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) gene, were identified 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 788 (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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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 × 10^6 d; 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. 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). The domains of vWF are in the following order from N- to C-terminus: D1-D2-D3-A1-A2-A3-D4-B1-B2-B3-C1-C2-CK. The functions of vWF have been located to specific domains. Regions essential for factor VIII binding reside in the D1 domain (amino acids 769 to 865 in pre-pro-vWF) and D3 domain (amino acids 866 to 1242 in pre-pro-vWF) domains.

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. Type 1 vWD is the most common form of the disease; it affects about 70% of cases and is characterized by a partial quantitative deficiency of vWF and normal multimers. Type 3 vWD occurs in 1 to 2 individuals per million and refers to a complete deficiency of vWF. Type 3 vWD is generally inherited in an autosomal recessive manner. Type 2 vWD refers to qualitative deficiency of vWF and is subdivided into types 2A, 2B, 2M, and 2N. 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.

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 788 and 1225 of 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 a severe impairment in its ability to form high molecular weight multimers. In contrast, rVWF C1225G showed a very mild reduction in its ability to form high molecular weight multimers 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). Seadgen agarose (HCT15p) was purchased from FMC BioProducts (Rockland, ME). All antibodies and antibody conjugates were purchased from Dako (Glostrup, Denmark). Monoclonal mouse anti-human vWF RFF-VIII R/I, which was purchased from Harlan Sera-Lab (Copthorne, UK). Nitrocellulose membranes were purchased from Biorad (Hertfordshire, UK). The Super Signal–enhanced chemiluminescence kit was purchased from Pierce (Rockford, IL). Recombinant factor VIII was purchased from Baxter (Deerfield, IL). Coats factor VIII chromogenic assay kits were purchased from Coatest (Hitchin, UK). T4 polynucleotide kinase was purchased from Helena International (Bucks, UK). Thermo sequence 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 (Glaskow, 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 2500 g for 10 minutes to separate plasma, which was stored at −70°C, and platelets. Blood samples and plasma were shipped to Aarhus, Denmark, where the laboratory assays for vWF antigen (vWF:Ag), ristocetin cofactor (RcoF), factor VIII:Ag, factor VIII:C and colorimetric multimer analysis were performed. Blood samples and plasma were then shipped to Sheffield, UK, where the mutation detection, chemiluminescent multi assay, and factor VIII-binding analysis were performed. All subsequent recombinant expression experiments, including laboratory assays, chemiluminescent multimer analysis, factor VIII–binding, and pulse-chase analysis, were performed in Sheffield, UK.

Phenotypic analysis and laboratory assays. Factor VIII coagulant activity (factor VIII:C), factor VIII antigen (FVIII:Ag), vWF antigen (vWF:Ag) and vWF ristocetin cofactor (RcoF) assays were performed with the use of the standard methods.10,11 Bleeding times were determined with the use of a Simplate-II device (General Diagnostics, Morris Plains, NJ). Phenotypic analysis and laboratory assays. Factor VIII coagulant activity (factor VIII:C), factor VIII antigen (FVIII:Ag), vWF antigen (vWF:Ag) and vWF ristocetin cofactor (RcoF) assays were performed with the use of the standard methods.10,11 Bleeding times were determined with the use of a Simplate-II device (General Diagnostics, Morris Plains, NJ).

FVIII binding assay. The affinity of vWF for factor VIII was assessed as described previously.12 Results were plotted as concentration of vWF:Ag (μU/mL) 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,13 were used as control samples.

Terminology. 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 BACCC2 DNA extraction kit and the protocol supplied by the manufacturer. DNA corresponding to the 2.2-kb promoter (nucleotides 1 through 2181)14 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.5,16 PCR products contained 0.5 ng 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° 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.17,18 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.19 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.20

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 Thermo sequence cycle sequencing kit with 3’P-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.21 Plasmid pSVWVFC788R contained a T-to-G transversion at nucleotide 788 of the vWF gene.22 pSVWVFC1225G contained a T-to-C transversion at nucleotide 2252 of the vWF cDNA predicted to result in substitution of cysteine by arginine at amino acid 788 in pre-pro-vWF. PSV vWF C788R was confirmed again by sequence analysis of amplified DNA and plasmid DNA was performed with the use of a Thermo sequence cycle sequencing kit with 3’P-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).

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Plasmid construction. Plasmid pSVWVFC1225G contained a T-to-G transversion at nucleotide 2252 of the vWF cDNA predicted to result in substitution of cysteine by arginine at amino acid 788 in pre-pro-vWF. PSV vWF C788R was constructed by mutagenesis of a shuttle vector pSP70BgI by PCR, which contains a 4.7-kb fragment (nucleotides 2248 through 6936) of the vWF cDNA obtained by BglI digestion of pSVWVWF cloned into pSP70. Mutagenesis was performed by means of the GeneEditor system and the mutagenic oligonucleotide: 5’GAAAGGCTCAGAGCTCAACAAACGTG3’ (nucleotides 2600 through 2625 in the vWF cDNA). Clones containing the appropriate mutation were confirmed by sequence analysis. The mutated fragment was subcloned into the BglI sites of pSVWVWF1 to obtain pSV vWF C788R. The presence of the appropriate mutation in pSVWVFC788R was confirmed again by sequence analysis.

Plasmid pSVWVFC1225G contained a T-to-G transversion at nucleotide 2252 of the vWF cDNA predicted to result in substitution of cysteine by arginine at amino acid 788 in pre-pro-vWF. PSV vWF C788R was constructed by mutagenesis of a shuttle vector pSP70 by PCR, which contains a 4.7-kb fragment (nucleotides 2248 through 6936) of the vWF cDNA obtained by BglI digestion of pSVWVWF1 to obtain pSV vWF C788R. The presence of the appropriate mutation in pSVWVFC788R was confirmed again by sequence analysis.

Plasmid pSVWVFC1225G contained a T-to-G transversion at nucleotide 2252 of the vWF cDNA predicted to result in substitution of cysteine by arginine at amino acid 788 in pre-pro-vWF. PSV vWF C788R was constructed by mutagenesis of a shuttle vector pSP70 by PCR, which contains a 4.7-kb fragment (nucleotides 2248 through 6936) of the vWF cDNA obtained by BglI digestion of pSVWVWF1 to obtain pSV vWF C788R. The presence of the appropriate mutation in pSVWVFC788R was confirmed again by sequence analysis.
vWF 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 pSBPb/I1, and finally the BglII fragment was subcloned into the BglII sites of pSHvWF1 to obtain pSVvWF1225G. The presence of the appropriate mutation in pSVvWF1225G was confirmed again by sequence analysis.

Expression of wild-type pSVHvWF1, pSVvWF1C788R, and pSVvWF1C1225G. COS-7 cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS). Cells, at 60% confluence, in 10-cm dishes were washed twice with serum-free DMEM 24 hours posttransfection and incubated for a further 48 hours 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, COS-7 cells were washed three 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 then incubated at 4°C with lysis buffer and 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.

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 then adjusted to 1 mL with lysis buffer and 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 preincubated with 100 µL and 200 µL of protein-A sepharose (10% wt/vol in PBS), respectively, at 4°C for 1 hour to preclarify the samples of protein-A binding components. Preclarified 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 bromophenol 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 bromophenol 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.

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

<table>
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<tr>
<th>Exon Number</th>
<th>Primer Name*</th>
<th>Location†</th>
<th>Primer Sequence</th>
<th>Product Size (bp)</th>
<th>Annealing Temperature (°C)</th>
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<td>1/1</td>
<td>GAATTCATCGTCAAGAGAGCT</td>
<td>691</td>
<td>57</td>
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<tr>
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<td>PE</td>
<td>1/691</td>
<td>GTAAGGAATATCTTTAAGG</td>
<td>593</td>
<td>60</td>
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<tr>
<td>Promoter</td>
<td>PF</td>
<td>1/634</td>
<td>CTTGCTTCAGGTCTTTAG</td>
<td>643</td>
<td>60</td>
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<tr>
<td>Promoter</td>
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<td>1/1226</td>
<td>CGAGGAGAGGAAGAGCAGCA</td>
<td>573</td>
<td>60</td>
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<tr>
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<td>57</td>
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<tr>
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<td>GACTACCAACTCTCTAT</td>
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<td>62</td>
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<tr>
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<td>1/1729</td>
<td>CCAATTGAGCTTCTGTATAGC</td>
<td>849</td>
<td>60</td>
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<tr>
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<td>231</td>
<td>57</td>
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<td>9</td>
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<td>6/101</td>
<td>GTTGGAGATGAGGCTACATGAG</td>
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<td>56</td>
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<tr>
<td>10</td>
<td>10As</td>
<td>7/85</td>
<td>GAGCCCTTAAATCCTATTG</td>
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<td>57</td>
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<td>15</td>
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<tr>
<td>15</td>
<td>15B</td>
<td>12/348</td>
<td>CACCCGCTTACGAGGCT</td>
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<td>62</td>
</tr>
<tr>
<td>26</td>
<td>26A</td>
<td>22/88</td>
<td>CAAGATTATCGGAGTATGAC</td>
<td>849</td>
<td>60</td>
</tr>
<tr>
<td>26</td>
<td>26B</td>
<td>22/388</td>
<td>CTTCCCCATTCACCGCATCT</td>
<td>231</td>
<td>57</td>
</tr>
<tr>
<td>28</td>
<td>28A</td>
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<td>56</td>
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<tr>
<td>28</td>
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<tr>
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<tr>
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<td>28D</td>
<td>24/1684</td>
<td>AGAGTAGAAAAAGGAGTCC</td>
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<td>56</td>
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* 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
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 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 propositus 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[i], 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 propositus 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[i] and Figure 1B[i], lanes 5 and 6). Only multimers of low molecular weight were detectable in plasma from the propositus 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 propositus 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 BII: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.
Table 2. Phenotypic data for family A and family B

<table>
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<tr>
<th>Individual</th>
<th>I:1</th>
<th>I:2</th>
<th>Ii:1</th>
<th>Ii:2</th>
<th>Normal Range</th>
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<td>Family A</td>
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<tr>
<td>Family member</td>
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<td>AI:2</td>
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<td>AI:2</td>
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<tr>
<td>Bleeding time (min)</td>
<td>3</td>
<td>3.5</td>
<td>4</td>
<td>10</td>
<td>&lt;8</td>
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<tr>
<td>vWF:Ag (IU/mL)</td>
<td>0.36</td>
<td>0.76</td>
<td>0.57</td>
<td>0.18</td>
<td>0.46-1.46</td>
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<tr>
<td>Factor VIII:Ag (IU/mL)</td>
<td>0.42</td>
<td>0.79</td>
<td>0.44</td>
<td>0.09</td>
<td>1.0 (mean value)</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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<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>Family B</td>
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<tr>
<td>vWF:Ag (IU/mL)</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>
</tr>
<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>
</tr>
<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>
</tr>
</tbody>
</table>

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 (AI: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 pSVvWFC788R and pSVvWFC1225G were used to transfect COS-7 cells either alone or in combination with pSVHvWF1 (wild-type) to generate mutant and wild-type hybrids. Intracellular rWF in the cell lysates and secreted rWF 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 rWF C788R and rWF C788R/wild-type hybrid were decreased to 41% ± 9% and 67% ± 5, respectively, relative to wild-type rWF. Secretion of rWF C1225G and rWF C1225G/wild-type hybrid were decreased to 58% ± 3% and 74% ± 10, respectively, relative to wild-type rWF. The intracellular levels of rWF C788R and rWF C788R/wild-type hybrid were 107% ± 8% and 97% ± 3%, respectively, relative to wild-type rWF. The intracellular levels of rWF C1225G and rWF C1225G/wild-type hybrid were 102% ± 4% and 96% ± 1, respectively, relative to wild-type rWF (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 rWF 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 rWF was detected in the medium after 2 hours, and all of the wild-type rWF 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 rWF C788R and rWF C1225G were immunoprecipitated from the cell lysates compared with the wild-type rWF (Figure 3). This indicated that amounts of rWF C788R and rWF C1225G equivalent to that of wild-type rWF were synthesized. Both rWF C788R and rWF 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 rWF C788R or rWF C1225G remained in the cells between 48 and 96 hours. These results indicate that both rWF C788R and rWF 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 rWFs to bind factor VIII (Figure 4). Wild-type rWF showed a dose-dependent increase in binding of factor VIII similar to that of vWF from normal pooled plasma (Figure 4). However, rWF C788R (Figure 4A) failed to bind factor VIII like plasma vWF from a patient known to be

![Figure 2. Steady-state analysis of rWF C788R, rWF C1225G, and mutant/wild-type hybrids expressed in COS-7 cells.](image-url)
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.\textsuperscript{23,33} The difference in multimer pattern of rvWF 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\textsuperscript{'} domain, possibly by disruption of intrachain 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\textsuperscript{34} is often followed by binding to the molecular chaperones of the endoplasmic reticulum, such as calnexin\textsuperscript{35,36} and BiP.\textsuperscript{37,38} 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.\textsuperscript{39} Previously, BiP has been shown to interact with type 2A vWD-causing mutations, which are associated with impaired intracellular transport and endoplasmic reticulum retention.\textsuperscript{23} 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\textsuperscript{'} domain. However, a recent study\textsuperscript{26} reported a mutation in the D3 domain, D879N, of pre-pro-vWF (D116N in mature vWF) that results in defective multimerization.\textsuperscript{26} None of the other previously reported type 2N mutations are known to be associated with abnormal multimer profiles. Multimer formation begins in the endoplasmic reticulum with the assembly of dimers in which 2 subunits are linked through cysteine residues located in the last 151 amino acids of the C-terminus. The dimers then form multimers on passage to the Golgi apparatus by interchain disulfide bond formation between the N-termini.\textsuperscript{27-29} Previous studies have shown the requirement of the propeptide, D\textsuperscript{'} and D3 domains for multimerization.\textsuperscript{30-32} As well as containing intrachain disulfide bonds, the D3 domain contains cysteine residues that participate in interchain disulfide bonds that covalently link multimer subunits.\textsuperscript{31,32} It has been shown that intersubunit disulfide bonds involving C1142 (C379 in mature vWF) and one or more of the cysteine residues at positions 1222, 1225, and 1227 (cysteines 459, 462, and 464 in mature vWF) connect the N-terminal ends of the vWF subunits in a parallel orientation.\textsuperscript{31} Mutation of C1225 alone to either glycine or alanine was shown in the previous study to have no effect on multimerization. However, in the present study we showed that mutation of C1225 to glycine gave rise to a very mild impairment in the ability of vWF to form multimers in vitro. The patient homozygous for the C1225G substitution showed a more severe reduction of high molecular weight vWF multimers in plasma than those of rvWF C1225G secreted by COS-7 cells. Examples of type 2A vWD causing mutations in the A2 domain have been documented in which platelet vWF and recombinant mutant vWF have normal
the monoclonal antibody to bind wild-type vWF and rWF C1225G, and there was no difference in the ability of rWF 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 C1225G. This study adds two mutations to a group of atypical type 2N vWD-associated mutations, including C788Y and D879N, that, in addition to causing defective factor VIII binding, also result in mild quantitative deficiencies and abnormal multimers.

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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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