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Mutation in the gene encoding the α chain of platelet glycoprotein Ib in platelet-type von Willebrand disease

(blood platelets/platelet disorders/genetic diseases)

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ABSTRACT **Platelet-type von Willebrand disease (PT-vWD) is an autosomal dominant bleeding disorder characterized by abnormally enhanced binding of von Willebrand factor (vWF) by patient platelets. Although the platelet glycoprotein (GP) Ib/IX complex is known to constitute the platelet's ristocetin-dependent receptor for vWF, a unique structural abnormality within this complex has not previously been identified in PT-vWD. Using the polymerase chain reaction to amplify genomic DNA coding for the α chain of GP Ib (GP Ibα) and then sequencing the amplified DNA following cloning into M13mp18 and M13mp19 phage vectors, we have found a single point mutation in the GP Ibα coding region of PT-vWD DNA resulting in the substitution of valine for glycine at residue 233. This substitution within the vWF-binding region of GP Ibα is likely to exert a significant influence on the conformation of the resulting protein. Competitive oligonucleotide primer assay for this mutation showed a homozygous wild-type pattern in genomic DNA from the 161 normal volunteers studied and from 6 phenotypically normal members of a PT-vWD family. All 7 affected members of this family studied were heterozygous for the mutant allele. Platelet GP Ibα mRNA reverse-transcribed and studied by competitive oligonucleotide primer assay showed similar expression of the mutant and wild-type alleles in the affected PT-vWD patients. Absence in the normal population, tight linkage with phenotypic expression of disease, and absence of any additional abnormality of GP Ibα in these patients identify the glycine-to-valine substitution as a point mutation underlying functional abnormality of the vWF receptor in PT-vWD.**

Platelet-type von Willebrand disease (PT-vWD) is an autosomal dominant bleeding disorder in which patients characteristically show prolonged bleeding times, borderline thrombocytopenia, and decreased von Willebrand factor (vWF) high molecular weight multimers and functional activity (1–5). PT-vWD appears to result from an abnormality of the platelet receptor for vWF, whereby patient platelets show an abnormally increased binding of circulating vWF. In the laboratory, this platelet hyperresponsiveness may be demonstrated with the use of low concentrations of ristocetin. Whereas normal platelets show little or no aggregation at ristocetin concentrations as low as 0.5 mg/ml, patient platelets typically show significant binding of vWF, together with strong aggregation, following stimulation by 0.5 mg/ml, or even lower, concentrations of ristocetin (1–3). The unique ability of desialylated vWF (asialo-vWF) to agglutinate patient platelets in the presence of the divalent-cation chelator EDTA has additionally been demonstrated (6). Platelets from patients with PT-vWD also show a characteristically increased binding of the monoclonal antibody C-34, which is directed against an epitope within the platelet glycoprotein (GP) Ib/IX complex (7). Although this complex is known to constitute the platelet's ristocetin-dependent receptor for vWF (8), identification of a unique structural abnormality within this complex that might underlie the functional abnormalities seen in PT-vWD has not yet been achieved.

The platelet GP Ib/IX receptor for vWF is believed to consist of a 1:1 heterodimeric complex (9) between GP Ib (160 kDa) and GP IX (17 kDa) in a noncovalent association. GP Ib in turn consists of a disulfide-linked 140-kDa α chain (GP Ibα) and 22-kDa β chain (GP Ibβ) (10). A full-length cDNA for GP Ibα was isolated from human erythroleukemia (HEL) cells by Lopez *et al.* (11). Absolute identity of the HEL GP Ibα sequence with that obtained from the sequencing of nearly 800 nucleotides of cDNA obtained from human platelets was subsequently reported by Wicki *et al.* (12). Moreover, the human gene for GP Ibα has now been sequenced, and the entire coding region for the resulting protein has been shown to reside within a single exon (13, 14).

Functional studies utilizing the water-soluble, extracellular portion of GP Ibα termed glycocalicin, and more particularly the 45-kDa amino-terminal region common both to glycocalicin and to the native GP Ibα molecule, strongly suggest that the actual binding of vWF occurs within this region (15–18). What roles the other constituents of the complex may play in the regulation of vWF binding to the receptor remain unknown. The present investigation was undertaken to determine whether a structural abnormality within GP Ibα itself might underlie PT-vWD. From studies at both the genomic DNA and platelet RNA levels, we now report a unique point mutation that results in an abnormality of primary structure of platelet GP Ibα in patients with PT-vWD.

METHODS

Subjects. We studied three generations of a previously described (1, 4) Caucasian family with PT-vWD. For all patients undergoing genetic analysis, presence or absence of the PT-vWD phenotype was assessed. The unique agglutination of patient platelet-rich plasma induced by asialo-vWF in the presence of 5 mM EDTA (6) was used to identify presence of the disease. Family members showing evidence of PT-vWD were clearly segregated from unaffected relatives by this test, with no equivocal results.

The normal population that was studied comprised 161 adults, all of whom denied any history of an increased bleeding tendency. This population consisted of 70 medical students and 91 hospital personnel, representing a variety of ethnic backgrounds, although predominantly Caucasian. Informed consent for these studies was given by each subject,

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Abbreviations: PT-vWD, platelet-type von Willebrand disease; vWF, von Willebrand factor; GP, glycoprotein; GP Ibα, α chain of GP Ib; COP, competitive oligonucleotide primer; PCR, polymerase chain reaction.

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as approved by the Institutional Review Board for the Protection of Human Subjects at the State University of New York Health Science Center at Syracuse.

Preparation and Analysis of DNA and Platelet RNA. Peripheral blood leukocytes were used as a source of genomic DNA. For our initial studies, 50 ml of blood was drawn, and DNA was purified by standard techniques (19, 20). DNA for use in the competitive oligonucleotide primer (COP) assay (see below) was isolated from 5–10 ml of peripheral blood by the method of Kawasaki (21). Total RNA was isolated from the platelets present in 50–100 ml of blood by using a guanidinium isothiocyanate/cesium chloride gradient as described (22).

Polymerase Chain Reaction (PCR). Genomic DNA was amplified by the PCR using a series of oligonucleotide primer pairs. Since the entire coding region of the resulting protein is contained within a single exon (13, 14), nucleotide positions corresponding to the GP Ibα cDNA sequence (11) have been used in the present study. The PCR primers are listed in Table 1. PCR amplifications were performed for 30–40 cycles in an Eppendorf Microcycler, with annealing at 55–60°C for 30–60 sec, extension at 72°C for 2 min, and denaturation at 94°C for 30 sec. To 100 μl of reaction buffer, 1 μg of genomic DNA, 2.5 units of *Taq* DNA polymerase (Perkin–Elmer/Cetus), 50 pmol of each primer, and 10 nmol of each dNTP were added. DNA amplified by PCR was subsequently cloned into M13mp18 and M13mp19 bacteriophage.

DNA Sequence Analysis. Complete sequence of the GP Ibα coding region (nucleotides 91–1920) was obtained by a combination of (*i*) standard dideoxy sequencing (23) of PCR products cloned into M13mp18 and M13mp19 with (*ii*) direct sequencing of PCR products by the method of Carothers *et al.* (24). Since the autosomal dominant genetics of PT-vWD suggested that any abnormality would be heterozygous, at least 9 individual clones and a pool of 30 or more clones were sequenced for each region that was read from cloned DNA. Oligonucleotide primer pairs and the amino acid residues of GP Ibα included in the amplified PCR products are shown in Table 1.

GP Ibα DNA sequence for nucleotides 80–727 and 754–1034 was read from cloned DNA amplified with primer pairs J8/J5a and J9/J10, respectively. Direct sequencing of DNA amplified with primer pair J8/J10 provided sequence in the 728–753 region for which amplified DNA would reflect the sequence of primers J5A and J9 themselves. In addition, direct sequencing of DNA amplified by primer pair J6/J14, which included the remaining sequence of the GP Ibα coding region, proved capable of resolving all but a few difficult reading regions. Cloned DNA containing the full-length GP Ibα coding sequence of both the wild-type and mutant alleles was obtained using J8 and J14 as a PCR primer pair later in the course of these studies. This cloned DNA provided definitive resolution and confirmation of sequence for those remaining regions in which analysis by direct sequencing had been difficult.

COP Assay. COP analysis (25) was developed for population screening for the presence of a single nucleotide point mutation. In brief, the 16-mer oligonucleotide sense primers J12 and J13, each differing by only a single base at position 8, were utilized in equimolar concentrations as competing primers in a PCR reaction, with J10 serving as a common antisense primer. To approximately 5 μg of genomic DNA were added 50 pmol each of primers J12, J13, and J10 and 12.5 nmol of each dNTP in a total volume of 100 μl. For each individual studied, the COP assay was run in duplicate: to one tube was added a trace amount ($\approx 8 \times 10^5$ cpm) of ^{32}P-labeled J12, and to the other tube an equivalent amount of ^{32}P-labeled J13. This technique was also applied to platelet RNA, as follows. The J9/J10 primer pair was used in the sequential reverse transcription and PCR amplification of platelet RNA (22). The PCR mixture was then diluted 1:50 with 0.1 mM EDTA/10 mM Tris hydrochloride, pH 7.4, and 10 μl of this diluted material was then used as the target for a subsequent COP procedure, with the addition of 50 pmol each of J12 and J13 as well as an additional 50 pmol of J10. Tracer amounts of ^{32}P-labeled J12 or J13 were added as in the case of genomic DNA COP analyses. COP amplifications were performed for 25 PCR cycles for genomic DNA and 15 cycles for reverse-transcribed platelet RNA. Annealing was at 49°C for 60 sec, followed by extension at 72°C for 3 min and denaturation at 94°C for 45 sec. Amplified products from the COP reactions were electrophoresed in agarose gels containing 2.5% NuSieve GTG agarose (FMC) and 1% low-electroendosmosis (EEO) agarose (Boehringer Mannheim). After electrophoresis, the gels were dried and autoradiography was performed.

Linkage Analysis. Logarithm-of-odds (lod) scores (26) for assessment of linkage between PT-vWD phenotypic expression and allele assignments resulting from DNA analysis were computed using the MENDEL program (27).

RESULTS

The nearly intronless nature of the gene coding for platelet GP Ibα (13, 14) permitted our initial studies to be performed

Table 1. Oligonucleotide PCR primers

Primer	Nucleotides included*	Direction	Sequence	Amino acids included†
J8	38–60	Sense	5′-TACTGAATTCCTCATGCCTCTCCTCCTCTTG-3′	−16–222
J5A	758–728	Antisense	5′-TTGTCCTGCAGCCAGCGACGAAAATAGAGGA-3′	
J9	737–753	Sense	5′-TTCGTCGCTGGCTGCAG-3′	217–320
J10	1051–1035	Antisense	5′-GGCTGTCTAGAGAAGCA-3′	
J12 (or J13)	781–796	Sense	5′-AAGCAAGG(or T)TGTGGACG-3′	231–320
J10	1051–1035	Antisense	5′-GGCTGTCTAGAGAAGCA-3′	
J6	748–777	Sense	5′-CTGCAGGACAATGCTGAAAATGTCTACGTA-3′	220–610
J14	1987–1964	Antisense	5′-CCGGATCCCAACTAGATTCCAATAGGAGAG-3′	

*The J8 primer additionally has sequence 5′ to nucleotide 38 that includes an *Eco*RI recognition site, and the J14 primer has sequence 3′ to nucleotide 1987 that includes a *Bam*HI recognition site.

†The J8/J5A primer pair includes the GP Ibα signal peptide (−16 to −1) in addition to residues 1–222 of the mature protein.

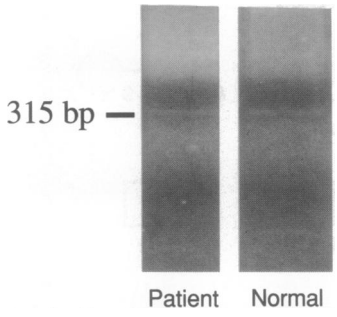

FIG. 1. Amplification of GP Ibα genomic DNA. The PCR primer pair J9/J10 was used to amplify nucleotides 737–1051 of the genomic DNA of patient II-2 or a normal individual. After electrophoresis in a 1% agarose gel, amplified products of 315 base pairs (bp) are seen by ethidium bromide staining in each case.

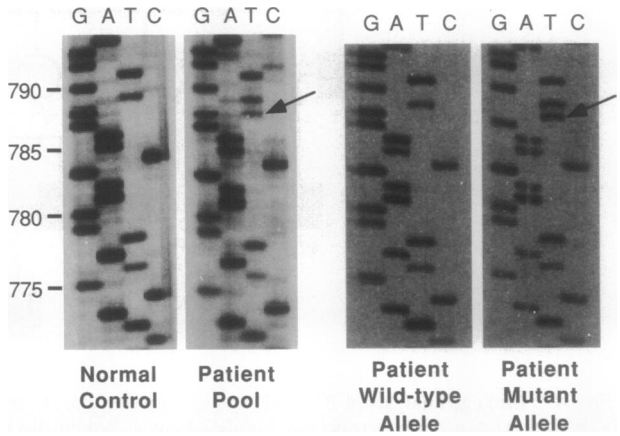

FIG. 2. DNA sequence analysis of the GP Ibα gene in PT-vWD. Genomic DNA amplified by the primer pair J9/J10 was cloned into M13mp18 and then sequenced. (*Left*) Products of sequencing reactions show the heterozygous presence of a G and a T at nucleotide position 788 (arrow) in a pool of 32 individual M13mp18 clones of amplified DNA from patient II-2, in contrast to the homozygous wild-type G seen in a normal individual. (*Right*) The corresponding sequences of the wild-type and mutant alleles from individual M13mp18 clones of amplified DNA from this patient are shown.

on genomic DNA obtained from the circulating leukocytes of PT-vWD patients. Using a series of oligonucleotide PCR primer pairs (Table 1), we observed identically migrating bands of amplified DNA from patients as compared with normal controls (Fig. 1). This finding suggested the absence of any major deletions within the gene but could not exclude the possibility of alterations affecting only one or several nucleotides within the amplified segments.

DNA sequencing of two PT-vWD patients confirmed the absence of any substitutions, additions, or deletions of nucleotides, as compared with the normal genome, throughout the entire protein coding region, with a single exception. In both PT-vWD patients, the substitution of T for G at position 788 of the nucleotide sequence was observed. This substitution was nonconservative, resulting in the substitution of valine for glycine at residue 233 of the mature GP Ibα molecule. The G-to-T substitution was heterozygous in the PT-vWD patients (Fig. 2). Only the wild-type G at position 788 was seen in normal individuals.

Substitution of T for G at position 788 did not create or destroy a recognition sequence for any known restriction enzyme. To investigate the occurrence of this substitution in a large number of individuals, we developed a screening assay based on the COP technique reported by Gibbs *et al.* (25). Using equal amounts of J12 and J13 and only a trace amount of either ^{32}P-labeled J12 or ^{32}P-labeled J13, we sought to identify the presence of alleles showing a perfect match with the individual primers. The COP technique correctly identified the presence of both alleles in the genomic DNA of all affected PT-vWD patients but identified only the wild-type allele in the genomic DNA of normal individuals (Fig. 3). We subsequently applied this technique to 161 unrelated normal volunteers and observed a homozygous wild-type pattern in each case, for a total of 322 normal alleles. Moreover, the COP assay indicated a homozygous wild-type pattern from the six phenotypically normal members of the PT-vWD family. All seven members of the family for whom phenotypic expression of PT-vWD could be demonstrated were heterozygous for the mutant allele by COP assay (Fig. 4). The association of the heterozygous G-to-T substitution at nucleotide 788 accordingly showed very tight linkage with clinical expression of the PT-vWD phenotype, with a maximum lod score of 2.53 at a recombination fraction of 0.

Gene expression was studied by PCR analysis of reverse-transcribed platelet mRNA. As in the case of genomic DNA, no gross deletions were seen in the patient samples (Fig. 5 *Left*). Application of the COP technique to cDNA reverse transcribed from platelet mRNA showed similar expression of the mutant and wild-type alleles in the affected PT-vWD patients (Fig. 5 *Right*). Normal individuals and phenotypically normal members of the PT-vWD family, in contrast, uniformly showed the homozygous wild-type pattern.

DISCUSSION

The present studies provide the characterization of a specific alteration of protein structure underlying the qualitative platelet disorder PT-vWD. The heightened responsiveness of the platelets of patients with PT-vWD is associated with a

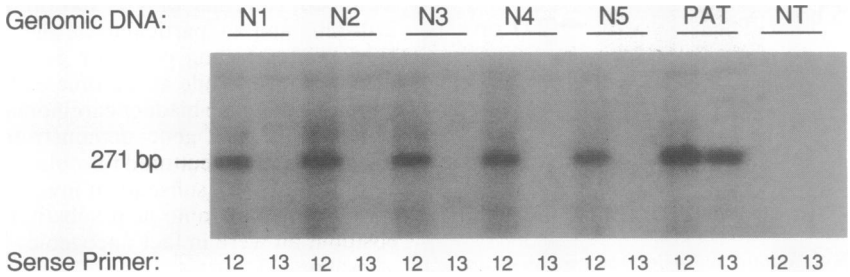

FIG. 3. COP assay for mutation in GP Ibα genomic DNA. Genomic DNA samples from each of five normal individuals (N1–N5) and from patient II-5 (PAT), as well as a "no template" control lacking any target DNA (NT), were amplified in parallel PCRs using the sense primers J12 and J13 and the antisense primer J10. Trace amounts of either ^{32}P-labeled J12 or ^{32}P-labeled J13 were added to each reaction, as noted by a 12 or a 13 below each lane. After 25 PCR cycles, the amplification product was electrophoresed in an agarose gel, the gel was dried, and autoradiography was performed. While ethidium bromide staining demonstrated a band of amplified DNA at 271 bp in all lanes (data not shown), the autoradiograph reveals that the amplified product is composed exclusively of the wild-type allele in all normal controls, but of both the wild-type and mutant alleles in the patient.

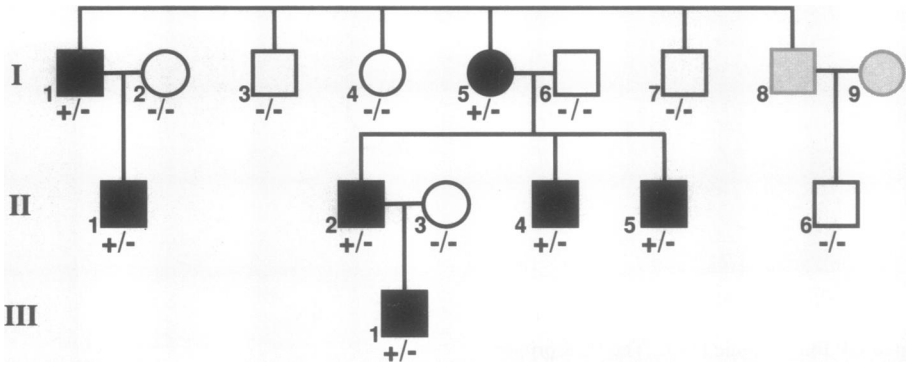

FIG. 4. Linkage analysis of PT-vWD and mutation of the GP Ibα gene. Solid symbols represent patients showing phenotypic expression of PT-vWD, and open symbols represent family members lacking disease expression. Individuals unavailable for study are shown by gray symbols. Squares denote males, and circles denote females. Based on COP assay results, mutant alleles (coding for valine at residue 233) are represented by plus signs, and wild-type alleles (coding for glycine) by minus signs.

substitution of valine for glycine at residue 233 of the mature GP Ibα protein. This substitution was not detected in the 322 chromosomes represented by the 161 individuals studied in the normal population. Absence in the normal population, tight linkage with phenotypic expression of disease, and absence of any additional abnormality of GP Ibα in these patients provide strong support for the glycine-to-valine mutation underlying the functional abnormality of the vWF receptor in PT-vWD.

Previous studies utilizing proteolytic fragments of GP Ibα and a variety of monoclonal antibodies have provided strong suggestions that the actual vWF-binding domain of the GP Ib/IX complex is contained within the 45-kDa amino-terminal region of GP Ibα (15–18). Studies of ristocetin- and botrocetin-mediated interaction of vWF with reconstituted GP Ib/IX complexes have provided further support for this concept (28, 29). Recent efforts to localize the vWF binding site more specifically within the 293 residues of the 45-kDa amino-terminal region of GP Ibα have employed a series of overlapping synthetic peptides (30). However, no single peptide was found that exerted inhibitory activity in competitive binding assays comparable to that exerted by the 45-kDa tryptic fragment itself or by the larger proteolytic fragment of GP Ibα, glycocalicin.

A report by Handin and Petersen (31) on the expression of GP Ibα *in vitro* provided further information concerning the vWF binding site. COS cells transfected with a cDNA coding for the amino-terminal region but terminating before the beginning of the serine/threonine-rich domain at residue 320

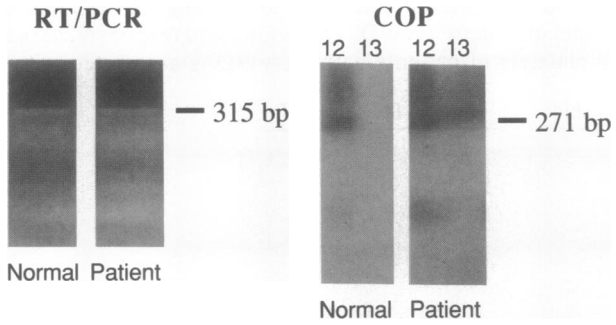

FIG. 5. Expression of both mutant and wild-type mRNA in the platelets of PT-vWD patients. Platelet mRNA from patient II-4 or from a normal control was reverse-transcribed (RT) and the resulting cDNA was amplified with the J9/J10 primer pair, producing the anticipated 315-bp products (*Left*). This cDNA was then used as starting material in the COP procedure. Autoradiography of COP products (*Right*) shows the presence of both mutant and wild-type expression in patient platelets, but only wild-type expression in normal platelets.

produced recombinant protein capable of inhibiting ristocetin-induced binding of ^{125}I-vWF to washed platelets. In contrast, a smaller recombinant product lacking nearly 100 additional amino acids at the carboxyl end (i.e., extending from the amino-terminal end through residue 220) lacked functional activity in this assay. The region extending from residue 220 to 320 of the mature GP Ibα accordingly appears to be critical for vWF binding. The glycine-to-valine mutation at residue 233 in the PT-vWD patients is thus well situated to produce an abnormality in vWF binding.

Substitution of a valine for a glycine may be anticipated to exert significant influence on the conformation of the resulting protein. For example, this precise amino acid substitution underlies the *ras* protooncogene-to-oncogene transformation (32, 33), where resulting conformational changes are believed to have profound functional sequelae (34, 35). Computerized conformational energy analysis of GP Ibα polypeptides has indeed shown that a glycine-to-valine mutation at residue 233 produces major changes in conformation of this region of the protein (M. R. Pincus, D. C. Dykes, R. P. Carty, and J.L.M., unpublished observation). Since this substitution is associated with an abnormally enhanced interaction of patient platelets with native vWF under a variety of conditions (1–3) as well as with the unique ability of asialo-vWF to bind to formalin-fixed patient platelets or to agglutinate fresh patient platelets in the presence of EDTA (6), further study of the structural consequences of this mutation may lead to increased understanding of the mechanisms involving vWF–platelet interactions.

Several other families with PT-vWD (or pseudo-vWD) have been reported (2, 3, 36). Although the affected individuals share many aspects of phenotypic expression, it is quite possible that the underlying genetic abnormalities are not identical. Analysis of GP Ibα in the region of residues 220–320, and in particular at the 233 locus, may be an appropriate starting point for genetic analysis in such patients. Indeed, while at the time of the discovery of the *ras* mutation in human bladder carcinoma the only substitution in codon 12 of this gene demonstrated to cause malignant transformation of cultured fibroblasts was a glycine-to-valine mutation (32, 33), subsequent investigations revealed that of the 19 possible amino acid substitutions for glycine at this position, all were in fact oncogenic, with the sole exception of proline (37).

We have shown that heterozygosity in the patient genome for the glycine-to-valine substitution is also reflected in the expression of both of the corresponding mRNAs in the patient platelets. It remains unknown, however, whether the resulting abnormal form of GP Ibα might have a selective advantage over the wild-type form in achieving membrane insertion and formation of the GP Ib/IX vWF receptor

complex. Since there are no patients known to be homozygous for PT-vWD alleles, it has not been possible to study the effects of gene dosage on the function of this receptor. Through the application of *in vitro* expression in tissue culture cells, however, in which mutant vWF receptor molecules may be precisely engineered, it may soon prove possible to reach a fuller understanding of the structure–function properties of the platelet vWF receptor that result in clinical bleeding disorders.

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1. Miller, J. L. & Castella, A. (1982) *Blood* **60**, 790–794.
2. Weiss, H. J., Meyer, D., Rabinowitz, R., Pietu, G., Girma, J. P., Vicic, W. J. & Rogers, J. (1982) *N. Engl. J. Med.* **306**, 326–362.
3. Takahashi, H. (1980) *Thromb. Res.* **19**, 857–867.
4. Miller, J. L., Kupinski, J. M., Castella, A. & Ruggeri, Z. M. (1983) *J. Clin. Invest.* **72**, 1532–1542.
5. Takahashi, H., Handa, M., Watanabe, K., Ando, Y., Nagayama, R., Hattori, A., Shibata, A., Federici, A. B., Ruggeri, Z. M. & Zimmerman, T. S. (1984) *Blood* **64**, 1254–1262.
6. Miller, J. L., Ruggeri, Z. M. & Lyle, V. A. (1987) *Blood* **70**, 1804–1809.
7. Miller, J. L., Hustad, K. O., Kupinski, J. M., Lyle, V. & Kunicki, T. J. (1990) *Br. J. Haematol.* **74**, 313–319.
8. George, J. N., Nurden, A. T. & Phillips, D. R. (1984) *N. Engl. J. Med.* **311**, 1084–1098.
9. Du, X., Beutler, L., Ruan, C., Castaldi, P. A. & Berndt, M. C. (1987) *Blood* **69**, 1524–1527.
10. Fitzgerald, L. A. & Phillips, D. R. (1989) in *Platelet Immunobiology: Molecular and Clinical Aspects*, eds. Kunicki, T. J. & George, J. N. (Lippincott, Philadelphia), pp. 9–30.
11. Lopez, J. A., Chung, D. W., Fujikawa, K., Hagen, F. S., Papayannopoulou, T. & Roth, G. J. (1987) *Proc. Natl. Acad. Sci. USA* **84**, 5615–5619.
12. Wicki, A. N., Walz, A., Gerber-Huber, S. N., Wenger, R. H., Vornhagen, R. & Clemetson, K. J. (1989) *Thromb. Haemostasis* **61**, 448–453.
13. Wenger, R. H., Kieffer, N., Wicki, A. N. & Clemetson, K. J. (1988) *Biochem. Biophys. Res. Commun.* **156**, 389–395.
14. Petersen, E., Wyler, B., Morton, C., Clevers, H., Bruns, G. & Handin, R. I. (1988) *Blood* **72**, 335a (abstr.).
15. Wicki, A. N. & Clemetson, K. J. (1985) *Eur. J. Biochem.* **153**, 1–11.
16. Brower, M. S., Levin, R. I. & Garry, K. (1985) *J. Clin. Invest.* **75**, 657–666.
17. Handa, M., Titani, K., Holland, L. Z., Roberts, J. R. & Ruggeri, Z. M. (1986) *J. Biol. Chem.* **261**, 12579–12585.
18. Vicente, V., Kostel, P. J. & Ruggeri, Z. M. (1988) *J. Biol. Chem.* **263**, 18473–18479.
19. Bell, G. I., Karam, J. H. & Rutter, W. J. (1981) *Proc. Natl. Acad. Sci. USA* **78**, 5759–5763.
20. Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Lab., Cold Spring Harbor, NY).
21. Kawasaki, E. S. (1990) in *PCR Protocols*, eds. Innis, M., Gelfand, D., Sninsky, J. & White, T. (Academic, San Diego), pp. 146–152.
22. Finch, C. N., Miller, J. L., Lyle, V. A. & Handin, R. I. (1990) *Blood* **75**, 2357–2362.
23. Sanger, F., Nicklen, S. & Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. USA* **74**, 5463–5467.
24. Carothers, A. M., Urlaub, G., Mucha, J., Grunberger, D. & Chasin, L. A. (1990) *Biotechniques* **7**, 494–499.
25. Gibbs, R. A., Nguyen, P. N. & Caskey, C. T. (1989) *Nucleic Acids Res.* **17**, 2437–2448.
26. Morton, N. E. (1955) *Hum. Genet.* **7**, 277–318.
27. Lange, K., Weeks, D. & Boehnke, M. (1988) *Genet. Epidemiol.* **5**, 471–472.
28. Berndt, M. C., Du, X. & Booth, W. J. (1988) *Biochemistry* **27**, 633–640.
29. Andrews, R. K., Booth, W. J., Gorman, J. J., Castaldi, P. A. & Berndt, M. C. (1989) *Biochemistry* **28**, 8317–8326.
30. Vicente, V., Houghten, R. A. & Ruggeri, Z. M. (1990) *J. Biol. Chem.* **265**, 274–280.
31. Handin, R. I. & Petersen, E. (1989) *Blood* **74**, 129a (abstr.).
32. Reddy, E. P., Reynolds, R. K., Santos, E. & Barbacid, M. (1982) *Nature (London)* **300**, 149–152.
33. Tabin, C. J., Bradley, S. M., Bargmann, C. I., Weinberg, R. A., Papageorge, A. G., Scolnick, E. M., Dhar, R., Lowy, D. R. & Chang, E. H. (1982) *Nature (London)* **300**, 143–148.
34. Varmus, H. (1987) in *The Molecular Basis of Blood Diseases*, eds. Stamatoyannopoulos, G., Nienhuis, A. W., Leder, P. & Majerus, P. W. (Saunders, Philadelphia), pp. 271–346.
35. Pincus, M. R. & Brandt-Rauf, P. W. (1985) *Cancer Invest.* **4**, 185–195.
36. Bryckaert, M. C., Pietu, G., Ruan, C., Tobelem, G., Girma, J. P., Meyer, D., Larrieu, M. J. & Caen, J. P. (1985) *J. Lab. Clin. Med.* **106**, 393–400.
37. Seeburg, P. H., Colby, W. W., Capon, D. J., Goeddel, D. V. & Levinson, A. D. (1984) *Nature (London)* **312**, 71–75.