Point Mutation in a Leucine-rich Repeat of Platelet Glycoprotein Ibα Resulting in the Bernard-Soulier Syndrome

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Abstract

Leucine-rich repeats are a conserved structural motif, of yet undefined significance, found in a group of proteins from different species. Among these are the four components of the human platelet glycoprotein Ib-IX-V complex, a membrane receptor that performs an essential role in the thrombogenic function of platelets by interacting with the adhesive protein, von Willebrand factor. We have found that a single amino acid substitution (Ala^{156} → Val) within one of the six leucine-rich repeats in the α-subunit of glycoprotein Ib results in a variant form of the congenital bleeding disorder, Bernard-Soulier syndrome, characterized by giant dysfunctional platelets. Genetic studies of the propositus and his family members were complemented by immunological and functional analysis of expressed recombinant GP Ibα fragments to demonstrate that the observed mutation is the cause of defective von Willebrand factor binding. These studies define the molecular basis of the Bernard-Soulier syndrome within this family and demonstrate that structural integrity of a leucine-rich repeat is necessary for normal function of the glycoprotein Ib-IX-V receptor complex and, possibly, for normal platelet morphology. (*J. Clin. Invest.* 1993. 92:1213–1220.) Key words: thrombosis • bleeding disorder • von Willebrand factor • platelet adhesion • giant platelets

Introduction

The Bernard-Soulier syndrome (1, 2) is a congenital bleeding disorder characterized by giant platelets with an absent or dysfunctional platelet membrane receptor, the glycoprotein (GP)[1] Ib-IX-V complex (3). At sites of vascular injury, the GP Ibα component of this receptor interacts with the adhesive protein, von Willebrand factor (vWF), to support platelet adhesion and initiate thrombus formation (3). The GP Ib-IX-V receptor is assembled from four distinct gene products into a hetero-oligo-

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1. *Abbreviations used in this paper:* CHO, Chinese hamster ovary; GP, platelet membrane glycoprotein; PCR, polymerase chain reaction.

meric complex composed of the disulfide-linked α- (4, 5) and β-subunits (6) of GP Ib associated in a noncovalent manner with GP IX (7) and GP V (8, 9). Most cases of Bernard-Soulier syndrome may be caused by a genetic defect within one of the subunits, preventing a coordinated assembly of the complex and, thus, membrane expression (10). The validity of this concept has been proven in a family bearing a nonsense mutation in the GP Ibα gene (11) and by the coordinated expression of a recombinant GP Ib-IX complex on the surface of heterologous cells (12).

In contrast to the classical form of the disease, a previously characterized variant of Bernard-Soulier syndrome, designated type Bolzano, was found to express a dysfunctional GP Ib-IX-V complex on the surface of giant platelets that lacked the ability to bind vWF (13). In this report we demonstrate that the latter abnormality is caused by an Ala^{156} → Val substitution within the conserved leucine-rich motif present in GP Ibα (14). Expression of an isolated recombinant GP Ibα analogue containing the mutation resulted in a structural and functional abnormality equivalent to that associated with type Bolzano platelets. These findings establish that the leucine-rich motif is an important structural element in the GP Ib-IX-V complex and is required for normal platelet activity in hemostasis.

Methods

Bernard-Soulier syndrome, type Bolzano. The propositus was previously described as a typical case of Bernard-Soulier syndrome, with giant platelets exhibiting a markedly decreased vWF-binding capacity (13, 15). Detailed immunochemical studies revealed also a major structural abnormality of GP Ibα characterized by the absence of conformation-dependent epitopes recognized by specific monoclonal antibodies (13).

Genetic studies. Blood samples were obtained from the propositus, other family members, and normal blood donors recruited through the General Clinical Research Center of Scripps Clinic. All individuals were informed of the purpose of these studies and gave their consent according to the Declaration of Helsinki. Genomic DNA was isolated from peripheral lymphocytes using standard procedures (16). Bacteriophage clones containing the propositus' GP Ibα gene were isolated from a library of EcoRI restriction fragments propagated in the cloning vector λZAP (Stratagene Cloning Systems, La Jolla, CA) (17). Purified bacteriophages were characterized by restriction enzyme mapping, and this analysis identified an EcoRI fragment of ~ 6,000 bp in length containing the entire GP Ibα gene; this fragment was subsequently subcloned into pBS/SK⁻ for DNA sequence analysis. The coding sequence for the propositus' GP Ibα was also amplified by polymerase chain reactions (PCR) (18) into a series of overlapping fragments that generated a composite array of the GP Ibα gene. PCR fragments were subcloned into the M13 vector before DNA sequence analysis. The latter was performed using GP Ibα-specific oligonucleotides synthesized according to published GP Ibα sequences (5, 19). Southern transfer and hybridization were performed essentially as described (17).

Two missense mutations corresponding to residues 145 and 156 of GP Ibα were identified during the course of these studies. A 591-bp fragment containing the coding sequence for GP Ibα residues 106–302 (residue numbers are referenced from His[1] of mature GP Ibα) was generated by PCR and used for the characterization of the two mutations. The oligonucleotide primers used in the PCR contained nucleotides 899–919 and 1470–1489, as numbered in the published sequence (19).

Monoclonal and polyclonal antibodies. The production and characterization of the anti-GP Ibα monoclonal antibodies used in these studies have been described elsewhere (20–22). Antibody LJ-Ibα1 recognizes an epitope located within residues 1–237 and reacts with SDS-denatured and reduced GP Ibα antigen. Antibodies LJ-Ib1, LJ-P3, and LJ-P19 react with epitopes that are destroyed by treatment with SDS and are located in an amino-terminal 45-kD tryptic fragment of GP Ibα composed of residues 1–293. A rabbit polyclonal antibody was generated using a synthetic peptide composed of GP Ibα residues Gly^{271}-Glu^{285}.

Expression of recombinant GP Ibα fragments. The characterization of a recombinant plasmid synthesizing a functional amino-terminal domain of GP Ibα has been described in detail elsewhere (22). Briefly, a mammalian cell expression plasmid was constructed from a fragment of the GP Ibα gene (coding for signal peptide and mature residues His^1-Ala^{302}) synthesized in a PCR that added BamHI restriction sites on the ends of the amplified fragment. The amplified GP Ibα fragment was cloned into M13mp19 as a BamHI fragment and sequenced to verify that no spontaneous mutations had arisen during the PCR. The GP Ibα insert was removed from the M13 construct by digestion with EcoRI and XbaI and cloned into the same restriction sites of the polylinker region of PBS/KS⁻ (Stratagene). As a result of cloning into PBS/KS⁻, restriction sites for XhoI (5′ to the GP Ibα initiating Met codon) and NotI (3′ to the Ala^{302} codon) were acquired from the polylinker region and were used to clone the fragment into the mammalian cell expression plasmid, pCDM8^{neo}. The latter is identical to pCDM8 (23) except that it contains a neomycin gene for conferring resistance to the aminoglycoside antibiotic, G418 or Geneticin (Sigma Chemical Co., St. Louis, MO).

Mutations within the expression plasmid were constructed using mutagenic oligonucleotides and site-directed mutagenesis on uracil-containing templates of the original M13 construct (24). After mutagenesis and subcloning into PBS/KS⁻ (described above), the mutant GP Ibα coding regions were completely sequenced to ensure that no additional mutagenic errors had occurred and then were cloned into the pCDM8^{neo} expression plasmid.

Chinese hamster ovary (CHO-K1) cells were maintained in 5% CO_2 and grown in DME supplemented with 10% fetal calf serum (10%-DME), 0.5 mM nonessential amino acids, and 2 mM L-glutamine (Whittaker Bioproducts, Walkersville, MD). DNA (10 μg per dish) was introduced into cells (subcultured at a density of 1.5×10^5 per 60-mm dish 24 h before transfection) using a calcium phosphate–mediated transfection procedure (25); cells were then maintained in 10%-DME.

After transformation of CHO-K1 cells with each plasmid, cell lines secreting the recombinant GP Ibα fragments were identified in preliminary experiments by evaluating immunoreactivity with the anti-GP Ibα monoclonal antibody LJ-Ibα1. Subsequent screening was performed with the rabbit polyclonal antibody recognizing a GP Ibα epitope within Gly^{271}-Glu^{285}; this linear epitope was assumed to be equally expressed in all the recombinant fragments regardless of the mutations at positions 145 and 156. This analysis allowed comparable amounts of each recombinant fragment to be tested for immunoreactivity with the conformation-dependent anti-GP Ibα monoclonal antibodies LJ-Ib1, LJ-P3, and LJ-P19. For the detection of recombinant GP Ibα antigen, a confluent flask of cells was cultured in the absence of fetal calf serum (24 h) and harvested for immunoblotting. The dot-blot technique used to evaluate the immunochemical reactivity of recombinant fragments has been described in detail elsewhere (22). In brief, proteins in the culture medium were immobilized onto nitrocellulose membranes by filtration, reacted with the appropriate specific anti-

body, and detected by autoradiography after incubation with ^{125}I-labeled rabbit anti–mouse IgG or ^{125}I-labeled goat anti–rabbit IgG (22).

vWF binding to immobilized recombinant fragments. The method used for this assay has been described in detail elsewhere (22). All reagents were filtered (in ∼ 5 min for each step) through a nitrocellulose membrane (0.45-μm pore size; Bio-Rad Laboratories, Hercules, CA) using a peristaltic pump applied to a device that delimits a circular area of application (22). All the steps of this assay were performed at room temperature (22–25°C). Serum-free culture media from different cell lines were either diluted with the appropriate amount of serum-free medium from nontransfected CHO-K1 cells or used in volumes (30–400 μl) inversely proportional to the content of recombinant protein so that, in either case, comparable amounts of GP Ibα fragment were immobilized onto the nitrocellulose membrane. The amount of each mutant fragment used in the binding assay was nonlimiting, such that doubling it caused < 10% increase in vWF binding. After binding the medium from the appropriate cell line, the membrane was blocked with three washes (200 μl each) of a solution containing 20 mM Hepes and 150 mM NaCl, pH 7.4 (Hepes buffer), with 1% bovine serum albumin (fraction V; Calbiochem Corp., La Jolla, CA) and 1% bovine γ-globulin (prepared from plasma, 99% electrophoretic purity; Sigma Chemical Co.). After blocking, 50 μl of ^{125}I-labeled vWF, preincubated for 30 min with either botrocetin or ristocetin as modulators of binding (22), was filtered through the nitrocellulose membrane. The specific activity of the different preparations of ^{125}I-vWF used varied between 13 and 140 μCi/mg. Ristocetin (Sigma Chemical Co.) was used at the final concentration of 1 mg/ml; botrocetin (two-chain form) was purified as previously described in detail (26, 27) from the crude venom of *Bothrops jararaca* (Sigma Chemical Co.) and was used at the final concentration of 10 μg/ml. When indicated, appropriate anti–GP Ibα monoclonal antibodies (the combination of LJ-Ib1 and LJ-P19, 100 μg/ml each) were added to these mixtures before filtering through the nitrocellulose; a solution of the same antibodies was also filtered through the membrane before application of the vWF-containing mixture. After two additional washes in the blocking solution, the membrane was dried, the portion of the membrane corresponding to each application well was cut out, and the bound radioactivity determined in a γ-scintillation spectrometer. In the assays using the modulator ristocetin, the latter was also present in the final washing fluid at the same concentration used during the incubation with vWF. It is important to note that the addition of bovine γ-globulin to the solution used to block the nitrocellulose membrane proved essential to reduce the level of nonspecific binding, particularly when ristocetin was utilized as modulator. Binding was considered nonspecific when it occurred in the absence of recombinant GP Ibα fragment and/or was not inhibited by appropriate anti-GP Ibα monoclonal antibodies. Indeed, in several experiments performed without the use of γ-globulin in the blocking solution, we found variable nonspecific vWF binding to one or more component present also in nontransfected CHO-K1 cell culture media. In total, of nine different control media tested for apparent vWF binding in the presence of ristocetin, only three exhibited values corresponding to background; four exhibited apparent binding that was ≥ 50%, and two > 100% of that measured with the medium containing normal GP Ibα fragment. The same variable level of vWF binding was observed with fragments containing the Ala^{156} → Val mutation; in either case, however, this nonspecific binding was essentially abolished when the nitrocellulose membrane was blocked with the solution containing bovine γ-globulin (see Results). Use of the latter, therefore, represents a crucial modification of the method previously described to measure vWF binding to recombinant GP Ibα fragments (22).

Results

Characterization of the GP Ibα gene associated with the Bernard-Soulier syndrome, type Bolzano phenotype. The structural GP Ibα defect in platelets from the propositus with the type Bolzano phenotype was previously demonstrated by the
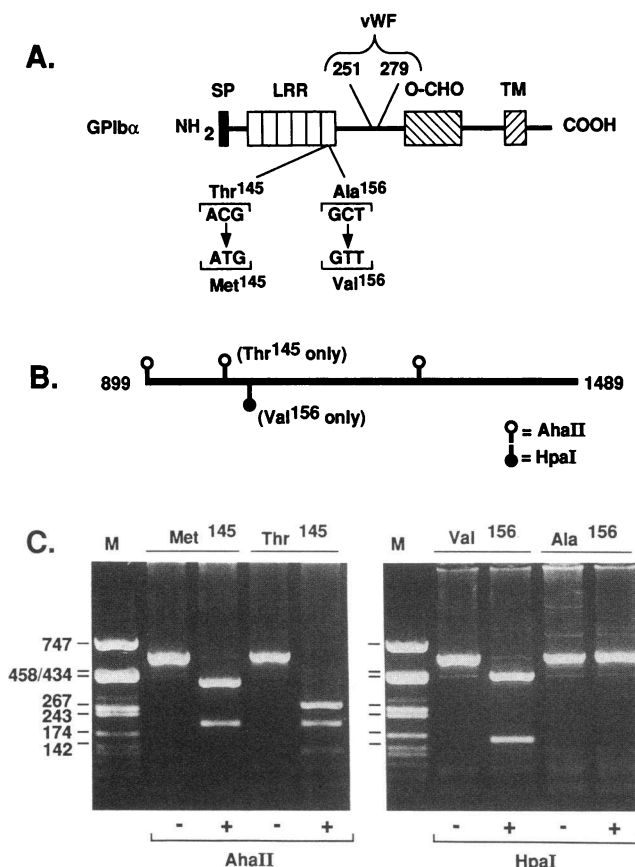
Figure 1. Primary structure of the GP Ibα protein, PCR strategy and restriction enzyme analyses. (A) The precursor α-subunit of GP Ib consists of a signal peptide (SP) and a 610-residue polypeptide both encoded within a single exon (19). The mature GP Ibα subunit contains six repeats of a leucine-rich motif (LRR) (residues 36–176), a binding site for von Willebrand factor (vWF), a carbohydrate-rich region (O-CHO), a transmembrane domain (TM), and an intracytoplasmic carboxyl-terminal tail (4, 5). As compared with the published GP Ibα gene sequences, genetic analysis of the propositus identified Thr^{145} → Met and Ala^{156} → Val codon substitutions, which abolish an AhaII restriction site and create a HpaI restriction site, respectively. This is documented in B and C by restriction enzyme digestion of a 591-bp fragment generated by PCR using the propositus' genomic DNA (Met^{145}/Val^{156}) and DNA from a normal control (Thr^{145}/Ala^{156}). The characterization of genomic DNA samples revealed that the Thr^{145} → Met codon substitution is a naturally occurring dimorphism that produces restriction fragments of 271, 201, and 116 bp for a Thr^{145}-containing allele, or fragments of 387 and 201 bp for a Met^{145}-containing allele. A 591-bp fragment containing a Val^{156} codon produces fragments of 439 and 152 bp after digestion with HpaI. Agarose electrophoresis of the amplified 591-bp fragment is shown either before (−) or after (+) digestion with the relevant restriction enzyme. Molecular weight standards (M) are indicated in base pairs.

abnormal interaction with specific monoclonal antibodies (13). Thus, to define the molecular basis of this variant form of Bernard-Soulier syndrome, we aimed our studies at a characterization of the gene encoding the α-chain of GP Ib. Nucleotide sequence analysis was obtained from restriction enzyme fragments of bacteriophage λ clones containing a complete copy of the propositus' GP Ibα gene as well as from DNA sequence analysis of PCR-generated fragments. Two C to T missense mutations were found on a continuous fragment derived from

the GP Ibα gene of the propositus, indicating that both were present on the same allele: the codon for Thr^{145} (ACG) was changed to Met (ATG), and the codon for Ala^{156} (GCT) was changed to Val (GTT). DNA analysis by restriction enzyme digestion demonstrated that the propositus was homozygous for the two mutations (Fig. 1), whereas his parents were both heterozygous with cosegregation of the Met^{145} and Val^{156} codons (not shown). The Thr^{145} → Met mutation was subsequently determined to be a genetic dimorphism found in the normal population with an allele frequency of 11% (n = 122) (28, 29); the Ala^{156} → Val mutation, in contrast, was not detected in DNA fragments generated from normal individuals. The propositus was also homozygous, and both his parents were heterozygous, for a triplication of 39 nucleotides encoding residues 399–411 (Fig. 2). This is another GP Ibα polymorphism, described also by others (30), that in our studies was found to occur with an allele frequency of 13% (n = 24).

Immunologic consequences of the α-subunit missense mutations. We have previously shown that the amino-terminal domain of GP Ibα is structurally and functionally mimicked by a 302-residue recombinant fragment that can be independently expressed and secreted as a soluble species from CHO-K1 cells (22). The expressed normal fragment is recognized by a panel of conformation-specific anti–GP Ibα monoclonal antibodies,
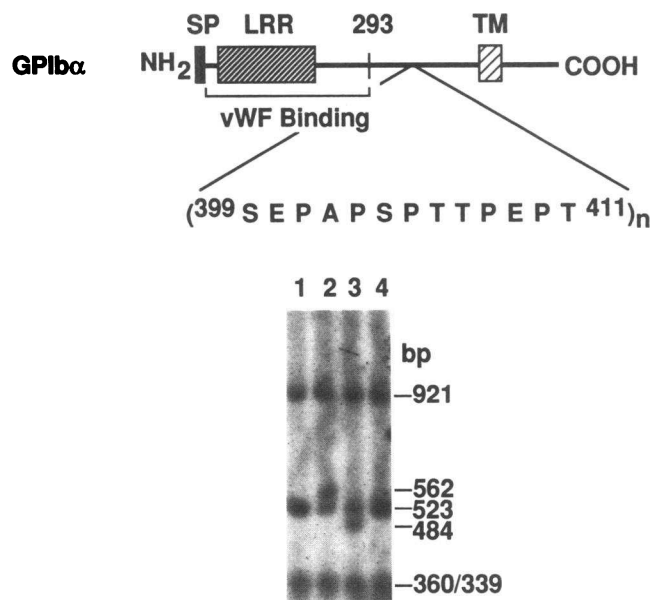

Figure 2. Southern blot analysis of a hypervariable motif within the extracytoplasmic carbohydrate-rich domain of GP Ibα. Genomic DNA from normal individuals was digested with AvaII and electrophoresed through a 1% agarose gel. The DNA was transferred to nitrocellulose via the Southern procedure and hybridized to a radiolabeled DNA fragment containing the entire coding sequence of GP Ibα. The autoradiograph illustrates a single band of 921 bp and a doublet of 360 and 339 bp identical in all samples. In contrast, the hypervariable region corresponding to the polymorphic motif encoding GP Ibα residues Ser^{399}-Thr^{411} produces fragments ranging in size from 484 to 562 bp, as shown by the four different DNA samples from normal individuals. A triplication of this 13-residue sequence was originally found in the propositus' DNA, corresponding to the 562-bp fragment seen in the normal sample in lane 2. The DNA samples shown in lanes 1 and 4 contain homozygous alleles for the 523-bp fragment; in lane 2, heterozygous alleles with the 562- and 523-bp fragments; and in lane 3, heterozygous alleles with the 523- and 484-bp fragments.
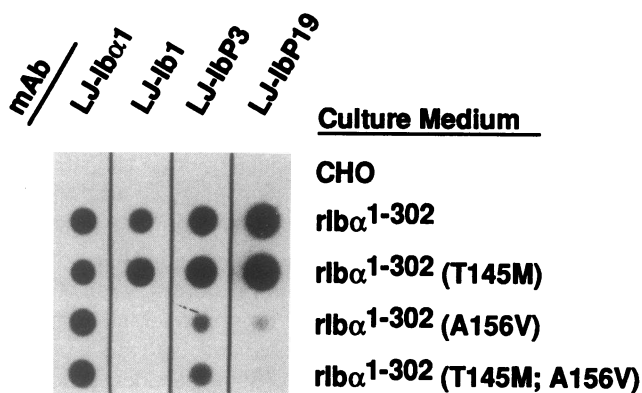
Figure 3. Epitope analysis of recombinant GP Ibα fragments by non-denaturing dot blot. Proteins in culture media were immobilized onto a nitrocellulose membrane; the latter was then saturated with a blocking solution and incubated with the appropriate mouse monoclonal antibody; positive reactivity was detected by incubation with ^{125}I-labeled rabbit anti–mouse IgG and autoradiography. A panel of anti-GP Ibα monoclonal antibodies was tested for immunoreactivity with culture media obtained from nontransfected CHO-K1 cells (CHO); cells secreting a recombinant fragment with the more common (Thr[145]) GP Ibα sequence, rIbα[1-302]; cells secreting the alternative Met[145] dimorphic residue, rIbα[1-302](T145M); cells secreting the Ala[156] → Val substitution, rIbα[1-302](A156V); cells secreting both the Thr[145] → Met and Ala[156] → Val substitution, rIbα[1-302](T145M;A156V). The monoclonal antibody LJ-Ibα1 recognizes an epitope present on reduced and denatured platelet GP Ibα; LJ-Ib1, LJ-P3, and LJ-P19 each recognize epitopes that are destroyed by denaturation. The LJ-Ib1 epitope was previously reported to be abnormal on the propositus' platelets (13), as it is in the mutant recombinant fragments containing Val[156]; in contrast, the LJ-Ibα1 epitope is normal in all the fragments. LJ-P19 is a new monoclonal antibody recognizing a distinct conformation-dependent epitope in the amino-terminal domain of GP Ibα and confirms the results obtained with LJ-Ib1 and LJ-P3.

each reacting with a distinct epitope within the amino terminus of the molecule (22). The epitope of one of these antibodies, designated LJ-Ib1, was previously found to be typically absent in platelets from the type Bolzano propositus (13). Therefore, to evaluate the immunological consequences of the identified mutations, we expressed fragments bearing the dimorphism (Thr[145] → Met) and the unique point mutation identified in the Bernard-Soulier variant (Ala[156] → Val) either independently or in association (the latter representing the genotype of the propositus) and compared their immunoreactivity to that of the GP Ibα fragment with normal sequence. Like platelets from the propositus (13), the GP Ibα fragment bearing the Thr[145] → Met and Ala[156] → Val mutations had markedly decreased reactivity with LJ-Ib1 and other conformation-dependent anti–GP Ib monoclonal antibodies (Fig. 3). The fragment bearing the single Ala[156] → Val mutation also demonstrated a similar abnormal reactivity, but a fragment with the single Thr[145] → Met mutation reacted normally (Fig. 3). This demonstrates that the Ala[156] → Val mutation is responsible for the alteration in antibody recognition associated with the propositus' abnormal GP Ib-IX-V complex on platelets, regardless of the dimorphic residue at position 145. All mutant fragments reacted equally well with the conformation-independent antibody, LJ-Ibα1, after reduction of intramolecular disulfide

bonds (Fig. 3); the latter, then, could be used to demonstrate the presence of immunoreactive GP Ibα-related protein in different culture media.

Functional consequences of the α-subunit missense mutations. In a previous publication we have proposed the concept that the vWF-binding function of the platelet GP Ib-IX-V complex can be recapitulated in the soluble recombinant GP Ibα fragment containing residues 1–302 (22). We based our conclusions on the following observations: (a) the binding of soluble vWF to immobilized recombinant fragment as well as to the intact GP Ib-IX-V receptor complex on platelets requires the presence of a modulator, either ristocetin or botrocetin; (b) the binding of purified vWF to immobilized recombinant fragment can be inhibited with the same monoclonal antibodies that inhibit binding to the GP Ib-IX-V complex; and (c) vWF binding to immobilized recombinant fragment and intact GP Ib-IX-V complex on platelets exhibits essentially the same apparent affinity (22). In agreement with this concept, expression of the mutation identified in the type Bolzano Bernard-Soulier variant resulted in a dysfunctional recombinant fragment lacking vWF-binding activity, similar to the abnormality observed with intact platelets from the propositus (13). Indeed, using either ristocetin or botrocetin as modulators of vWF binding, it was apparent that the GP Ibα fragment bearing the Thr[145] → Met dimorphic substitution and the Ala[156] → Val mutation (thus, with sequence identical to GP Ibα in the propositus) had negligible vWF binding function, not distinguish-
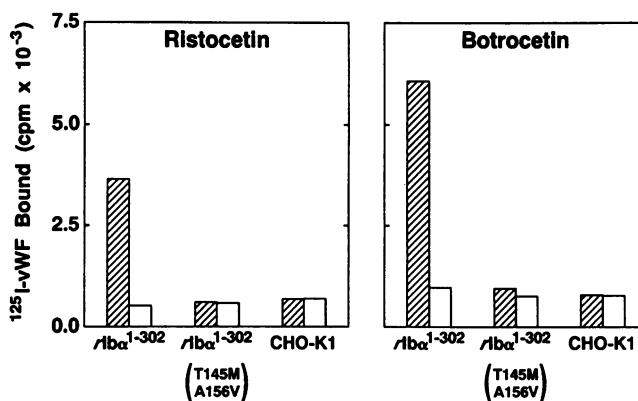

Figure 4. Interaction of ^{125}I-labeled vWF with insolubilized recombinant GP Ibα fragments. Appropriate volumes (30–400 μl) of different culture media were used to yield comparable amounts of recombinant fragments insolubilized onto the nitrocellulose membrane. After blocking of the membrane with a solution containing 1% bovine serum albumin and 1% bovine γ-globulin, the binding of ^{125}I-labeled vWF (10 μg/ml) was measured in the presence of either ristocetin (1 mg/ml; left) or botrocetin (10 μg/ml; right). The fragment designated as rIbα[1-302] represents the published normal GP Ibα sequence (threonine at position 145 and alanine at position 156); the fragment designated as rIbα[1-302](T145M;A156V) contains methionine at position 145 and valine at position 156, as in the Bernard-Soulier syndrome type Bolzano propositus. The sample designated as CHO-K1 represents control culture medium of nontransfected cells. Only the normal fragment supports vWF binding, both in the presence of ristocetin and botrocetin (■); such binding is markedly inhibited in the presence of appropriate anti-GP Ibα monoclonal antibodies (LJ-Ib1 and LJ-P19, each at 100 μg/ml; □). This experiment is representative of at least four additional ones, performed with different preparations of culture media; all results were essentially identical.
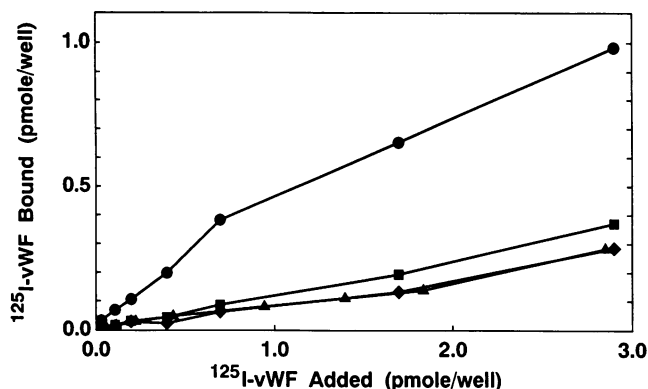
Ristocetin-Induced Binding

Figure 5. Dose-dependent ^{125}I-vWF binding to recombinant GP Ibα fragments in the presence of ristocetin. Recombinant fragments were immobilized onto a nitrocellulose membrane and the latter was blocked with a solution containing 1% bovine serum albumin and 1% bovine γ-globulin, as described in the legend to Fig. 4. Increasing concentrations of ^{125}I-labeled vWF were then filtered through the membrane in the presence of the modulator ristocetin (1 mg/ml). Bound vWF was detected in a γ-scintillation spectrometer. Four different samples were tested in this assay: rIbα$^{1-302}$ with normal sequence and the more common Thr^{145} dimorphic residue (●); rIbα$^{1-302}$ with the Bernard-Soulier type Bolzano genotype, containing the Thr^{145} → Met dimorphism and the Ala^{156} → Val mutation (♦); rIbα$^{1-302}$ with the Ala^{156} → Val mutation but the more common Thr^{145} dimorphic residue instead of Met^{145} (▲); culture medium of nontransfected CHO-K1 cells (■). Note that only the normal recombinant fragment supported vWF binding; presence of the Ala^{156} → Val mutation, regardless of the dimorphic residue at position 145, resulted in markedly decreased vWF binding. Three separate experiments with the normal fragment and the type Bolzano mutant gave essentially the same results.

able from that of nontransfected CHO-K1 cells; in the same assays, a recombinant fragment with normal sequence supported specific vWF binding that could be inhibited > 80% by anti-GP Ibα monoclonal antibodies (Fig. 4).
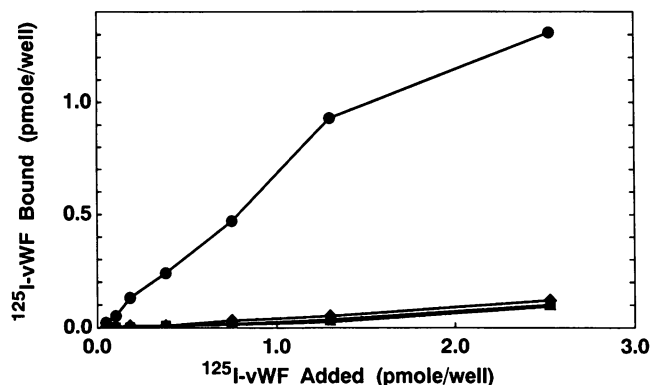
Botrocetin-Induced Binding

Figure 6. Dose-dependent ^{125}I-vWF binding to recombinant GP Ibα fragments in the presence of botrocetin. The experiment was performed as described in the legend to Fig. 5, except that botrocetin (10 μg/ml) was used as a modulator of binding instead of ristocetin. The samples and corresponding symbols are the same as described in the legend to Fig. 5; the results obtained with botrocetin are comparable to those obtained with ristocetin.

Additional binding experiments performed with increasing ligand concentrations confirmed that the mutant fragment expressing the type Bolzano phenotype fails to interact with vWF both in the presence of ristocetin (Fig. 5) and botrocetin (Fig. 6). Moreover, a GP Ibα fragment containing the Ala^{156} → Val mutation but the more common threonine at position 145 exhibited the same loss of activity as the fragment containing Met^{145} (Figs. 5 and 6), confirming that the effects of the mutation at position 156 are not influenced by the dimorphism at position 145. In this regard, therefore, vWF-binding studies and immunochemical studies (Fig. 3) gave concordant results. It should be noted that, under the experimental conditions used, the interaction of vWF with recombinant GP Ibα fragments is not reversible; thus, since binding is not at equilibrium, we chose not to submit the data to Scatchard-type analysis. The results obtained in this study with the normal GP Ibα fragment, however, are similar to those reported previously and calculated to give apparent dissociation constant comparable to that measured for vWF binding to intact GP Ib-IX complex on platelets (22).

Discussion

The pathogenesis of the Bernard-Soulier syndrome may parallel, in most instances, congenital abnormalities of other multisubunit receptor complexes, such as integrins (31, 32), to the extent that a defect in a single subunit prevents assembly and surface expression of the entire complex (11, 12). Among the reported cases of Bernard-Soulier syndrome, however, type Bolzano represents a unique example since the GP Ib-IX-V complex is present on the platelet surface, albeit with a reduced number of copies, but is dysfunctional, showing normal ability to bind α-thrombin but defective vWF binding function (13). In previous studies (13), we suggested that the genetic defect resulting in the type Bolzano phenotype was likely to be localized in the α-subunit of GP Ib for two reasons: (a) vWF binding to the receptor, the function defective in platelets from the propositus, is an intrinsic property of the amino terminus of GP Ibα (21, 22); and (b) immunological characterization of the propositus' platelets demonstrated loss of the epitope recognized by the monoclonal antibody LJ-Ib1, located within residues 1-293 of the GP Ibα-chain (20). Indeed, we have now found that the propositus' GP Ibα gene contains a unique Ala^{156} → Val substitution as well as two polymorphic markers, a Met^{145} codon instead of the more common Thr^{145} and coding sequence for 26 additional residues within the carbohydrate-rich domain. In the previous characterization of the propositus' family (13), the binding of antibody LJ-Ib1 to the mother's platelets was reported to be 50% of normal, a finding consistent with the possibility of genetic heterozygosity. Indeed, it is now apparent that both parents of the propositus are obligatory carriers for the Bernard-Soulier syndrome, since they have both a mutant (Val^{156}) and a normal (Ala^{156}) GP Ibα allele. The presence of the same two polymorphic markers in the alleles containing the Val^{156} codon in both parents suggests that the type Bolzano phenotype is the consequence of a consanguineous mating.

Immunological characterization of recombinant fragments corresponding to the 45-kD amino-terminal domain of GP Ibα established a direct correlation between presence of the Val^{156} mutation and loss of the epitope recognized by the monoclonal antibody LJ-Ib1, the same defect exhibited by platelets from

$_1$L X L X X N X L$_8$ X X L P X G L$_{15}$ X X X L X X L$_{22}$ X X$_{24}$ **Leucine-rich consensus**

$_{153}$L S L A N N L T E L P A G L L N G L E N L D T$_{176}$ **Platelet glycoprotein Ibα**

↓
V$_{156}$ **Bernard-Soulier syndrome, type Bolzano**

Figure 7. Sequence alignment (single-letter designation) of the leucine-rich repeat consensus sequence and the sixth leucine-rich repeat of platelet GP Ibα. Leucine-rich repeats form the basis for a family of proteins containing a 24-residue motif with leucine residues at every seventh position and other conserved hydrophobic and hydrophilic residues interspersed among them (14). The variant residues of the leucine-rich consensus sequence are designated by X. The molecular defect resulting in the Bolzano variant of the Bernard-Soulier syndrome is an Ala^{156} → Val substitution and is shown at its corresponding position within a leucine-rich repeat. Sequence alignment of 138 leucine-rich repeats from 16 different proteins in the Protein Information Resource database revealed that the fourth position of the repeat, which corresponds to the identified mutation within platelet GP Ibα, is occupied by noncharged amino acids in 65% of the repeats. 71% of the noncharged residues are Ser or Gly. None of the 138 complete leucine-rich repeats examined exhibited the hydrophobic residues Val, Leu, Ile, and Pro at the fourth position of the consensus sequence, whereas every other amino acid was represented at that position in at least one repeat.

the type Bolzano propositus (13). This amino acid substitution is not likely to affect selectively the antibody contact site but, rather, appears to have a more complex effect on the conformation of the expressed molecule. In fact, two other anti–GP Ibα monoclonal antibodies, each recognizing distinct epitopes sensitive to denaturation (20, 22), showed decreased interaction with recombinant fragments containing the Val^{156} mutation. Similar results have also been observed with platelets from the type Bolzano propositus, supporting the conclusion that the effects of the Ala^{156} → Val mutation are essentially the same in the isolated GP Ibα amino-terminal fragment as they are in the GP Ib-IX-V complex on intact platelets. It is also clearly indicated by our experimental findings that the mutation at residue 156 by itself explains the abnormal immunochemical reactivity of the type Bolzano GP Ibα molecule, without relevant contribution by the dimorphism at position 145. It should be remembered, however, that the Thr/Met dimorphism, per se, can independently create a new antigenic site on GP Ibα as well as on the expressed recombinant fragment (29).

The results of studies on the functional properties of the expressed mutant GP Ibα fragments support the concept that the Ala^{156} → Val mutation, in addition to causing a structural abnormality resulting in the loss of conformation-sensitive epitopes, is also responsible for decreased vWF binding. Two exogenous modulators were used in these assays, ristocetin and botrocetin, as commonly done to measure the binding of native soluble vWF to platelets. These modulators may mimic events that occur at a site of vascular injury where soluble vWF becomes adsorbed onto exposed subendothelial structures and then promotes the attachment of platelets through the GP Ib-IX-V complex (33). Botrocetin forms a bimolecular complex with vWF that, in turn, binds specifically to GP Ibα (27); ristocetin acts in a less well-defined manner, but is thought to require interaction with both vWF and the platelet membrane in order to promote vWF binding to GP Ibα (34). It has been demonstrated that changes in GP Ibα conformation induced by reduction of disulfide bonds have different effects on vWF binding mediated by either ristocetin or botrocetin (21), indicating that each modulator elicits a distinct mode of ligand–receptor interaction. The correspondence between the effects of ristocetin and botrocetin, on one side, and the mechanisms physiologically relevant for vWF binding to platelets in vivo remains to be clarified. Nevertheless, studies in patients with von Willebrand disease have provided examples that support the use of both ristocetin (35) and botrocetin (36) for reproducing phenotypic abnormalities of the vWF-GP Ib interac-

tion. This suggests that neither modulator by itself may be sufficient to probe the vWF binding function of GP Ibα in its complexity and that their concurrent use may be necessary to obtain useful information in this regard. In the present studies, the functional consequences of the Ala^{156} → Val mutation could be demonstrated using both ristocetin and botrocetin as modulators of vWF binding to immobilized recombinant GP Ibα fragments, providing strong evidence that the identified mutation represents, indeed, the molecular basis of the type Bolzano variant of Bernard-Soulier syndrome. Moreover, it is apparent from all our results that the structural and functional effects of the Ala^{156} → Val mutation are independent of the Thr/Met dimorphism at residue 145.

The identification of the effects caused by the Ala^{156} → Val mutation in GP Ibα may have implications for understanding the structure and function of other proteins. Residue 156 is part of a conserved repetitive motif originally described in leucine-rich α$_2$-glycoprotein (37) and present in a growing list of diverse molecules with functions related to cell–cell, cell–protein, or protein–protein interactions (14). All four subunits of the GP Ib-IX-V complex contain this motif characterized by a conserved spacing of leucine residues within a 24–amino acid sequence (4–8). There are similarities and dissimilarities between the leucine-rich repeats and the well-characterized leucine-zipper motif (14). The most striking similarity is the predominance of leucine residues and their similar spacing at every seventh position (Fig. 7); however, the leucine-rich repeats have a high content of proline residues that are likely to interfere with the formation of the regular coiled-coil structure that serves as the backbone for the leucine-zipper (38). The three-dimensional conformation of leucine-rich repeats is unknown, but it has been proposed that they form amphipathic β sheets (39). Perhaps, the local increase in hydrophobicity caused by the Ala^{156} → Val mutation may alter the amphipathic structure and interfere with the surface orientation of residues involved in interactions that are essential for the structural and functional integrity of GP Ibα. It is noteworthy that in > 100 homologous sequences present in the protein database, the position corresponding to residue 156 in GP Ibα is predominantly occupied by polar residues; thus far, valine, leucine, isoleucine, and proline have never been found in that position (Fig. 7). It has been proposed that leucine-rich repeats may be involved in protein–membrane and protein–protein interactions (37, 39–41). With regard to the GP Ib-IX-V complex, however, we have now demonstrated that they affect the activity of the vWF binding site even when expressed in an

isolated soluble fragment of GP Ibα. Thus, such function does not require interactions with the platelet membrane; moreover, it is clearly intrinsic to the α-chain and independent of association with homologous sequences in the other components of the receptor complex.

Previous results obtained with synthetic peptides (42) and recombinant fragments (22) indicate that a domain relevant for vWF-binding function is located between residues 221 and 318 of GP Ibα, with a predominant role for a cluster of negatively charged residues between positions 251 and 279 (Fig. 1). The leucine-rich repeats may provide the conformation necessary for the function of this site, e.g., affecting the orientation of charged residues, without necessarily having a direct participation in establishing contact with the ligand. Such structural effects may be achieved through self-interactions of the homologous leucine-rich sequences, a function that may be regulated through the number of repeats present in a given molecule. That the Ala^{156} → Val substitution may indeed alter the conformation of the amino terminal domain of GP Ibα is suggested by the evidence that the type Bolzano mutant has not only an abnormal interaction with vWF but also markedly decreased reactivity with three different monoclonal antibodies each recognizing distinct and nonoverlapping epitopes (20, 22, 42). Appropriate conformation maintained by the leucine-rich repeat region in the extracytoplasmic domain may also influence the linkage of the intracytoplasmic tail of GP Ibα to the cytoskeleton (43), so that the Ala^{156} → Val mutation may be responsible for both the ligand binding defect and the aberrant morphology of giant platelets in the type Bolzano variant.

Acknowledgements

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