Identification of Three Candidate Mutations Causing Type IIA von Willebrand Disease Using a Rapid, Nonradioactive, Allele-Specific Hybridization Method

By Aida Inbal, Talma Engliender, Nurtik Kornbrot, Anna M. Randi, Giancarlo Castanam, Pier M. Mannucci, and J. Evan Sadler

Type IIA von Willebrand disease (vWD), the most common type II vWD variant, is characterized by decreased binding of von Willebrand factor (vWF) to platelet glycoprotein Ib (GpIb) and by a decrease in large and intermediate vWF multimers. Mutations reported to cause vWD type IIA are clustered within the Aβ domain of vWF, which is encoded by exon 28. Genomic DNA from affected members of 12 unrelated families with type IIA vWD were screened for these mutations by a rapid, nonradioactive, allele-specific oligonucleotide (ASO) hybridization method. Oligonucleotides containing each of eight mutations were cross-linked onto a nylon membrane by UV irradiation. A fragment of vWF exon 28 was amplified from peripheral blood leukocyte DNA using biotinylated primers and hybridized to the immobilized oligonucleotides. Positive signals were detected with an avidin-alkaline phosphatase conjugate and chemiluminescent substrate. Thus, in a single hybridization reaction, a patient sample could be analyzed for a large number of mutations simultaneously. Polymerase chain reaction (PCR) products from four patients did not contain any of the tested mutations and therefore were sequenced. Three additional candidate missense mutations, two of them novel, were identified: Arg(834)→Gln in one patient, Gly(846)→Arg in one patient, and Val(902)→Glu in three ostensibly unrelated patients. By ASO hybridization, the mutations were confirmed in the affected patients and excluded in unaffected relatives and 50 normal controls. In one family, the Val(902)→Glu mutation was shown to be a de novo mutation. This rapid screening method is applicable to other subtypes of vWD for which mutations have been identified.

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VON WILLEBRAND factor (vWF) is a high-molecular weight disulfide-linked glycoprotein that circulates in plasma as a series of multimers ranging from 400,000 to greater than 20 million daltons.1,2 vWF plays a dual role in hemostasis: it mediates platelet adhesion to the subendothelium at sites of vascular injury1,2 and it serves as a carrier protein for coagulation factor VIII, permitting normal survival of this factor in the circulation.3

Quantitative and qualitative defects of vWF cause the most common hereditary bleeding disorder in humans, known as von Willebrand disease (vWD). The most common qualitative disorder of vWF, type IIA vWD, is characterized by autosomal-dominant inheritance, a decrease in large and intermediate vWF multimers, and diminished vWF binding to platelet glycoprotein Ib (GpIb).4

Thirteen missense mutations causing type IIA vWD have been reported,5-12 and all but one are within the Aβ domain of vWF. Expression of selected mutant vWF sequences by transfection in mammalian cells showed that type IIA vWD is caused by at least two distinct mechanisms in specific subgroups of these mutations.13 In one group, represented by the Arg(834)→Trp mutation, a full range of vWF multimers is produced, but they are abnormally sensitive to proteolytic degradation in the circulation, resulting in a type IIA multimeric pattern and decreased vWF function. In the second group, represented by the Val(844)→Asp mutation, there is abnormal intracellular processing and consequent secretion of vWF with a type IIA multimeric pattern and decreased function.11

The sensitivity and specificity of the current standard diagnostic tests for vWD may be as low as 60%.13 Additional variability is contributed by a number of other factors, including ABO blood group and estrogen level.14,15 The complexity of vWD classification and the limitations of the available diagnostic tests present major problems for the practicing clinical hematologist.

These problems could be overcome by the direct detection of vWF mutations. Since the mutations that are known to cause type IIA vWD are localized to vWF exon 28, differential hybridization with a panel of oligonucleotide probes containing type IIA mutations would permit accurate diagnosis and classification at the DNA level for this common vWD subtype.

Nonradioactive hybridization with sequence-specific oligonucleotide probes has become a widely used technique for the detection of genetic mutations and polymorphisms.15-19 An efficient approach suggested by Saki et al20 is to cross-link the oligonucleotides onto a nylon membrane with UV light after addition of a long deoxyribothymidine homopolymer tail to the 3' end of the oligonucleotide. The target segment DNA is amplified by polymerase chain reaction (PCR) with biotin-labeled primers and then hybridized to the immobilized oligonucleotides. Hybridization signals are detected after binding of streptavidin-horseradish peroxidase to the biotinylated primers. Thus, in a single hybridization reaction, a DNA sequence can be analyzed for many mutations simultaneously.

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Submitted January 26, 1993; accepted March 31, 1993.

Supported by Israeli Ministry of Health Grant No. 2134, Grant No. HLBI 14147 (Specialized Center of Research in Thrombosis) from the National Institutes of Health, Bethesda, MD, and by CNR Grant, P.F. Ingegneria Genetica.

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0006-4971/93/8203-0040$3.00/0

Blood, Vol 82, No 3 (August 1), 1993: pp 830-836
We used such a method to screen for eight type IIA mutations in representative patients from 12 unrelated families. Five unrelated patients were negative for the panel of tested type IIA mutations, and among them three additional mutations were identified by further DNA sequencing. One mutation occurred independently in three unrelated families.

**MATERIALS AND METHODS**

**Materials.** Polynucleotide kinase, terminal deoxynucleotidyl transferase (TdT), and One-Phor-All Buffer Plus (OPA) were obtained from Pharmacia (Piscataway, NJ). Taq DNA polymerase was purchased from Perkin Elmer-Cetus (Norwalk, CT). Oligonucleotides were synthesized on an Applied Biosystems synthesizer model 380A or 380B (Foster City, CA).

Deoxyadenosine 5'-[32P]triphosphate ([32P]dATP) and [γ-32P]ATP ([γ-32P]ATP) were purchased from New England Nuclear (Boston, MA). Sequencing kit, including Sequenase. Sequencing, was purchased from United States Biochemical (Cleveland, OH).

**Patients.** The patients studied were from 12 unrelated families from Italy affected with type IIA vWD. Each of the affected family members showed a prolonged bleeding time, decreased vWF antigen and ristocetin cofactor activity, and a plasma vWF multimer pattern consistent with type IIA vWD.

**PCR.** The PCR was performed with a Perkin Elmer-Cetus DNA Thermal Cycler as previously described. Briefly, to 30 pmol oligonucleotides were added 500 μmol/L dTTP, 2 μL 10X OPA, and 40 U TdT in a total 20-μL vol reaction. According to the manufacturer’s protocol (TdT protocol: simplified tailing of DNA in OPA), those conditions provide a nominal tail length of approximately 400-dT residues. After a 1-hour incubation at 37°C, 1/10 vol of 0.5 mol/L EDTA was added to stop the reaction. The tailed oligonucleotides were transferred onto nylon membrane (Genescreen, New England Nuclear) using a slot manifold (Schleicher & Schuell, Keene, NH; 5 pmol/well) and immobilized by UV light in a UV cross-linker (Stratalinker; Instruction Manual, Stratagene, La Jolla, CA). Approximately 120 to 150 mJ/cm² was applied. The filters were air-dried and stored at room temperature.

**Nonradioactive ASO hybridization of biotinylated DNA.** The membrane was prehybridized for 15 minutes at 50°C in 8 mL of hybridization buffer (5× SSPE/5× Denhardt’s/0.5% SDS, 5× SSPE = 0.9 mol/L NaCl, 0.05 mol/L NaH₂PO₄ pH 6.8, 0.005 mol/L EDTA; 5× Denhardt’s = 0.1% polyvinylpyrrolidone, 0.1% Ficoll, 0.1% bovine serum albumin; SDS = sodium dodecyl sulfate). Biotinylated amplified DNA (50 μL of a 100-μL PCR reaction, ~150 ng) was denatured in a boiling water bath for 10 minutes and then added to the membrane. Hybridization continued for 2 hours at 50°C. The membrane was washed twice for 20 minutes in 2× SSPE/0.1% SDS/3 mol/L tetramethyl ammonium chloride at 50°C. To permit visualization of all immobilized oligonucleotides, control hybridizations were performed under low-stringency conditions: 20-minute wash in 2× SSPE/3 mol/L tetramethylammonium chloride at 37°C. Positive signals were detected using avidin-biotinylated complex (Dynabeads, M-280; Dynal AS, Oslo, and Sequenase. Sequencing). Peripheral blood leukocyte DNA was amplified with primers 373 and 375a as described above, except that only one of the primers was biotinylated. The amplified DNA was electrophoresed on 0.8% Sea-Plaque agarose gels (FMC Bio Products, Rockland, ME) and visualized by staining with ethidium bromide. The desired fragment was excised and purified on GeneClean glass beads (Bio Lab, La Jolla, CA). Biotinylated strands were separated from nonbiotinylated by binding to streptavidin-magnetic beads complex (Dynabeads, M-280; Dynal AS, Oslo, Norway) and sequenced directly with Sequenase. Sequencing. Sequences were identified on products from several independent PCRs, and were confirmed by sequencing both strands. Additional mutations were excluded by DNA sequencing of representative cloned PCR products. The sequence obtained corresponded to cDNA nucleotides 4504 to 5033, which encode amino acid residues 739 to 921. This segment includes the entire domain A₂.

**ASO hybridization using [γ-32P]ATP.** The Val(902)→Glu, Gly(846)→Arg, and Arg(834)→Gln substitutions were evaluated by ASO hybridization. Oligonucleotides (17 mer) were synthesized with either normal sequences (4994T, 4825G, and 4790G, Table 1) or mutant sequences (4994A, 4825A, and 4970A, Table 1).

Samples of genomic DNA from patients and controls were am-

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Table 1. Sequences of Oligonucleotides Primers and Probes

<table>
<thead>
<tr>
<th>Name*</th>
<th>Function or Mutation</th>
<th>Nucleotide Position†</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>373</td>
<td>PCR primer</td>
<td>4484</td>
<td>TGG TCT TGG ATG TGG CTT TC</td>
</tr>
<tr>
<td>375a</td>
<td>PCR primer</td>
<td>5113</td>
<td>TCT TGG CAT AGT CAT GTA GC</td>
</tr>
<tr>
<td>742E</td>
<td>Gly(742)→Glu</td>
<td>4508</td>
<td>GTG GAA GAA TGG GAC AA</td>
</tr>
<tr>
<td>742R</td>
<td>Gly(742)→Arg</td>
<td>4506</td>
<td>C GTG CAG GAA TGG GAC A</td>
</tr>
<tr>
<td>743L</td>
<td>Ser(743)→Leu</td>
<td>4508</td>
<td>GTG GAA GAA TGG GAC AA</td>
</tr>
<tr>
<td>742GS</td>
<td>Normal</td>
<td>4506</td>
<td>GTG GAA GAA TGG GAC AA</td>
</tr>
<tr>
<td>834W</td>
<td>Arg(834)→Trp</td>
<td>4881</td>
<td>AGT GAC TGG GAC GAG</td>
</tr>
<tr>
<td>834R</td>
<td>Normal</td>
<td>4881</td>
<td>AGT GAC TGG GAC GAG</td>
</tr>
<tr>
<td>777P</td>
<td>Leu(777)→Pro</td>
<td>4711</td>
<td>AGC GTG CGG CAC TAC T</td>
</tr>
<tr>
<td>777L</td>
<td>Normal</td>
<td>4711</td>
<td>AGC GTG CGG CAC TAC T</td>
</tr>
<tr>
<td>844D</td>
<td>Val(844)→Asp</td>
<td>4803</td>
<td>CAC CTC CGC TAC ATG G</td>
</tr>
<tr>
<td>844V</td>
<td>Normal</td>
<td>4803</td>
<td>CAC CTC CGC TAC ATG G</td>
</tr>
<tr>
<td>865T</td>
<td>Ile(865)→Thr</td>
<td>4875</td>
<td>GTG GGC CCT AGA GAC G</td>
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<tr>
<td>865I</td>
<td>Normal</td>
<td>4875</td>
<td>GTG GGC CCT AGA GAC G</td>
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<tr>
<td>879K</td>
<td>Glu(875)→Lys</td>
<td>4905</td>
<td>GTC GAG CGA CGG GAC A</td>
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<tr>
<td>875E</td>
<td>Normal</td>
<td>4905</td>
<td>GTC GAG CGA CGG GAC A</td>
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<tr>
<td>4790G</td>
<td>Normal</td>
<td>4782</td>
<td>GCT GGC GGG GAC GAT</td>
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<tr>
<td>4790A</td>
<td>Arg(834)→Gln</td>
<td>4782</td>
<td>GCT GGC GGG GAC GAT</td>
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<tr>
<td>4825G</td>
<td>Normal</td>
<td>4817</td>
<td>TGG GTC ACC AGA AAT CCT</td>
</tr>
<tr>
<td>4825A</td>
<td>Gly(846)→Arg</td>
<td>4817</td>
<td>TGG GTC ACC AGA AAT CCT</td>
</tr>
<tr>
<td>4994T</td>
<td>Normal</td>
<td>4986</td>
<td>T GAC CGT CGT CGT CGA A</td>
</tr>
<tr>
<td>4994A</td>
<td>Val(902)→Glu</td>
<td>4986</td>
<td>T GAC CGT CGT CGT CGA A</td>
</tr>
</tbody>
</table>

* Antisense denoted by "a."
† Numbers indicate the position of the first nucleotide of the corresponding primer within the vWF sequence, numbering from the first nucleotide of the initiation codon.
Fig 1. ASO analysis of type IIA vWD mutations. Constant amounts of tailed oligonucleotides with normal or candidate mutant sequences were cross-linked to nylon filters by UV irradiation, hybridized with biotinylated amplified genomic DNA from unrelated type IIA vWD patients, washed at high stringency, and treated for color development. The name of each oligonucleotide (Table 1) is given next to the corresponding position on the filter. Line 1, filter strip with oligonucleotides containing a representative normal sequence; lines 2, 3, 4, 5, filter strips with oligonucleotides containing mutant sequences hybridized with DNA from patients that appear heterozygous for specific mutations.

plified with nonbiotinylated primers 373 and 375a as described above. PCR product (200 ng) was denatured in 100 μL of 0.25 mol/L NaOH/0.125× SSC (1× SSC = 0.15 mol/L NaCl + 0.015 mol/L sodium citrate) for 10 minutes at room temperature and applied to a nylon filter (GeneScreen Plus, New England Nuclear) using a dot blot manifold (Bio-Rad, Richmond, CA). Filters were hybridized with oligonucleotides labeled with [γ-32P]ATP using T4 polynucleotide kinase. The final wash was performed in 6× SSC and 0.1% SDS for 20 minutes at an optimal temperature empirically determined for each ASO probe (5°C below the melting temperature [Tm] of the corresponding oligonucleotide).

RESULTS

Screening of patients with type IIA vWD. A “reverse” ASO hybridization strategy was used to screen human genomic DNA samples for mutations that cause type IIA vWD. When these studies were performed, nine candidate type IIA vWD mutations had been reported, and eight of them were selected for study. Preliminary comparisons indicated that specific hybridization signals were easily obtained with 17-mer oligonucleotides, but not with 15-mer, and 17-mer oligonucleotides were used for subsequent experiments. Oligonucleotides (17 mers) containing each of these mutations or the corresponding normal sequences (Table 1) were elongated with (dT)n tails using terminal transferase and cross-linked to nylon membranes by UV irradiation. All eight tested mutations are within the A2 domain of vWF, which is encoded by a portion of exon 28. This segment was amplified from genomic DNA by PCR with biotinylated primers 373 and 375a, yielding a 627-bp product that encodes amino acid residues 732 to 921 of the mature vWF subunit. These primers permit selective amplification of vWF exon 28 without interference from the highly conserved sequences of the vWF pseudogene. The PCR products were hybridized with membranes containing immobilized target oligonucleotides. Membranes were washed in the presence of tetramethyl ammonium chloride, which permits the discrimination of mismatches independent of base composition, and thus allows the simultaneous analysis of multiple hybridization reactions with a single high-stringency wash condition. An avidin-alkaline phosphatase conjugate was then bound to the biotinylated primers of the hybridized PCR probe, and hybridization signals were visualized with a chemiluminescent phosphatase substrate. Control hybridizations under low-stringency conditions confirmed that all target oligonucleotides were successfully immobilized on the membrane and were available for annealing with the PCR probes (data not shown).

Genomic DNA samples from 12 families with type IIA vWD were screened with this assay, and four previously reported mutations were identified in seven unrelated patients (Fig 1). All patients gave hybridization signals with every normal oligonucleotide, but only one mutant oligonucleotide, suggesting that they were heterozygous with one apparently normal and one mutant allele. The Val(844)→Asp and Arg(834)→Trp mutations occurred independently in three and two unrelated families, respectively. The Ile(865)→Thr and Gly(742)→Arg mutations each occurred in one family. DNA probes of patients from the remaining five families hybridized only with the normal oligonucleotides, suggesting the existence of previously un-
known mutations. Therefore, the PCR products from affected family members were sequenced, and additional candidate mutations were identified in all five families: Val(902)→Glu in three families and Gly(846)→Arg and Arg(834)→Gln in one family each. 

Candidate mutation Val(902)→Glu. In three unrelated families (representative members BA, PG, and CR), a T→A transversion was identified at position 4994 resulting in Val→Glu substitution at amino acid residue 902 of the mature vWF subunit (Fig 2). This substitution was confirmed by ASO hybridization (Fig 3). All of the affected patients from the three families were heterozygous for this candidate mutation, consistent with the dominant inheritance of type IIA vWD. This substitution was not observed either in the unaffected family members or in 50 normal controls.

In one type IIA vWD family, the Val(902)→Glu substitution was not observed in the unaffected father (CC), mother (CCV), or sister (CV) (Fig 3). The assessment of paternity in this family was performed by HLA-DR genetic typing and with the polymorphic marker VNTR pYNZ22 (D17S5). The results showed that patient CR inherited two different HLA-DR alleles (one paternal and one maternal) and the same VNTR allele from both parents, indicating that she is almost certainly the daughter of parents CC and CCV. Thus, the Val(902)→Glu change appears to be a de novo mutation in this family.

Candidate mutation Arg(834)→Gln. A representative affected member of the fourth type IIA vWD family was heterozygous for the transition of G(4790)→A. This changes the encoded amino acid sequence from Arg to Gln at residue 834 (Fig 4). This candidate mutation was detected only in the affected patient and not in 50 normal controls by ASO hybridization (Fig 5).

Candidate mutation Gly(846)→Arg. A representative affected member from the fifth type IIA vWD family was heterozygous for the transition of G(4825)→A. This changes the encoded amino acid sequence from Gly to Arg at residue 846 (Fig 6). This candidate mutation was detected only in the affected patient and not in 50 normal controls by ASO hybridization (Fig 7).

**DISCUSSION**

Screening of genomic DNA from 12 patients with type IIA vWD by a nonradioactive ASO hybridization method identified five type IIA patients who did not contain a previously reported mutation. By further sequencing, three additional candidate missense mutations were detected within the A2 domain encoded by exon 28. The mutations were confirmed by ASO hybridization in the affected patients and excluded in unaffected relatives and 50 normal controls. One potential missense mutation resulting in Val(902)→Glu substitution occurred independently in three unrelated patients. The Gly(846)→Arg and Arg(834)→Gln substitutions each occurred in a single patient.

Since these studies were performed, four additional vWD type II A mutations were reported; one of them was identical to the Arg(834)→Gln mutation identified in one of our families, suggesting that it occurred independently. This mutation could be explained by deamination of methylcytidine in a CG dinucleotide, causing a C→T transition on the noncoding strand. Such methylated CG dinucleotides are often hot spots for mutation in the human genome. C→T transition in the same CG dinucleotide, but on the coding strand, results in the substitution Arg(834)→Trp, which is the most common vWD type IIA mutation. The Gly(846)→Arg candidate mutation can also be explained by a C→T transition in a CG dinucleotide on the noncoding strand.

In one type IIA vWD family (Figs 2 and 3, Table I), the Val(902)→Glu substitution was not detected in the unaffected parents. Since correct assignment of parentage was supported by studies with DNA polymorphic markers, the Val(902)→Glu mutation in the affected daughter (CR) may represent a new mutation. Germinal mosaicism for the mutation in one of the parents could also account for the de novo appearance of the mutation, as has been reported for one type IIB vWD family. Among the three families in the present study, the Val(902)→Glu candidate mutation appears to have occurred independently at least twice.

The results of this study together with previous reports indicate that the vast majority of type IIA vWD mutations cluster within A2 domain of vWF. These mutations appear to cause vWD by at least two mechanisms. Some directly impair the biosynthesis of vWF multimers. Others are...
compatible with apparently normal multimer synthesis and secretion when expressed as recombinant mutant proteins. These latter mutations are proposed to cause vWD type IIA by increasing the sensitivity of vWF to proteolysis in vivo. No obvious structural principle appears to unite the mutations of either class. Therefore, the mechanism is unknown by which the mutations Arg(834)→Gln, Gly(846)→Arg, and Val(902)→Glu may cause vWD type IIA.

Since many mutations outside of domain A2 might be expected to impair vWF multimer biosynthesis or stability, and thereby cause a phenotype-like vWD type IIA, this apparent clustering of type IIA mutations requires an explanation. It is possible, for example, that sequence variations in domain A2 may not be causative mutations, but may simply be linked to mutations elsewhere. This is not a problem for mutations that affect multimer biosynthesis, because the corresponding recombinant proteins have an appropriate abnormal phenotype. It is a potential problem for candidate mutations that cause no apparent abnormal phenotype upon expression as recombinant mutant proteins.

However, substantial indirect genetic evidence indicates that mutations in the A2 domain of vWF do cause vWD type IIA without directly impairing vWF multimer biosynthesis. The evidence includes the following: (1) candidate mutations have not been found in a single unaffected relative or normal control; (2) identical candidate mutations have been found repeatedly in unrelated patients who otherwise have distinguishable vWF genes, indicating multiple independent origins; (3) candidate mutations and vWD type IIA have appeared together de novo in a single generation; and (4) amino acid residues affected by candidate mutations are highly conserved. For example, sequence for pig vWF recently was reported that corresponds to amino acid residues 473 to 891 of human vWF. Fourteen of 15 type IIA mutations (including the mutations in the present study) occur in this region, and all 14 of them affect amino acid residues that are identical in human and pig vWF. Similarly, a partial bovine vWF cDNA sequence was reported that corresponds to amino acid residues 262 to 777 of human vWF; within this segment are five amino acid residues altered by type IIA vWD mutations, and all are conserved in the bovine sequence.

vWD is an extremely heterogeneous disorder, with more than 20 distinct clinical subtypes described. The complexity of vWD classification and the limitations of the current diagnostic tests suggest that a convenient screening assay for known DNA mutations could be useful. As described herein, a nonradioactive "reverse" ASO hybridization method permits the rapid screening of patient samples.
for a panel of type IIA vWD mutations. The same approach can be applied to other variants for which clustered mutations have been reported, such as vWD type IIB33,35 and vWD "Normandy."36 Identification of known mutations can rapidly diagnose these specific vWD subtypes, and also select patients in whom further studies may identify new vWD mutations.

ACKNOWLEDGMENT

This work was performed in partial fulfillment of PhD thesis requirements of Talma Englender. We thank Dr. Tirza Klein for performing paternity tests.

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