Mutation of Leucine-57 to Phenylalanine in a Platelet Glycoprotein Ibα Leucine Tandem Repeat Occurring in Patients With an Autosomal Dominant Variant of Bernard-Soulier Disease

By Jonathan L. Miller, Vicki A. Lyle, and David Cunningham

The primary sequences of the three individual glycoprotein (GP) chains, GPIbα, GPIbβ, and GPIX, comprising the normal platelet GPIb/IX receptor for von Willebrand factor (vWF) have recently been determined, opening the possibility for characterization of disorders of this receptor at the molecular level. The presence of a leucine tandem repeat in each of these chains is of particular interest, because such repeats may be involved in associations between polypeptide segments. We now report an autosomal dominant variant of Bernard-Soulier disease associated with the heterozygous substitution of phenylalanine for a highly conserved leucine residue within the GPIbα leucine tandem repeat. Affected individuals experienced a moderate bleeding tendency, thrombocytopenia, and an increased mean platelet volume. Platelet aggregation was decreased only in response to ristocetin or to asialo-vWF. The kd for ^{125}I-vWF binding to patient platelets was significantly increased over control values at 0.5 mg/mL ristocetin, but was normal at 1.0 or 1.5 mg/mL ristocetin. While sodium dodecyl sulfate-polyacrylamide gel electrophoresis showed an essentially normal complement of all components of the GPIb/IX complex, a minor amount of a putative proteolytic fragment was identified that migrated faster than GPIb and was immunoreactive with polyclonal anti-GPIbα antibody, but not with a monoclonal antibody directed against the 45-Kd amino-terminal region of GPIbα. However, because the great majority of patient GPIbα comigrates with normal GPIbα, the major functional abnormalities of the patient platelets are most likely a consequence of the altered structure of the nonproteolyzed protein. Full concordance within the studied family between phenotypic expression and a heterozygous single nucleotide substitution in genomic DNA coding for a phenylalanine in place of the wild-type leucine at residue 57 of the mature GPIbα, absence of this substitution in 266 alleles from the normal population, and the lack of any other abnormality of patient DNA throughout the entire coding sequence for GPIbα provide strong support that this substitution may constitute a pathologic point mutation responsible for the observed phenotypic abnormalities. While the roles that leucine tandem repeats may normally play within the GPIb/IX complex are not yet known, the perturbation of such a repeat in GPIbα may impair interaction with other components of the complex and/or with the binding of vWF.

THE STUDY OF FUNCTIONAL platelet disorders has traditionally consisted of a description of the abnormal responsiveness of patient platelets in a variety of experimental conditions, together with a genetic analysis of the families involved. In most cases, however, a precise correlate of abnormal functions with underlying abnormal structure has not been possible. Recently, the entire cDNA sequences encoding the protein chains comprising the platelet glycoprotein (GP) IIb/IIIa receptor[1-6] and the platelet GPIb/IX receptor[7-10] have been obtained, opening the possibility for study of the molecular basis of disorders affecting these receptors. Bray and Shuman[11] first identified an abnormal gene coding for GPIIIa in one Glanzmann thrombasthenia family. A major deletion of genomic DNA coding for GPIIb[12] was subsequently described in another thrombasthenic patient. Most recently, Newman et al[13] have identified an 11-base deletion of the GPIIIa gene leading to premature protein termination in thrombasthenic patients of Iraqi-Jewish origin, as well as a 13-base deletion of the GPIIb gene producing a 6-amino acid deletion of the GPIIb protein in thrombasthenic patients of Arabic origin. A nonsense mutation has also been reported in the GPIbα gene, leading to incomplete synthesis of the GPIbα protein in a patient with Bernard-Soulier diseasee (BSD).[14] A point mutation in the GPIbα gene coding for a valine in place of the wild-type glycine in the GPIbα of patients with platelet-type von Willebrand disease has also recently been identified.[15]

Absence of the GPIb/IX receptor for von Willebrand factor (vWF) is the hallmark of the autosomal recessive bleeding disorder, BSD. We have previously described in preliminary form[16] a family in whom attributes of BSD appear to be transmitted in an autosomal dominant manner, and in whom there appears to be a qualitative, rather than quantitative, abnormality of GPIbα. Descriptions of apparently qualitative abnormalities of GPIbα have also been reported in patients of Italian[17] and Norwegian[18] origin. We now present a molecular characterization of the abnormality in the American family we have studied.

MATERIALS AND METHODS

Subjects. Five patients representing two generations of a Caucasian family from central New York State (patients II-2, III-1, III-2, III-3, and III-4) were available for study. The proband (patient III-1) was a 13-year-old male who was referred for evaluation of thrombocytopenia (80,000/μL) noted prior to dental extractions. The patient had had frequent episodes of epistaxis, one episode severe enough to result in hospitalization. Otherwise, he was in good health. The proband's mother (patient II-2) had a long history of increased bleeding, most notably menorrhagia severe enough to result in an eventual hysterectomy, epistaxis continuing into adulthood, profuse bleeding associated with tonsillectomy, and most recently gastrointestinal tract bleeding of undefined origin. Of three daughters of patient II-2 by a second

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marriage, one (patient III-2) also had a history of menorrhagia and epistaxis. Patient III-3 was felt to have had exaggerated bleeding after tonsillectomy in early childhood, but was otherwise normal. Patient III-4 had an essentially negative bleeding history.

The father of the proband was not available for study, but has a negative bleeding history. The proband's maternal grandfather and grandmother were also unavailable for testing. The latter has a negative bleeding history, while the maternal grandfather has carried a history of easy bruising and excessive bleeding from minor trauma.

The normal population studied was composed of 133 adults, all of whom denied any history of increased bleeding. This population consisted of medical students and hospital personnel, representing a variety of ethnic backgrounds, although primarily Caucasian. Informed consent for these studies was given by each subject, as approved by the Institutional Review Board for the Protection of Human Subjects at the SUNY Health Science Center at Syracuse.

Routine hemostatic studies. Bleeding times were obtained using the Simplate device (Organon Teknika, Durham, NC). Platelet count and mean platelet volume were determined with a Coulter Electronics (Hialeah, FL) S-Plus IV on EDTA-anticoagulated whole blood, with reference intervals based on the study of 120 normal adults.

Platelet function studies. Blood from normal volunteers or from patients was collected into 1/10 volume of 3.8% sodium citrate and the platelet-rich plasma (PRP) prepared by centrifugation at room temperature for 50 to 90 seconds at 900*g*. Platelet aggregation and secretion studies were performed on this citrated PRP, using a Chronolog (Havertown, PA) lumi-aggregometer. For studies with asialo-vWF, native human vWF was purified from human cryoprecipitate (provided by the Greater Syracuse Chapter of the American National Red Cross) and digested with proteinase-free neuraminidase from Vibrio cholerae (Calbiochem, La Jolla, CA), as previously described.[19]

Platelet membrane GP analysis. Blood from normal donors was collected in acid-citrate-dextrose (ACD), platelets washed multiple times in phosphate-buffered saline (PBS) containing 2 mmol/L EDTA, and ^{3}H-labeled by the periodate–^{3}H-borohydride procedure.[20] For immunoprecipitation studies, labeled platelets were solubilized with 0.5% (vol/vol) Nonidet P40 (NP-40) in the presence of the inhibitors iodoacetamide (10 mmol/L), phenylmethylsulfonylfluoride (1 mmol/L), and aprotinin (1%) (all obtained from Sigma Chemical Co, St Louis, MO) and further processed as described by Miller et al.[21] Immunoprecipitates were subsequently prepared using 10^{8} platelet equivalents of NP-40 lysate incubated for 18 hours at 4°C either with 7.5 μg of the anti-GPIb monoclonal antibody (MoAb) AS-7[22] or with the anti-GPIX MoAb Beb-1[23] (a kind gift of Dr John Kelton, Hamilton, Ontario, Canada), together with 150 μL of goat antimouse Ig coupled to agarose beads (Sigma). The agarose beads were washed exhaustively in Tris-buffered saline with 0.1% NP-40, and the immunoprecipitate complexes were then eluted from the agarose beads in 5% sodium dodecyl sulfate (SDS) in 10 mmol/L Tris, pH 6.8, and electrophoresed either nonreduced or after reduction with 2% β-mercaptoethanol on 5% to 15% exponential gradient SDS-polyacrylamide gel electrophoresis (SDS-PAGE), using the discontinuous buffer method of Laemmli.[24]

For Western blotting, gels were electrophoretically transferred to nitrocellulose by the method of Towbin et al,[25] put into 0.9% NaCl-10 mmol/L Tris-HCl, pH 7.4 (TBS) containing 5% fetal bovine serum (FBS) and 0.05% Tween 20 (Bio Rad, Richmond, CA) to saturate any free binding sites, and then incubated with polyclonal rabbit antibody raised against the purified α-chain of human platelet GPIb (a kind gift of Dr Gerald Roth, Seattle, WA) for 1.5 hours at 22°C. After three washes in TBS-Tween, the blots were then incubated with a 1:750 dilution of peroxidase-conjugated $F(ab')_2$ goat antirabbit IgG (Cooper Biomedical, Malvern, PA) in TBS-Tween-FBS for an additional 1.5 hours, then washed again three times in TBS-Tween, and finally incubated at 22°C in TBS containing 0.5 mg/mL 4-chloro-1-naphthol (Sigma) and 0.015% H_2O_2 (wt/vol) for color development.

Radioligand binding studies. Platelets from normal or patient blood anticoagulated with ACD were washed by the albumin density gradient method of Walsh et al[26] and resuspended to 50,000 platelets/μL in modified Tyrode's buffer containing 2% bovine serum albumin (BSA), pH 7.3. To 90 μL of this platelet suspension was added 25 μL of Tyrode-BSA buffer, pH 7.3, and 25 μL of serial dilutions (20 to 0.625 μg/mL) of ^{125}I-vWF labeled by the method of Fraker and Speck.[27] Ristocetin was then added (15 μL) at 0.5, 1.0, or 1.5 mg/mL, and after 1 hour of incubation at room temperature under nonstirring conditions, 50 μL of the platelet suspension was centrifuged through 300 μL of 20% sucrose in modified Tyrode-BSA buffer[28] and the platelet-associated radioactivity counted. Scatchard analysis of binding data was performed with the LIGAND (Scafit) computer program,[29] Fortran version 2.3.10 for the IBM PC.

Preparation of DNA and RNA for analyses. The preparation of genomic DNA from peripheral blood leukocytes and cDNA from platelet RNA was as previously described.[15] DNA was amplified by the polymerase chain reaction (PCR) using primer pairs based on the published genomic DNA sequence of GPIbα.[7] Primers J5a, J8, and J14 have been described elsewhere[15] and correspond to nucleotide positions 728-758, 38-60, and 1964-1987, respectively. For DNA sequence analysis the full-length coding region for mature GPIbα was amplified with primers J8 and J14. Each 100 μL reaction was in buffer consisting of 10 mmol/L Tris-HCl pH 8.3, 0.5 mmol/L $MgCl_2$, 50 mmol/L KCl, 10 pmoles of each dNTP, 50 pmoles of each primer, 5 U AmpliTaq DNA polymerase (Perkin Elmer Cetus, Norwalk, CT), and 1 μg of genomic DNA. Thermocycling was in an Eppendorf MicroCycler (Eppendorf Inc, Fremont, CA) for 35 cycles of 20 seconds at 96°C, 1 minute at 55°C, and 4 minutes at 75°C. Products from four individual PCR reactions were pooled, purified by agarose gel electrophoresis, and cloned into M13mp18 and M13mp19. Single-stranded DNA templates were then prepared from pools of 70 clones in each vector.

For allele-specific hybridizations, the GPIbα coding region between bases 60 and 728 was amplified from either genomic DNA or cDNA with primers J8 and J5a. PCR reactions were as above except that $MgCl_2$ was at 2 mmol/L and each reaction contained 1 U Taq polymerase and 50 pmol each of J8 and J5a. Thermocycling was for 40 cycles of 1 minute at 94°C, 2 minutes at 56°C, and 2 minutes at 72°C.

DNA sequence analysis. Dideoxy sequence analysis was performed by standard methods[30] using Sequenase (US Biochemicals, Cleveland, OH), α-^{35}S-dATP (Amersham Corp, Arlington Heights, IL), and the appropriate primers.

Allele-specific hybridizations. Allele-specific oligonucleotides (16-mers) were purchased from Genosys (The Woodlands, TX). J18 is an antisense oligonucleotide probe (5′-CTGAGTGAGGC-GAGTG-3′) that is the complement of the published GPIbα sequence from nucleotides 252 to 267. J19 (5′-CTGAGTGAAGC-GAGTG-3′) differs from J18 only at the position corresponding to nucleotide 259, where an A (complement of T) is present instead of a G (complement of the wild-type C), reflecting the single base difference observed in the GPIbα coding sequence of patient DNA. Probes were end-labeled using α-^{32}P-ATP and T4 polynucleotide kinase,[30] and had a specific activity of 3 μCi/pmol. Amplified DNA was denatured in 0.4N NaOH, 25 mmol/L EDTA. Approximately 25 ng of each sample was then applied to each duplicate Gene Screen Plus (Dupont New England Nuclear, Boston, MA)

nylon membrane using a Bio-Dot spotting apparatus (Bio Rad). The DNA was fixed to the nylon by ultraviolet irradiation of damp membranes for 5 minutes (Model TM-20 Transilluminator, UV Products, San Gabriel, CA). Membranes were then prehybridized for 1 hour at 65°C each in 10 mL 6X SSC, 0.5% SDS, 10 mmol/L sodium phosphate, pH 6.8, 1 mmol/L EDTA containing 120 μg/mL denatured salmon sperm DNA. Labeled probe (either J18 or J19) was then added at 0.5 pmol/mL, and membranes were hybridized for 2 hours at 49°C (for J18) or 46°C (for J19). Membranes were then washed once for 5 minutes at room temperature in 2X SSC, 0.1% SDS, followed by a high stringency wash in 6X SSC, 0.1% SDS for 5 minutes at 49°C (for J18) or 46°C (for J19). Autoradiography was then performed on the air-dried membranes.

RESULTS

The history of phenotypic expression in this kindred (Fig 1), and in particular the involvement of both the proband (patient III-1) and a half-sister (patient III-2)—who are the offspring of a symptomatic mother (patient II-2) but of two unrelated (and asymptomatic) fathers—provides strong evidence that the bleeding disorder in this family follows a pattern of autosomal dominant transmission. The results of routine hemostatic studies are shown in Table 1. Affected individuals experienced a moderate bleeding tendency, which appeared to vary in severity over the course of multiple clinic visits. However, they consistently exhibited thrombocytopenia and an increased mean platelet volume. Platelet aggregation responses to ADP, collagen, and γ-thrombin were normal in all patients studied. In contrast, ristocetin-induced aggregation was characteristically decreased. However, even with respect to a single patient, the degree of this decrease was quite variable over time. Thus, at the time of his initial study the proband's platelets exhibited 6% aggregation in response to 1.2 mg/mL ristocetin, yet in an identical study 2 years later, showed 43% aggregation. In each case, the addition of exogenous vWF did not significantly affect this response. The platelets from patients II-2 and III-2 showed 28% to 48% aggregation in response to 1.2 mg/mL ristocetin, whereas those from the clinically unaffected siblings, patients III-3 and III-4, showed much stronger responses (91% to 100%). The platelets from patients III-1 and III-2 additionally showed a decreased responsiveness to aggregation by asialo-vWF (Fig 2). In contrast, platelets from patients III-3 and III-4 produced full aggregation in response to concentrations of asialo-vWF (2 to 10 μg/mL) that produce identical results in normal individuals.[19]

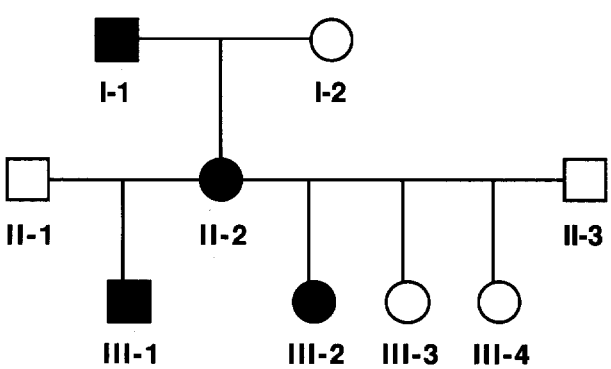

Fig 1. Family pedigree showing autosomal dominant inheritance pattern. The proband (patient III-1) and other relatives with a history of clinically significant bleeding are shown in solid symbols. Asymptomatic individuals are shown in open symbols.

To study possible quantitative abnormalities of the vWF receptor, we performed binding studies of native vWF to patient and normal platelets. In the presence of ristocetin, ^{125}I-vWF showed saturable binding kinetics over the range of 0.5 to 1.5 mg/mL ristocetin both in normals and in patients. At 1.0 and 1.5 mg/mL ristocetin, the apparent kd of ^{125}I-vWF binding to patient platelets was indistinguishable from that to normal platelets (Table 2). In contrast, at 0.5 mg/mL ristocetin, a significantly ($P < .001$) higher apparent kd was observed with patient platelets. Although total vWF bound to normal platelets typically exceeded that bound to patient platelets, such a difference was not statistically significant at any of the three ristocetin concentrations studied.

Platelets from the affected patients showed an essentially normal complement of the components of the GPIb/IX complex, as detected by SDS-PAGE of immunoprecipitates with AS-7, an MoAb recognizing an epitope in the amino-terminal region of GPIbα[22] (Fig 3), or of whole detergent lysates (data not shown). While not seen in AS-7 immunoprecipitates, an additional band of 140 Kd nonreduced (bands of 115 and 105 Kd reduced) coprecipitated with an antibody directed against GPIX in patient lysates (Fig 3, arrows). These new bands consistently represented a relatively small proportion of the total GP precipitable by the anti-GPIX MoAb, as determined by scanning densitometry of the gels. For example, the 140-Kd band (nonreduced) seen in the experiment shown in Fig 3 represented only 23% as much density as the patient band comigrating with normal GPIb, and the 115- and 105-Kd bands (reduced) showed only 4% and 13%, respectively, as much density as the patient band comigrating with normal GPIbα. When platelet lysates immunoprecipitated with the anti-GPIX MoAb were electrophoresed and subsequently transfered to nitrocellulose, Western blotting with a polyclonal anti-GPIbα antibody confirmed that the 140-Kd (nonreduced) patient band was indeed a derivative of the GPIbα chain (Fig 4). The polyclonal anti-GPIbα antibody also stained a band migrating immediately below that of intact GPIb. While the intensity of staining of this band was greater in the patient than in the normal control, this immunoreactive derivative of GPIbα, unlike the 140-Kd band, was not unique to patient platelets and likely reflects normal proteolytic degradation of GPIb, although possibly at a heightened rate.

Because the entire protein coding region of the GPIbα gene is contained within a single exon,[31,32] we were able to perform DNA sequence analysis using genomic DNA obtained from circulating leukocytes. Using a series of oligonucleotide PCR primer pairs,[15] we consistently observed identically migrating bands of amplified DNA in patients as compared with normal controls, suggesting the absence of any major deletions within the gene. DNA sequencing of patient III-2 confirmed the absence of any

Table 1. Family Studies

	II-2	III-1	III-2	III-3	III-4
Bleeding episodes	Tooth extractions Tonsillectomy Menorrhagia	Severe epistaxis Tooth extractions	Epistaxis Menorrhagia	Negative history	Negative history
Bleeding time (2-8 min)	5	8.5-23	5.5-14.5	8	4
Platelet count (150-400 × 10^3/L)	65-81	63-128	87-109	306	403
Platelet volume (6.9-10.4 fL)	18.3-21.2	19.9-21.1	14.5-15.9	8.9	7.1
PRP platelet aggregation					
Ristocetin 1.2 mg/mL	Decreased	Decreased	Decreased	Normal	Normal
Ristocetin 1.2 mg/mL + 2 U/mL vWF	NP	Decreased	Decreased	NP	NP
Collagen 20 μg/mL	Normal	Normal	Normal	Normal	Normal
ADP 8 μmol/L	Normal	Normal	NP	Normal	NP
γ-Thrombin 135 nmol/L	Normal	Normal	Normal	Normal	Normal

Abbreviation: NP, not performed.

substitutions or deletions of nucleotides, as compared with the normal genome, throughout the entire protein coding region, with a single exception. At nucleotide 259 the patient DNA showed a heterozyzous substitution of a T for the C normally present at this position (Fig 5). This substitution was nonconservative, resulting in the replacement of a phenylalanine for a leucine at residue 57 of the mature GPIbα molecule. With respect to the recently described[33,34] normal polymorphic variation of a 13 amino acid sequence within the heavily glycosylated macroglycopeptide region, patient III-2 was homozygous for the duplicated form.

The single base substitution at nucleotide 259 did not create or destroy the recognition sequence of any known restriction enzyme. Allele-specific hybridization was used to determine the distribution of this substitution in family members and in the normal population. As shown in Fig 6A, probe J19, which detects the substituted T at position 259, hybridized with genomic DNA from all affected patients studied, but not from normal controls or from the phenotypically normal patient III-3; probe J19 similarly did not hybridize with genomic DNA from patient III-4 (data not shown). In contrast, probe J18, which detects the wild-type C at position 259, hybridized to the genomic DNA from all affected patients, from the unaffected family members (patients III-3 and III-4), and from the normal controls. This technique accordingly confirmed that the substitution was heterozygous in the affected family members. Allele-specific hybridization with probes J18 and J19 was studied with the genomic DNA from 133 normal individuals. In all cases only probe J18 showed hybridization, indicating a wild-type pattern for all 266 alleles.

Expression of this substitution in patient platelets was investigated by PCR analysis of reverse-transcribed platelet messenger RNA (mRNA). As in the case of genomic DNA, no gross deletions were seen in patient samples. Allele-specific hybridization of cDNA reverse-transcribed from the platelet mRNA of patient III-2 identified expression of both the wild-type and substituted sequences, whereas only the wild-type sequence was identified in normals (Fig 6B).

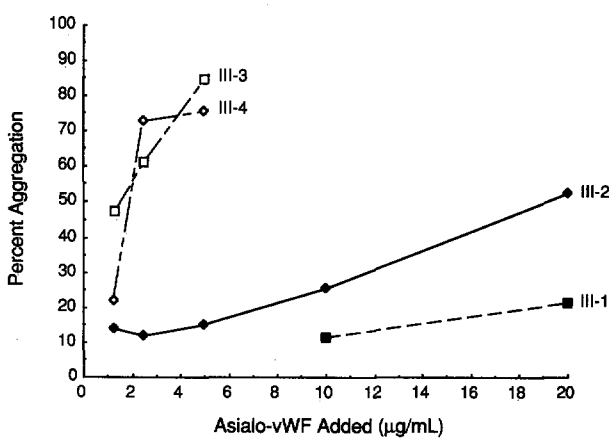

Fig 2. Asialo-vWF–induced aggregation of platelets. PRP was maintained at 37°C and stirred at 1,200 rpm. Asialo-vWF was added at the indicated final concentrations. Percent aggregation (ie, maximal extent of aggregation) of patients III-3 and III-4 is indistinguishable from that of normal controls, whereas that of patients III-1 and III-2 is decreased.

Table 2. ^{125}I-vWF Binding to Washed Platelets

Ristocetin	Patient III-1	Patient III-2	Patient Mean	Control Group	*P* Value
0.5 mg/mL					
kd*	3.70	4.34	4.02	1.39 ± 0.29	<.001
B_{Max}†	1.24	2.03	1.64	1.95 ± 0.60	.535
1.0 mg/mL					
kd	1.00	0.73	0.87	0.91 ± 0.22	.798
B_{Max}	1.38	1.41	1.40	2.25 ± 0.83	.213
1.5 mg/mL					
kd	0.83	0.74	0.79	0.75 ± 0.18	.773
B_{Max}	1.36	1.61	1.48	2.44 ± 1.01	.253

Platelets from freshly drawn blood were washed by albumin density gradient centrifugation, suspended in modified Tyrode medium, and incubated with varying dilutions of ^{125}I-vWF and ristocetin, as described in Materials and Methods. The platelets were than centrifuged through a layer of 20% sucrose containing 2% BSA, and the platelet-associated radioactivity counted. Data represent the specific binding of vWF, based on estimates of 0.3% to 2.6% nonspecific binding by the LIGAND[28] nonlinear curve fitting program. Control group data are mean ± SD, n = 6. Statistical significance for difference of patient mean from control mean was analyzed by the unpaired, two-tailed *t*-test; a significant probability (*P* value) was achieved only for the kd at a ristocetin concentration of 0.5 mg/mL.

*Apparent dissociation constant in micrograms per milliliter.

†Maximal binding in micrograms per 10^8 platelets.

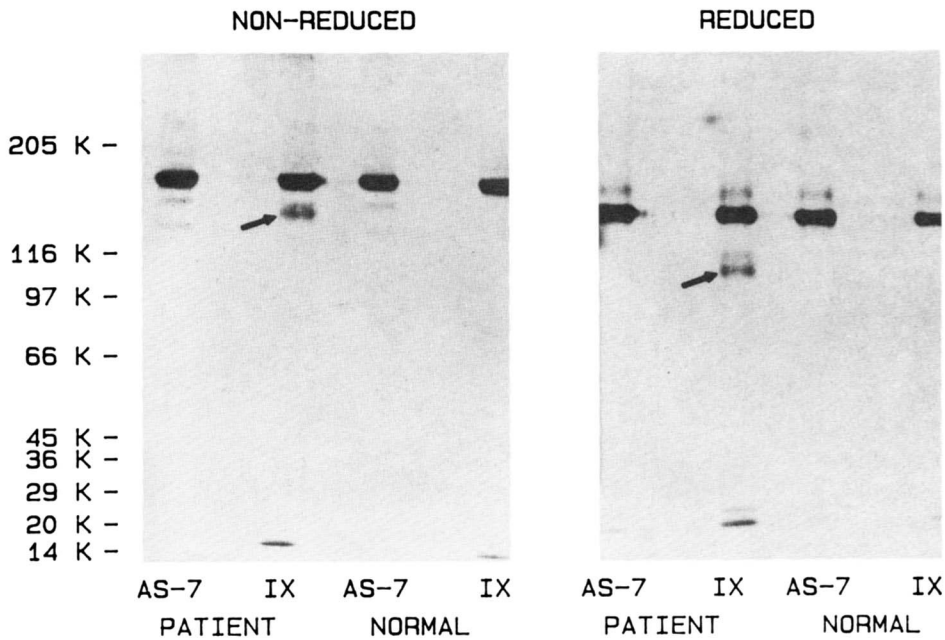

Fig 3. Autoradiogram of SDS-PAGE (5% to 15% exponential gradient) showing immunoprecipitates with an MoAb directed against GPIb (AS-7) or an MoAb directed against GPIX (IX) obtained with ^{125}I-labeled platelets from patient II-2 (PATIENT) or from a normal control. The arrows point to bands (140 Kd nonreduced, 115 and 105 Kd reduced) observed only in the anti-GPIX immunoprecipitates of the patient.

DISCUSSION

This study provides the first demonstration of a specific amino acid substitution in a component of the platelet GPIb/IX receptor complex for vWF in patients exhibiting a BSD phenotype. Full concordance within the studied family between phenotypic expression and a heterozygous single nucleotide substitution in genomic DNA coding for a phenylalanine in place of the wild-type leucine at residue 57 of the mature GPIbα, absence of this substitution in 266 alleles from the normal population, and the lack of any other abnormality of patient DNA throughout the entire coding sequence for GPIbα provide strong support that this substitution may constitute a pathologic point mutation responsible for the observed phenotypic abnormalities.

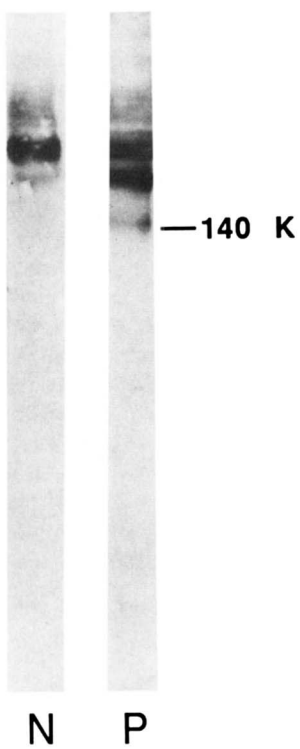

Fig 4. Western blot of normal (N) and patient (P) GPIX immunoprecipitates of platelet lysates run on 5% to 15% SDS-PAGE (nonreduced), electrophoretically transferred to nitrocellulose, and immunoblotted with polyclonal rabbit antibody directed against the α-chain of human platelet GPIb. The line indicates a band of 140 Kd, identified in the patient sample only, that migrates at a faster rate than the GPIb seen above this band in both the patient and normal samples.

GP analyses of patient platelets showed the presence of a 140-Kd band (nonreduced) that migrates faster than normal GPIb on SDS-PAGE gels, but that shows immunologic reactivity with polyclonal anti-GPIbα antibody. Because an MoAb directed against GPIX is able to immunoprecipitate this abnormal band from detergent lysates of patient platelets, while AS-7, an MoAb directed against the amino-terminal portion of GPIbα, is not, the new band appears to represent an incomplete portion of GPIbα lacking the amino-terminal region of the peptide chain. Aberrant de novo synthesis of this region of the GPIbα chain appears unlikely, particularly in view of the absence of any DNA sequence abnormalities within the coding region of this gene, other than the single point mutation of a C to a T at nucleotide position 259. A more likely possibility would appear to be that this mutation, resulting in the substitution of a phenylalanine for the wild-type leucine at residue 57 of the mature GPIbα, alters the susceptibility of the chain to proteolytic degradation, resulting in GPIbα derivatives lacking the normal amino-terminal region. The presence of immunoreactive GPIbα migrating with faster mobility than native GPIbα was also observed in an Italian BSD kindred by DeMarco et al,[17] who postulated increased susceptibility to proteolysis within a protease-sensitive region of the chain.

In the present study, localization of the mutation to leucine-57 is particularly interesting, because this involves a highly conserved leucine residue within the leucine tandem repeat region of GPIbα.[7,8,35] As Roth has recently reviewed,[35] GPIbα is a member of a family of leucine-rich GPs in which leucine residues appear at regular intervals within

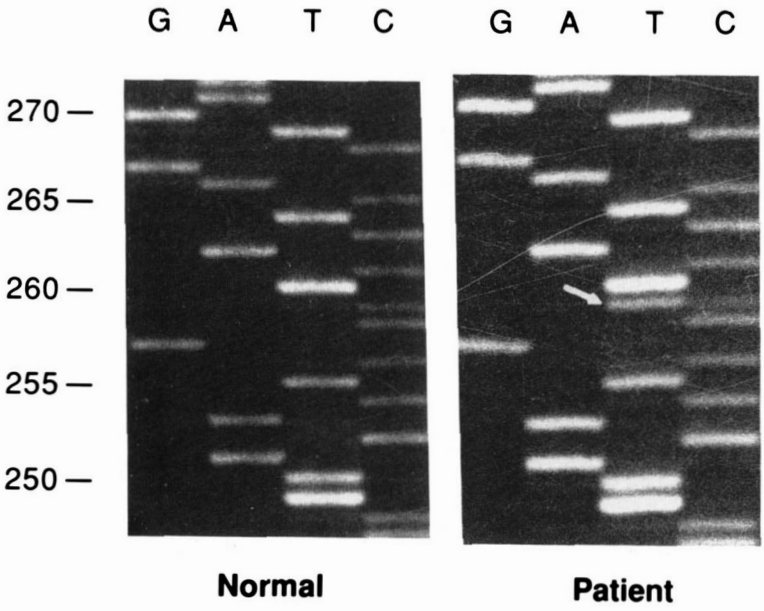

Fig 5. DNA sequence analysis of GPIbα gene. Genomic DNA amplified by the primer pair J8/J14 was cloned into M13mp18 and then sequenced. The heterozygous presence of a C and a T at nucleotide position 259 (arrow) in a pool of 70 individual M13mp18 clones of amplified DNA from patient III-2 (Patient) contrasts with the homozygous wild-type C seen in a normal individual (Normal).

sequential 24 amino acid repeating segments ("tandem repeats"). The leucine tandem repeats must be distinguished from "leucine zippers" in which leucine residues occuring regularly at every seventh position are believed to promote association between polypeptide segments,[36,37] apparently through the formation of coiled coils.[38,39] While functional implications of the leucine tandem repeat motif are still largely speculative, the presence of hydrophobic and hydrophilic regions contributing to potentially amphipathic structures suggests that this motif may also be involved in associations between polypeptide segments.[40-43] If the leucine tandem repeats of GPIbα were indeed involved in such associations, then disruption of the tandem repeat by substitution of a phenylalanine for a highly conserved leucine might result in an abnormally increased exposure of the chain, with the possibility of increased susceptibility to proteolysis. If the substitution of phenylalanine at residue 57 produced a large enough perturbation of protein three-dimensional structure, increased sensitivity to proteolysis might even occur at sites distant from the mutation itself. Finally, the identification of bands at both 115 and 105 Kd by SDS-PAGE, after the reduction of all disulfide bonds, raises the possibility that proteolytic cleavage may be occuring at more than a single site.

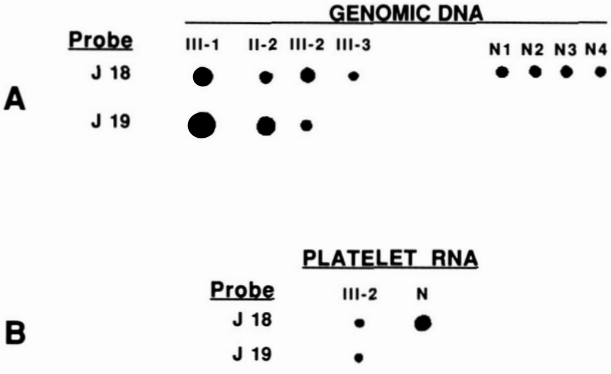

Fig 6. Allele-specific oligonucleotide hybridization for C to T mutation at nucleotide 259 of GPIbα. (A) Genomic DNA of patients II-2, III-1, III-2, and III-3, or of normal controls (N1 through N4), was hybridized, as described in Materials and Methods, both with the wild-type probe J18 and with probe J19 that detects the substitution of a T for the wild-type C at nucleotide position 259. (B) DNA obtained by RT/PCR of platelet RNA from patient III-2 or from a normal control (N) was hybridized with probes J18 and J19, as described in Materials and Methods.

We have consistently observed that the 140-Kd (nonreduced) immunoreactive GPIb found in these patients represents only a relatively small proportion of the total GPIb—never equal in amount to that comigrating with normal GPIb. If the hypothesis of increased susceptibility to proteolysis is correct, then this observation may be explained by the normally migrating GPIb from patient platelets representing protein coded for by both the normal and the mutant alleles, but where the abnormal GPIbα containing phenylalanine-57 has not yet undergone proteolytic degradation.

The major functional abnormalities of the patient platelets seen in vitro are a decreased binding affinity for native vWF demonstrable at low (0.5 mg/mL) ristocetin concentration, decreased vWF-dependent aggregation demonstrable at even relatively high (1.2 mg/mL) ristocetin concentration, and a decreased aggregation response to asialo-vWF. It is possible that the substitution of phenylalanine for leucine-57 produces a conformational change that does not favor the binding of vWF at relatively low ristocetin concentration and that impedes platelet agglutination or aggregation after the initial binding of vWF at higher ristocetin concentrations. The increased bleeding tendency of the affected patients may thus be related to impaired interaction between platelets and vWF in vivo. However, because all affected patients in this family are only heterozy-

gous for the leucine to phenylalanine substitution, the presence of one normal allele may well allow the platelets to retain a degree of functional integrity sufficient to prevent the more severe bleeding tendency typically associated with classic BSD.

As discussed above, it is not currently known what role(s) the leucine tandem repeats may normally play within the GPIb/IX complex[35]; indeed, the present study represents the first example of a perturbation of such a repeat within this complex. It is possible that GPIbα chains normally self-associate through the leucine tandem repeats, and that the phenylalanine-57 mutation reduces the extent of such self-association. A second possibility is that high affinity binding of vWF to the platelet GPIb/IX complex might be dependent on heterodimers forming between leucine tandem repeats of GPIbα and those present in GPIbβ,[8] GPIX,[9] or possibly even GPV.[44] Alternatively, the leucine to phenylalanine mutation in GPIbα might produce changes in the three-dimensional structure of GPIbα directly affecting the binding sites for vWF. The development of in vitro systems in which mutant GPIbα may be expressed with and without the concomitant production of GPIbβ or GPIX may eventually permit direct evaluation of these various possibilities. Additionally, because the natural occurence of the phenylalanine-57 mutation has so far been observed only in heterozygous expression in patients showing austosomal dominant transmission of the disorder, in vitro production of this mutation may provide a means to observe the effects of an essentially homozygous expression upon vWF-platelet interactions.

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