of the 5′ nucleotides are from genomic sequence data of the vWF gene.2

**Pulse-chase analysis of von Willebrand factor secretion.** For pulse-chase analysis of vWF secretion, at 48 hours posttransfection, COS-7 cells were washed twice with cysteine- and methionine-free DMEM and pre-incubated with the same medium for 1 hour. The cells were then incubated for 20 minutes with 250 µCi of Promix [35S]-cysteine and [35S]-methionine mixture per 10-cm dish in 5 mL of cysteine- and methionine-free DMEM. Labeling medium was removed, and the cells were incubated for various times up to 96 hours with 5 mL of chase medium consisting of serum-free DMEM supplemented with 0.5% BSA and a 1% insulin/transferrin/selenium supplement. The medium was then removed, and the cells were washed twice with ice-cold PBS before lysis with 0.75 mL of ice-cold Triton lysis buffer on ice for at least 1 hour. Lysates were adjusted to 1 mL with lysis buffer and then centrifuged for 15 minutes at 12 000g to pellet nuclei and cell debris. To each 5 mL medium sample, 0.5 mL of 10% concentrated Triton lysis buffer was added.
Results

Patient histories and phenotypic results

The propositus from family A (AII:2, Figure 1A[i]) was a 5-year-old Turkish girl with a history of easy bruising and a moderate bleeding tendency. On 2 occasions, bleeding persisted for 4 days after tooth extractions. The parents of the propositus (AI:1 and AI:2, Figure 1A[i]) were related. The father (AI:1) had prolonged bleeding following circumcision but has had no bleeding episodes since then. Menorrhagia was reported in the mother (AI:2). The phenotypic data for family A is summarized in Table 2. The propositus (AII:2) had a prolonged bleeding time, and her plasma showed reduced vWF:Ag (0.18 IU/mL), factor VIII:Ag (0.09 IU/mL), and RCoF (0.1 IU/mL) levels.

The propositus from family B (BII:1, Figure 1B[i]) was a 7-year-old Turkish girl with severe bleeding symptoms, which included frequent epistaxis, spontaneous gum bleeding, and easy bruising. The phenotypic data for family B is summarized in Table 2. The propositus had a prolonged bleeding time, reduced vWF:Ag (0.07 IU/mL), factor VIII:Ag (0.24 IU/mL), and RCoF (< 0.1 IU/mL) levels. Both parents, who were related (BI:1 and BI:2, Figure 1B), and another sibling (BII:2) had mild bleeding symptoms.

Multimer analysis of plasma von Willebrand factor

Multimer analysis of equal volumes (2 µL) of plasma from the family members of family A (Figure 1A[ii], lanes 1-4) and family B (Figure 1B[ii], lanes 1-4) failed to detect multimers in plasma from the propositi of family A (AII:2, Figure 1A[i], lane 1) and family B (BII:1, Figure 1B[ii], lane 1). All sizes of vWF multimers were detected in plasma from other family members (AI:2 and AII:1, Figure 1A[ii], lanes 2 and 3) (BI:2 and BII:2, Figure 1B[ii], lanes 2 and 3). To determine whether failure to detect multimers in plasma from the two propositi was due to the low concentration of vWF in the plasma or an absence of vWF multimers, we analyzed an appropriate volume of plasma that contained an identical number of units (0.5 mU) of vWF compared with normal plasma (Figure 1A[ii] and Figure 1B[ii], lanes 5 and 6). Only multimers of low molecular weight were detectable in plasma from the propositi of family A (AII:2, Figure 1A[ii], lane 5) and family B (BII:1, Figure 1B[ii], lane 5).

Factor VIII binding analysis of plasma von Willebrand factor

The results of factor VIII binding analysis performed on plasma samples from the propositi of families A and B are shown in Figures 1A(iii) and 1B(iii); vWF in plasma from the propositus AII:2 (Figure 1A[iii]) failed to bind factor VIII similar to plasma vWF from a patient known to be homozygous for the type 2N mutation T791M. A moderate reduction in the factor VIII binding capacity of plasma vWF was shown for the sibling of AII:2 (AII:1, Figure 1A[iii]). The vWF in plasma from the propositus BI:1 (Figure 1B[iii]) showed a severe reduction in factor VIII binding, which was only slightly increased compared with plasma vWF from the patient homozygous for the T791M mutation.

Figure 1. (i) Pedigrees of family A and family B. Squares denote males; circles denote females. In family A (Ai), the intron 40 VNTR-1 is shown as number of ATCT repeat units.17,18 In family B (Bi), the intron 40 VNTR-2 is shown as number of TCTA repeat units.19 (ii) Multimer analysis of vWF from plasma of members of family A and family B. Plasma samples were electrophoresed on 2% (A[ii], lanes 1-6; B[ii], lanes 5 and 6) or 3% (B[ii], lanes 1-4) SDS-agarose gels. Lanes 1 through 4 of A(ii) and B(ii) contain equal volumes of plasma (2 µL). Lanes 5 and 6 of A(ii) and B(ii) contain the appropriate volume of plasma containing 0.5 mU vWF as determined by ELISA. The vWF was transferred onto nitrocellulose by electroblotting. Lanes 1 through 4 of A(ii) and B(ii) were developed with the use of polyclonal rabbit anti-human vWF antibody and a secondary alkaline phosphatase-conjugated swine anti-rabbit IgG polyclonal antibody and colorimetric staining. Lanes 5 and 6 of A(ii) and B(ii) were developed with the use of rabbit anti-human vWF polyclonal antibody and a secondary horseradish peroxidase–conjugated swine anti-rabbit IgG polyclonal antibody and enhanced chemiluminescence. NP denotes plasma pooled from 20 normal individuals. (iii) Factor VIII binding to vWF from plasma of members of family A and family B. Mouse anti-vWF monoclonal RFF-VIII R/1 was used to coat the wells of a 96-well microtitre plate. Serial dilutions of patient plasma of known vWF:Ag (U/dL) were incubated in the wells. Then 5 mU of recombinant factor VIII was added, and the amount of factor VIII bound was determined by means of a chromogenic assay. Factor VIII binding expressed as absorbance at 405 nm was plotted against the concentration of vWF. Plasma from a patient with no factor VIII binding to vWF owing to homozygosity for the type 2N vWD mutation T791M.
Candidate mutations C788R and C1225G

CSGE and CCMA were used to analyze all exons, exon/intron boundaries, and the promoter of the vWF gene in DNA from affected members of both families. In family A, CSGE analysis detected a mutation in amplified DNA corresponding to exon 18, which on sequencing was shown to have a T-to-C transition at position 2612 of the vWF cDNA, predicting substitution of cysteine by arginine at amino acid 788 in pre-pro-vWF. The propositus (AII:2) was homozygous for the T2612C transition. The father (AI:1), mother (AI:2), and sibling (AII:1) were heterozygous for the same defect. In all cases, the mutation was inherited with the 6 repeat allele of the intron 40 VNTR-1.

In family B, CSGE analysis detected a mutation in amplified DNA corresponding to exon 27, which on sequencing was shown to have a T-to-G transversion at position 3923 of the vWF cDNA, predicting substitution of cysteine by glycine at amino acid 1225 in pre-pro-vWF. The propositus (BII:1) was homozygous for the T3923G transition. The father (BI:1), mother (BI:2), and sibling (BII:2) were heterozygous for the same defect. In all cases, the mutation was inherited with the 4 repeat allele of the intron 40 VNTR-2.

Steady-state analysis of recombinant von Willebrand factor C788R and recombinant von Willebrand factor C1225G secretion

The T2612C (C788R) and T3923G (C1225G) mutations were introduced into the full-length vWF cDNA in the expression vector pSVH vWF1. To investigate the synthesis and secretion of vWF containing these substitutions, the expression vectors pSVvWF C788R and pSVvWF C1225G were used to transfect COS-7 cells either alone or in combination with pSVHvWF1 (wild-type) to generate mutant and wild-type hybrids. Intracellular rvWF in the cell lysates and secreted rvWF in the conditioned media of the mutated vWF transfectants were quantified by ELISA and compared with that of wild-type vWF (Figure 2). Secretion of rvWF C788R and rvWF C788R/wild-type hybrid were decreased to 41% ± 9% and 67% ± 5%, respectively, relative to wild-type rvWF. Secretion of rvWF C1225G and rvWF C1225G/wild-type hybrid were decreased to 58% ± 3% and 74% ± 10, respectively, relative to wild-type rvWF. The intracellular levels of rvWF C788R and rvWF C788R/wild-type hybrid were 107% ± 8% and 97% ± 3%, respectively, relative to wild-type rvWF. The intracellular levels of rvWF C1225G and rvWF C1225G/wild-type hybrid were 102% ± 4% and 96% ± 1, respectively, relative to wild-type rvWF (Figure 2).

Pulse-chase analysis of recombinant von Willebrand factor C788R and recombinant von Willebrand factor C1225G secretion

A pulse-chase approach was adopted to further investigate the variation in steady-state levels of secreted rvWF having the C788R and C1225G amino acid substitutions. Transfected cells were pulse-labeled for 20 minutes and then chased for various periods of time in unlabeled growth medium. The labeled vWF in cell lysates and medium samples was immunoprecipitated and analyzed by SDS-PAGE and autoradiography (Figure 3). Wild-type rvWF was detected in the medium after 2 hours, and all of the wild-type rvWF was chased out of the cells between 48 and 96 hours as shown previously. Immediately after the pulse (0 hours of chase period, Figure 3), identical amounts of rvWF C788R and rvWF C1225G were immunoprecipitated from the cell lysates compared with the wild-type rvWF (Figure 3). This indicated that amounts of rvWF C788R and rvWF C1225G were synthesized. Both rvWF C788R and rvWF C1225G were detected in the medium after 2 hours. However, over the course of the 96-hour chase, the rates of secretion of both variants were significantly reduced. None of the radiolabeled rvWF C788R or rvWF C1225G remained in the cells between 48 and 96 hours. These results indicate that both rvWF C788R and rvWF C1225G underwent impaired intracellular transport and secretion.

Factor VIII binding analysis of recombinant von Willebrand factor C788R and recombinant von Willebrand factor C1225G

To determine if the C788R and C1225G amino acid substitutions alone could account for the lack of binding of vWF to factor VIII, medium supernatants from the steady-state secretion analysis were assayed for the ability of the rvWFs to bind factor VIII (Figure 4). Wild-type rvWF showed a dose-dependent increase in binding of factor VIII similar to that of vWF from normal pooled plasma (Figure 4). However, rvWF C788R (Figure 4A) failed to bind factor VIII like plasma vWF from a patient known to be

Table 2. Phenotypic data for family A and family B

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<td>AI:2</td>
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<td>Bleeding time (min)</td>
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<td>4</td>
<td>10</td>
<td>&lt;8</td>
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<tr>
<td>vWF:Ag (IU/mL)</td>
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<td>0.76</td>
<td>0.57</td>
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<td>Factor VIII:Ag (IU/mL)</td>
<td>0.42</td>
<td>0.79</td>
<td>0.44</td>
<td>0.09</td>
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<tr>
<td>Factor VIII:C (IU/mL)</td>
<td>0.47</td>
<td>0.60</td>
<td>0.43</td>
<td>0.15</td>
<td>0.52-1.40</td>
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<tr>
<td>RCoF (IU/mL)</td>
<td>0.52</td>
<td>0.78</td>
<td>0.84</td>
<td>0.10</td>
<td>0.5-1.72</td>
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<tr>
<td>Bleeding time (min)</td>
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<td>nd</td>
<td>&gt;20</td>
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<tr>
<td>vWF:Ag (IU/mL)</td>
<td>0.82</td>
<td>0.72</td>
<td>0.07</td>
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<td>Factor VIII:Ag (IU/mL)</td>
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<td>0.60</td>
<td>0.24</td>
<td>0.71</td>
<td>1.0 (mean value)</td>
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<tr>
<td>Factor VIII:C (IU/mL)</td>
<td>0.78</td>
<td>0.60</td>
<td>0.22</td>
<td>nd</td>
<td>0.52-1.40</td>
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<tr>
<td>RCoF (IU/mL)</td>
<td>0.74</td>
<td>0.54</td>
<td>&lt;0.1</td>
<td>0.35</td>
<td>0.5-1.72</td>
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homozygous for the type 2N mutation T791M (Figure 4). Similarly, rvWF C1225G (Figure 4B) showed a severe reduction in its ability to bind factor VIII, which was only slightly increased compared with vWF from the patient homozygous for the T791M mutation (Figure 4). The rvWF C788R/wild-type hybrid (Figure 4A) showed a dose-dependent increase in binding of factor VIII similar to that of vWF from normal pooled plasma and wild-type rvWF. In contrast, rvWF C1225G/wild-type hybrid (Figure 4B) exhibited a moderate reduction in factor VIII binding.

Multimer analysis of recombinant von Willebrand factor C788R and recombinant von Willebrand factor C1225G

To determine the effects of the individual C788R and C1225G amino acid substitutions on vWF multimer structure, we used vWF expressed in COS-7 cells. The COS-7 medium samples containing wild-type rvWF, rvWF C788R, rvWF C1225G, and hybrids of mutant and wild-type vWF were separated by SDS-agarose electrophoresis (Figure 5). The wild-type rvWF (Figure 5, lane 1) exhibited a full range of multimers. The rvWF C788R/wild-type hybrid (Figure 5, lane 2) showed a multimer pattern similar to that of wild-type rvWF from normal pooled plasma and wild-type rvWF. In contrast, rvWF C1225G/wild-type hybrid (Figure 5, lane 3) and rvWF C1225G (lane 5) showed very mild reductions in high molecular weight multimers compared with wild-type rvWF (lane 1), which were observed on repeated occasions. In contrast, rvWF C788R (lane 4) showed only low molecular weight multimers.

Discussion

In the classification of vWD,7 type 2N vWD is defined as a variant of vWD associated with defective binding of vWF to factor VIII. Type 1 vWD is defined as a quantitative form of the disease associated with reduced vWF:Ag level and normal binding of vWF to factor VIII. We report here 2 novel cases of atypical type 2N vWD in 2 families initially classified as having type 1 vWD owing to the quantitative deficiency of vWF:Ag in plasma from affected members of both families. The binding of plasma vWF to factor VIII was completely absent in the individual homozygous for the C788R mutation and virtually absent in the individual homozygous for the C1225G mutation. Similarly, the binding of rvWF C788R to factor VIII was completely absent, and the binding of rvWF C1225G to factor VIII was severely reduced.

The novel C788R and C1225G mutations presented here were detected after screening all exons, exon/intron boundaries, and the promoter of the vWF gene for nucleotide changes. Confirmation that these mutations were directly responsible for the phenotypes observed in the patients was provided when expression in vitro of these single-point mutations resulted in rvWFs‘ having phenotypic characteristics that matched those of the patient vWFs. Both mutations have pleiotropic effects on vWF as they affect the level of vWF secreted, the binding of vWF to factor VIII, and the ability of vWF to form multimeric structures. A recent study25 reported a similar mutation resulting in the substitution in the D’ domain, C788Y, of pre-pro-vWF (C25Y in mature vWF), causing type 2N vWD and abnormal vWF...
multimers whereas plasma vWF exhibits an abnormal multimer profile. This has been explained by the increased sensitivity to proteolytic cleavage in plasma. The difference in multimer pattern of rWF C1225G in vitro and the multimer pattern observed in the plasma from the propositus from family B may suggest a similar effect on vWF stability in plasma, resulting from the C1225G mutation.

Although no formal proof is provided here that C788 is essential for multimerization by direct involvement in interchain disulfide bond formation, it may be that conformational change brought about by its substitution with arginine is enough to disrupt the normal folding of the D' domain, possibly by disruption of interchain disulfide bonding in this domain. This, in turn, may significantly lower the efficiency of interchain disulfide bond formation. As well as showing loss of a normal multimer profile, the C788R mutant undergoes a partial reduction in level of secretion like the C1225G mutant. In both cases, this may arise owing to the effect of misfolding of the domains in which they occur, leading to prolonged interaction with the quality-control apparatus of the endoplasmic reticulum. The C788R and C1225G mutations may lower the efficiency of folding of monomeric subunits to conformations that are necessary for the formation of endoplasmic reticulum to Golgi transport competent dimers. The misfolding induced in a number of other mutated proteins associated with inherited human diseases is often followed by binding to the molecular chaperones of the endoplasmic reticulum, such as calnexin and BiP. As well as playing a role in retention of normal immature proteins in the endoplasmic reticulum, molecular chaperones have been shown to be involved in the retrograde transport of misfolded mutant proteins from the endoplasmic reticulum into the cytosol, where the misfolded proteins are destroyed by the proteasome complex. Previouly, BiP has been shown to interact with type 2A vWD-causing mutants, which are associated with impaired intracellular transport and endoplasmic reticulum retention. We anticipate that future studies in this area will provide a better understanding of the role of resident secretory-compartment proteins in the pathway of vWF biosynthesis. In turn, this should provide insight into the molecular basis of vWD resulting from defects in secretion.

Until recently, 7 of the 8 reported missense mutations associated with type 2N vWD were located in the D' domain. However, a recent study reported a mutation in the D3 domain, D879N in pre-pro-vWF (position 116 in mature vWF) that resulted in decreased factor VIII binding. Evidence that the D879N mutation induced a conformational change in the D' domain was obtained by means of antigen-capture experiments with a monoclonal antibody that recognized an epitope in the D' domain (epitope amino acids 829 through 839 in pre-pro-vWF). Like the D879N mutation, the C1225G mutation occurs within the D3 domain and causes type 2N vWD. Because of the close proximity of this mutation to the epitope recognized by the monoclonal antibody (RFF-VIII R/1) used in our factor VIII binding assay, we reasoned that the apparent lack of factor VIII binding by rWF C1225G and vWF in plasma from the patient homozygous for the C1225G mutation may have been due to failure of vWF to bind to the monoclonal antibody rather than to factor VIII. To investigate this possibility, we performed antigen-capture experiments that compared the ability of the monoclonal antibody to bind wild-type rWF and rWF C1225G. There was no difference in the ability of the monoclonal antibody to bind wild-type rWF and rWF C1225G.
the monoclonal antibody to bind wild-type rVWF and rVWF C1225G, and there was no difference in the ability of rVWF C1225G to bind either the monoclonal antibody or the polyclonal antibody used for ELISA determination of vWF antigen (results not shown). We therefore concluded that the C1225G mutation was responsible for the reduced factor VIII binding of plasma VWF in the patient.

In conclusion, we have identified 2 novel type 2N vWD-associated vWF mutations, C788R and D879N, that, in addition to causing defective factor VIII binding, also result in mild quantitative deficiencies and abnormal multimers.

Acknowledgments

We thank Dr. C. Mazurier (Lille, France) for supplying the plasma from a patient homozygous for the T791M mutation, Dr. A. Inbal (Tel Aviv, Israel) for providing pSVH vWF1, Barbara Sampson (Sheffield, UK) for advice and assistance with the mutliner analysis, and Hazel Holden (Sheffield, UK) for oligonucleotide synthesis and automated sequencing of mutated plasmid DNA.

References

Two novel type 2N von Willebrand disease–causing mutations that result in defective factor VIII binding, multimerization, and secretion of von Willebrand factor

Simon Allen, Adel M. Abuzenadah, Joanna L. Blagg, Joanna Hinks, I. Mandy Nesbitt, Anne C. Goodeve, Turkiz Gursel, Jørgen Ingerslev, Ian R. Peake and Martina E. Daly