Pseudo–von Willebrand Disease: A Mutation in the Platelet Glycoprotein Iba Gene Associated With a Hyperactive Surface Receptor

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Pseudo (platelet-type)–von Willebrand disease is an autosomal dominant bleeding disorder caused by the hyperfunction of a receptor on the platelet surface. The abnormal receptor, glycoprotein Ibα, displays increased affinity for its ligand, von Willebrand factor. Four members (normal mother/affected father/two affected daughters) of a family with pseudo–von Willebrand disease were studied to determine the molecular genetic basis for their congenital platelet defect. Segments of the platelet glycoprotein Iba gene were amplified by means of the polymerase chain reaction, cloned, and sequenced. A point mutation (A to G, codon 239) was found in segments from the affected individuals but not from the normal. The mutation results in a single amino acid substitution (valine-mutant for methionine-normal) at residue 239 within the Iba binding site for von Willebrand factor. Both the mutant and the normal sequence were found in affected individuals, suggesting a heterozygous state. Amplified DNA from family members and from 58 normal individuals was analyzed by allele-specific oligonucleotide hybridization. Only the normal sequence was found in the mother and the normal individuals, whereas both the normal and the mutant alleles were found in the affected family members. The described mutation is associated with the pseudo–von Willebrand disease phenotype seen in this kindred. The resultant single amino acid substitution in glycoprotein Ibaα relates to increased receptor function and to excessive binding of von Willebrand factor to the platelet surface.

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In this study, we set out to determine the molecular genetic defect in the pseudo-vWD family described by Weiss et al, focusing initially on the genomic sequence that encodes the vWF binding region of GP Iba. This portion of the gene was analyzed by nucleotide sequencing, and a heterozygous point mutation was identified in the genomes of the family members affected by the disorder. We show that an A to G change in codon 239 of the GP Iba gene (valine substituting for methionine) correlates with the pseudo-vWD defect observed in this family.

MATERIALS AND METHODS

Patient material. Four members of a family affected with pseudo-vWD were studied: a normal mother (MoR), an affected father (AR) and two affected daughters (TR, MaR). The affected family members have intermittent thrombocytopenia, increased ristocetin-induced platelet aggregation, and a deficiency of high molecular weight forms of plasma vWF. However, in type IIB vWD, the patients’ platelets are normal while their plasma vWF is abnormal, binding to normal platelets in the absence of ristocetin.

Glycoprotein Iba consists of disulfide-linked alpha (GP Ibα, molecular weight 143 Kd) and beta (GP Ibβ, Mr 22 Kd) subunits. A second glycoprotein, GP IX (Mr 20,000), is noncovalently attached to GP Iba, giving rise to the GP Iba-IX complex that constitutes the receptor for vWF on the platelet surface, whereas a third glycoprotein, GP V (Mr 82,000), associates more loosely with the complex. All four proteins (Ibα/Ibβ/IX) contain leucine-rich glycoprotein (LRG) segments, and all are deficient in a congenital disorder of the platelet surface membrane, Bernard-Soulier syndrome (BSS). The primary structures of the three proteins in the GP Ibα-IX complex have been deduced from cDNA sequences. The largest member of the group, GP Ibα, has a complex primary structure with three extracellular domains: an LRG region on the NH2-terminal side, an O-linked carbohydrate region on the COOH-terminal side, and an intervening charged, hydrophilic region (“hinge” region) that contains the binding site for vWF. Because the functional defect in pseudo-vWD involves increased vWF binding by GP Ibα, the structural defect in this disease may involve the vWF binding site within GP Ibα.

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nomic DNA was also isolated from 58 control individuals who had no history of bleeding.

**DNA sequence analysis.** The polymerase chain reaction (PCR) was used to amplify genomic glycoprotein Iba sequences.29 Initially, PCR primers were synthesized to amplify the sequence encoding the vWF binding region: Stu-1 caggacttctgataacatcaacggc (+ strand; nucleotides 637-660, extended with an EcoRI site on the 5' end) and Stu-2 caggacttctgataacatcaacggc (− strand; nucleotides 1087-1110, extended with a HindIII site on the 5' end [Fig 1]). Nucleotide numbers refer to the reported cDNA sequence, and codon 239 corresponds to amino acid residue 239 of the mature protein.16

Seven additional primer pairs were synthesized to amplify the entire open reading frame encoding GP Iba (2.1 kb), each pair amplified a segment of approximately 330 bp and overlapped with the adjacent pair(s). PCR was performed with 1 ug of genomic DNA (partially digested with EcoRI; 10 U, 37°C, 5 ng of each primer, and 1 U of Taq-DNA polymerase (Promega, Madison, WI). Thirty cycles were performed as follows: denature 95°C for 1 minute, anneal 55°C for 1 minute, extend 72°C for 1.5 minutes. Amplified fragments were extracted with phenol/chloroform, precipitated with ethanol, digested with EcoRI/HindIII, and cloned into Bluescript SK+ (Stratagene, La Jolla, CA). Plasmids (2.5 μg) were prepared by adding 0.2 N NaOH/0.2 mol/L EDTA, incubating 5 minutes 22°C, and neutralizing with 2.0 mol/L NH₄OAc. After ethanol precipitation, plasmid DNA was resuspended in H₂O, and sequencing was performed by the dideoxy-chain termination method using Sequenase 2.0 (United States Biochemical, Cleveland, OH).22

Allele-specific oligonucleotide hybridization. Genomic DNA was amplified as described above, using the Stu-1/2 primers. Two oligonucleotides (15mers) were synthesized to hybridize with the seven bases on either side of the mutation site plus the central nucleotide of interest (first base/codon 239): the normal nucleotide at position eight (probe 8A:WT caggacttctgataacatcaacggc) or the mutant nucleotide at position 239 (probe 8G:Mut caggacttctgataacatcaacggc). PCR product (3 μl) was denatured in 0.3 mol/L NaOH/1 mmol/L EDTA, applied to a nitrocellulose membrane (Minifold II slot-blot system; Schleicher and Schuell, Keene, NH), and baked (2 hours). Following a described protocol,28 membranes were prehybridized in 8 ml 5X SSPE (20X SSPE: 3.0 mol/L NaCl/0.2 mol/L Na₂HPO₄/0.02 mol/L EDTA, pH 7.4, 5X DET (10X DET: 0.2% Denhardt's solution; 2 mmol/L Tris-HCl, pH 8.2/2 mmol/L EDTA, pH 8), 0.5% sodium dodecyl sulfate (SDS), and 0.1 mg/mL salmon sperm DNA, 30 minutes, 63°C. Probes 8A and 8G were end-labeled with 32P-ATP and T4 polynucleotide kinase,32 added to the prehybridization mix, and hybridized: 60 minutes, 63°C. Membranes were rinsed (twice 2X SSPE, 0.1% SDS, 22°C), washed (5X SSPE, 0.1% SDS, 63°C, 10 minutes), and autoradiographed.

**RESULTS AND DISCUSSION**

Platelet GP Iba interacts with vWF in a highly specific manner.20 Normal platelet GP Iba will bind to subendothelial vWF under conditions of rapid blood flow ("arterial circulation").29 Conversely, under conditions of stasis ("venous circulation"), the vWF binding site in GP Iba is nonfunctional. One can simulate the "off-and-on" nature of vWF binding by GP Iba through the addition of nonphysiologic agents such as ristocetin that induce a specific interaction between vWF and GP Iba.6,7,30

In pseudo-vWD, the mutant form of the GP Iba-vWF interaction lacks the critical "off-and-on" nature, and platelet GP Iba assumes an "activated" form that binds vWF regardless of flow, vasculature, or exogenous stimulants.14 Such indiscriminant vWF binding leads to thrombocytopenia and the loss of high molecular weight vWF multimers from the plasma. The structural abnormality in the mutant (pseudo-vWD) form of GP Iba is likely to be subtle because "hyperfunction/activation" suggests that the bulk of the mutant molecule may remain unaltered. Accordingly, this study pursues the hypothesis that the molecular genetic defect in the pseudo-vWD kindred is a "subtle" change in the genome such as a point mutation leading to a single amino-acid substitution. The search for the defect was focused initially on the vWF binding site in GP Iba (residues 220-310) as the most likely place for a structural change.20-22 The strategy is illustrated in Fig 1. Because the disease is transmitted in an autosomal dominant fashion, the further assumption was made that affected individuals would be heterozygotes, carrying both a normal and a mutant allele.

The gene segment of interest was amplified by PCR and sequenced, showing the presence of a point mutation (A to G, codon 239, methionine to valine, Fig 2) in the genomes of the affected individuals. Because normal and mutant sequences (heterozygous state) are randomly distributed during cloning, approximately equal numbers of mutant and normal sequences were present in DNA segments from affected individuals: 25% from the affected father (AR, 1 of 4), 25% from one affected daughter (TR, 6 of 12). Seven independent clones from the unaffected mother did not contain the mutation, nor has the mutation appeared in additional sequences of the segment from two unrelated normals.

Portions of the GP Iba gene encoding regions outside the vWF binding site were also evaluated, and these sequences from both the mother and one affected daughter (TR) were found to be identical to the published sequence.16 The genes encoding the other members of the GP Ib-V-IX system (Ibβ, V, IX) were not analyzed in this study, and abnormalities in these genes could contribute to the mutant phenotype in the

**Fig 1.** PCR strategy: Targeting of primers to the region of the GP Iba gene that encodes the vWF-binding domain of the Iba chain. A diagram of the extracellular domains of the 610 amino acid (aa) platelet GP Iba chain (leucine-rich glycoprotein; LRG, O-linked carbohydrate: O-CHO, intervening "hinge" domain binding vWF) and the 1.9-kb open reading frame (ORF) of the GP Iba gene indicates the location of polymerase chain reaction (PCR) primers used to amplify the genomic sequence of interest.
A MUTATION IN PSEUDO-vWD MUTATION

Fig 2. The candidate mutation associated with the pseudo-vWF phenotype in the R family.¹ The sequences of the normal (left) and candidate mutant (right) alleles differ at the first base of codon 239 of the platelet glycoprotein IIbα gene.¹⁰,²⁶ The A to G transition (→) results in the substitution of valine (239-mutant) for methionine (normal) within the region of the GP IIbα polypeptide that binds to vWF.

kindred. However, evidence for or against this possibility is not currently available.

The presence of the mutation in the kindred and in 58 controls was assessed by allele-specific oligonucleotide hybridization. Amplified gene segments were slot-blotted onto nitrocellulose and hybridized with 15-mer probes, 8A and 8G (Fig 3). The wildtype oligonucleotide, 8A, hybridized with DNA from both the family members and the controls (Fig 3). However, the mutant oligonucleotide, 8G, hybridized only with the DNA of the three affected family members (heterozygotes) and not with that of the unaffected mother or the controls (116 alleles), suggesting that the G for A transition in codon 239 in the R family is a mutation and not a polymorphism (polymorphism defined as present in >1% of normal alleles). Because it is present in affected family members only, the described mutation is associated with the functional abnormality that marks the pseudo-vWD phenotype. A direct demonstration that the mutation is responsible for the functional change in the receptor will require transfection of the mutant cDNA and functional characterization of the expressed mutant polypeptide. Related studies have been published.³¹

A different point mutation in the GP IIbα gene, associated with pseudo-vWD, was reported recently.³² In this unrelated kindred, a transversion (G to T, codon 233, glycine-normal to valine-mutant) was found in seven affected heterozygous family members but not in six unaffected family members and 161 controls. This mutation resembles that described in the current study in several aspects: (1) both result in a valine substitution; (2) both are located in the same region of the GP IIbα chain (within 6 amino acids): and (3) both result in a similar change in GP IIbα receptor function (enhanced vWF binding). The findings suggest that alteration of one amino acid in one region of the primary structure of the GP IIbα
chain can lead to a marked change in vWF binding. Similar arguments apply to type IIB vWD in which mutations within a defined region of the vWF subunit are associated with an “activated” or “hyperactive” mutant ligand (type IIB vWF), capable of binding spontaneously to normal platelet GP Iba.33,35

In summary, this study defines a novel “hyperfunction” mutation in platelet GP Iba that may alter the conformation of the vWF binding site and lead to excessive vWF binding. In the light of other information from related p-vWD and type IIB-vWD mutations,33,35 this study shows that single amino acid substitutions within a critical region of either the GP Iba receptor or the vWF ligand can produce a marked change in the affinity of one molecule for the other. The precise relationships between the effects of the mutations and the effects of flow/vasculature/ristocetin on the individual molecules, in vivo and in vitro, remain to be defined.

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