A new variant of Glanzmann's thrombasthenia (Strasbourg I). Platelets with functionally defective glycoprotein IIb-IIIa complexes and a glycoprotein IIIa 214Arg----214Trp mutation.

F Lanza, A Stierlé, D Fournier, M Morales, G André, A T Nurden, J P Cazenave

J Clin Invest. 1992;89(6):1995-2004. https://doi.org/10.1172/JCI115808.

Research Article

We describe a new variant of Glanzmann's thrombasthenia (variant Strasbourg I). The patient (M.S.) showed an absence of platelet aggregation to ADP, thrombin, and collagen, and a decreased clot retraction. Platelet fibrinogen was approximately 20% of normal levels. ADP-stimulated platelets bound markedly reduced amounts of soluble fibrinogen and platelet adhesion to surface-bound fibrinogen was defective. Normal to subnormal amounts of glycoprotein (GP) IIb-IIIa (alpha IIb beta 3) complexes, the platelet fibrinogen receptor, were revealed by SDS-PAGE, crossed immunoelectrophoresis, and antibody binding. However, the complexes were unusually sensitive to dissociation with EDTA at room temperature. Furthermore, flow cytometry showed that the platelets failed to bind the activation-dependent monoclonal antibody, PAC-1, after stimulation. In contrast, an RGDS-containing peptide induced significant binding of the anti-ligand-induced binding site antibody, D3GP3, suggesting the presence of a functional RGD binding domain on the patient's GPIIb-IIIa complex. Sequence analysis was performed after polymerase chain reaction amplification of selected patient's GPIIIa exons, and of the patient's platelet GPIIb and GPIIIa mRNAs. A point mutation (C to T) was localized in exon D (iv) of GPIIIa that resulted in an 214Arg to 214Trp amino acid substitution. The defect has been inherited from the parents who are heterozygous for the same mutation. This substitution points to an essential amino acid in a region of GPIIIa involved in the binding of […]

A New Variant of Glanzmann's Thrombasthenia (Strasbourg I)

Platelets with Functionally Defective Glycoprotein IIb–IIIa Complexes and a Glycoprotein IIIa ^{214}Arg → ^{214}Trp Mutation

François Lanza, Anita Stierlé, Dominique Fournier,* Martine Morales, Gabriel André, Alan T. Nurden,* and Jean-Pierre Cazenave

*Institut National de la Santé et de la Recherche Médicale Unité 311, Centre Régional de Transfusion Sanguine, 67085 Strasbourg, France; and *Unité de Recherche Associée 1464 Centre National de la Recherche Scientifique, Université de Bordeaux II, Hôpital Cardiologique, 33604 Pessac, France.*

Abstract

We describe a new variant of Glanzmann's thrombasthenia (variant Strasbourg I). The patient (M.S.) showed an absence of platelet aggregation to ADP, thrombin, and collagen, and a decreased clot retraction. Platelet fibrinogen was ~ 20% of normal levels. ADP-stimulated platelets bound markedly reduced amounts of soluble fibrinogen and platelet adhesion to surface-bound fibrinogen was defective. Normal to subnormal amounts of glycoprotein (GP) IIb–IIIa ($\alpha_{IIb}\beta_3$) complexes, the platelet fibrinogen receptor, were revealed by SDS-PAGE, crossed immunoelectrophoresis, and antibody binding. However, the complexes were unusually sensitive to dissociation with EDTA at room temperature. Furthermore, flow cytometry showed that the platelets failed to bind the activation-dependent monoclonal antibody, PAC-1, after stimulation. In contrast, an RGDS-containing peptide induced significant binding of the anti-ligand-induced binding site antibody, D3GP3, suggesting the presence of a functional RGD binding domain on the patient's GPIIb–IIIa complex. Sequence analysis was performed after polymerase chain reaction amplification of selected patient's GPIIIa exons, and of the patient's platelet GPIIb and GPIIIa mRNAs. A point mutation (C to T) was localized in exon D (*iv*) of GPIIIa that resulted in an ^{214}Arg to ^{214}Trp amino acid substitution. The defect has been inherited from the parents who are heterozygous for the same mutation. This substitution points to an essential amino acid in a region of GPIIIa involved in the binding of fibrinogen and influencing the Ca^{2+}-dependent stability of the GPIIb–IIIa complex. (*J. Clin. Invest.* 1992. 89:1995–2004.) Key words: bleeding disorder • calcium • fibrinogen • integrin • polymerase chain reaction

Introduction

Glanzmann's thrombasthenia is a rare autosomal recessive disorder characterized clinically by mucocutaneous bleeding due to a defective hemostatic plug formation (1). Platelets of these patients fail to aggregate in response to physiologic agonists such as ADP, thrombin, or collagen, and have a defective fibrinogen binding. Early studies demonstrated that Glanzmann's thrombasthenia is due to an absence or dysfunction of the platelet glycoprotein (GP)[1] IIb–IIIa complex (2, 3). Various thrombasthenic phenotypes have been described, and have been tentatively classified in three categories: (*a*) type I patients with a severe GPIIb–IIIa deficiency (< 5% of normal), (*b*) type II patients with a moderate GPIIb–IIIa deficiency (5–20% of normal), and (*c*) variants with half-normal to normal amounts of dysfunctional GPIIb–IIIa complexes (4, 5). However, it is not known whether this classification corresponds to the molecular defects being assigned to different patients.

The platelet GPIIb–IIIa complex, also referred to as $\alpha_{IIb}\beta_3$, belongs to a large family of adhesive receptors named integrins (6). Other integrins have sequence homology with GPIIb–IIIa, are also formed by the noncovalent association of an α and a β subunit, and are involved in cell–cell and ligand–cell interactions (7, 8). Recent molecular biology studies have made available both the primary amino acid and the genomic sequences of GPIIb (9, 10) and GPIIIa (11–15), thus allowing genetic studies on Glanzmann's thrombasthenia patients. Knowledge of the molecular defects in this disorder may identify elements involved in the expression and assembly of GPIIb–IIIa. In the case of variants this can also lead to the identification of functional domains within GPIIb–IIIa. These may include the determinants responsible for ligand binding, for the Ca^{2+}-dependent stability of the GPIIb–IIIa heterodimer, and for the interaction of the complex with the platelet cytoskeleton.

Two genetic defects have been described for GPIIb (16, 17), and three defects have been attributed to GPIIIa (17–19). Only one molecular defect giving rise to a Glanzmann's variant has been described so far. Loftus et al. (19) performed studies on a Guam family, patients whose platelets have a normal GPIIb–IIIa content but failed to bind fibrinogen or synthetic RGD-containing peptides. In addition, these platelets have a constitutive expression of an epitope on GPIIb–IIIa, the PMI-1 epitope, that is only expressed in normal platelets exposed to low (< 0.1 μM) concentrations of Ca^{2+} (20). Polymerase chain reaction (PCR) analysis of their platelet mRNA revealed a single ^{119}Asp → ^{119}Tyr substitution on the GPIIIa molecule. This mutation resides in a putative RGD recognition domain of GPIIIa (21, 22), and was reproduced by site-directed mutagenesis (19), resulting in the synthesis of a defective GPIIb–IIIa complex incapable of binding fibrinogen and RGD-containing peptides.

This study describes a second type of Glanzmann's thrombasthenia variant, named variant Strasbourg I, characterized by an absence of platelet aggregation to all agonists and defec-

Address reprint requests to Dr. Lanza, INSERM U 311, Centre Régional de Transfusion Sanguine de Strasbourg, 10 rue Spielmann, 67085 Strasbourg, France.

Received for publication 24 October 1991 and in revised form 30 January 1992.

J. Clin. Invest.

0021-9738/92/06/1995/10 $2.00
Volume 89, June 1992, 1995–2004

1. *Abbreviations used in this paper:* CIE, crossed immunoelectrophoresis; GP, glycoprotein; LIBS, ligand-induced binding site; PCR, polymerase chain reaction.

tive fibrinogen binding. This patient (M.S.)[2] has subnormal amounts of platelet GPIIb–IIIa complexes with an increased sensitivity to dissociation by EDTA. Three patients with more easily dissociable GPIIb–IIIa have been described so far (23–25), but the genetic defects associated with these variants have not been reported. In order to identify the molecular abnormalities of the Strasbourg I variant, we sought to determine the sequence of the patient's GPIIb and GPIIIa after PCR amplification of the platelet mRNA and genomic DNA. We report a novel mutation in GPIIIa which provides evidence for an active site in GPIIIa that may be involved in fibrinogen binding and influence the Ca^{2+}-dependent stabilisation of the GPIIb–IIIa complex.

Methods

Case history. The patient (M.S.) is a caucasian female now 19 yr old who was initially diagnosed as a typical case of Glanzmann's thrombasthenia. She has been examined on four occasions over a 7-yr period. Bleeding episodes started at birth and mainly consisted of unprovoked bruising. She suffered a traumatic intracerebral hematoma when 6 yr old. Hematologic examination revealed a prolonged bleeding time (> 10 min), and an absence of platelet aggregation in response to all agonists tested: i.e., ADP, collagen, platelet-activating factor–acether, thrombin, and arachidonic acid. However, platelet shape change and the release reaction were both normal. Ristocetin-induced platelet agglutination was normal. Clot retraction occurred but was subnormal in comparison with that of control donors. Routine tests showed no other hematological or coagulation factor abnormalities. The parents, who are direct cousins have been studied in parallel. Paternity was established by human leukocyte antigen typing. There is no family history of bleeding. Preliminary results concerning this case have been reviewed in George et al. (5) under patient number 49.

Polyclonal and monoclonal antibodies (MAbs). Polyclonal antisera against purified GPIIb and GPIIIa were provided by Dr. Dominique Pidard (INSERM U 150, Paris, France) (26). The following MAbs were used: AP-2, specific for the GPIIb–IIIa complex (27), was provided by Dr. Thomas Kunicki (Blood Center of Southeastern Wisconsin, Milwaukee, WI); Tab, directed against an epitope on GPIIb (28), was provided by Dr. Roger McEver (Department of Medicine, University of Oklahoma Health Sciences Center, Oklahoma City, OK); PAC-1, directed against an activation-dependent epitope on the GPIIb–IIIa complex (29), was provided by Dr. Sanford Shattil (University of Pennsylvania, Philadelphia, PA); CS14 and D12A, specific for the GPIIb–IIIa complex, were provided by Dr. Gérard Marguerie (INSERM U 217, Commissariat à l'Energie Atomique, Grenoble, France); and D3GP3, directed against a ligand-induced binding site (LIBS) epitope on the GPIIb–IIIa complex (30), was provided by Dr. Lisa Jennings (University of Tennessee, Memphis, TN). Other MAbs, including those specific for GPIIb–IIIa (P2), GPIb (SZ2), GMP-140 (CLB-Thromb-6), and CD63 (GRAN 12), were purchased from Immunotech SA, Luminy, France.

Platelet preparation. Platelets were isolated from acid-citrate-dextrose (ACD) anticoagulated blood by differential centrifugation, and washed at 37°C according to a modification (31) of the method described by Kinlough-Rathbone et al. (32). Unless otherwise stated, platelets were washed in Tyrode's buffer, pH 7.3, 295 mosmol/kg, containing 5 mM Hepes, 0.35% (wt/vol) purified human albumin (Centre Régional de Transfusion Sanguine, Strasbourg, France), (Tyrode-Hepes-albumin), containing 1 μM prostacyclin (PGI_2). Platelets were finally suspended in Tyrode-Hepes-albumin buffer containing 2 μM apyrase, and adjusted to 3×10^8 platelets/ml.

Radiolabeling of platelet membrane proteins. Platelets washed according to the standard procedure were finally suspended at 10^9/ml in 10 mM Tris-HCl, 0.15 M NaCl, 5 mM glucose, pH 7.3. Lactoperoxidase-catalyzed ^{125}I-labeling of the platelet surface proteins was then performed as previously described (33, 34).

Single-dimension SDS-PAGE. Unlabeled or ^{125}I-labeled platelets were resuspended at 2×10^9/ml in 50 mM Tris-HCl, 2% (wt/vol) SDS, 2.5% (vol/vol) glycerol, pH 6.8, and for nonreducing conditions 2 mM *N*-ethylmaleimide. The samples were solubilized by heating at 100°C for 10 min with (reduced) or without (nonreduced) 5% (vol/vol) 2-mercaptoethanol. Solubilized proteins (40 μg) were electrophoresed on a 5–10% gradient acrylamide slab gel (33, 34). ^{125}I-labeled proteins on dried gels were located by autoradiography.

Western blot analysis of platelet GPIIb–IIIa. Platelet proteins (50 μg) were electrophoresed on a nonreduced 7% SDS-PAGE gel and electrotransferred on Immobilon membranes (Millipore Corp., Molsheim, France). The blots were incubated with rabbit polyclonal antisera specific for GPIIb or GPIIIa (26), and the immunoreactive bands were revealed with ^{125}I-protein A (Amersham Corp., Les Ulis, France).

Crossed immunoelectrophoresis (CIE). Washed unlabeled or ^{125}I-labeled platelets were resuspended at 5×10^9/ml in 38 mM Tris-HCl, 0.1 M glycine, pH 8.7, and solubilized at 4°C for 30 min after the addition of 1/10 vol of 10% (vol/vol) Triton X-100. CIE of the solubilized proteins was carried out as described previously (34) with separation by a first-dimension electrophoresis in agarose followed by a second-dimension electrophoresis across an intermediate gel into an upper gel containing a rabbit antibody to human platelets. In some experiments, ^{125}I-labeled MAbs (10^6 cpm/cm^2) were incorporated in the intermediate gel (27, 34). Immunoprecipitates were located by CB-R250 (Bio-Rad SA, Ivry sur Seine, France) staining and by autoradiography when ^{125}I-labeled platelets or ^{125}I-labeled antibodies were used.

Binding of ^{125}I-labeled MAbs to intact platelets. AP-2 and Tab IgGs were radiolabeled using the chloramine-T method (27) and the labeled IgGs were purified on a Sephadex G25 column (Pharmacia, Uppsala, Sweden). Specific activities ranged from 150 to 300 cpm/ng protein. Platelets isolated according to the standard procedure were resuspended in Tyrode-Hepes-albumin buffer or in the same buffer where divalent cations were omitted and replaced by 2 mM EDTA, pH 7.3. Aliquots (0.3 ml) of platelets (1.5×10^8/ml) were incubated at 22°C for 30 min. Binding was performed in the presence of 0.5–5 μg/ml ^{125}I-labeled antibody. After incubation for 30 min at room temperature, duplicate samples (0.16 ml) of each reaction were layered over 0.5 ml of 20% (wt/vol) sucrose and centrifuged at 12,000 *g* for 2 min. Total counts per minute, and counts per minute in the pellet and supernatant fractions, were measured and Scatchard analysis was performed (27).

Flow cytometry analysis. Aliquots (0.1 ml) of washed platelets (3×10^8/ml) were incubated at 37°C for 3 min, with or without 1 U/ml thrombin, or at 37°C for 15 min with or without 1 mM GRGDSP (Appligène, Strasbourg, France). Samples were then incubated with 2% (wt/vol) paraformaldehyde, pH 7.3, for 1 h at 37°C. The fixed platelets were centrifuged at 6,000 *g* for 1 min, resuspended in 50 μl of Tyrode's buffer, and incubated with 2 μg of the different purified MAbs. Excess unbound antibodies were removed by washing in Tyrode's buffer. Bound antibodies were revealed by incubation with a 1/5 dilution of a FITC-conjugated goat anti–mouse antibody (Becton, Dickinson, & Co., Pont de Claix, France). Cell sorting was performed on a FACScan analyzer (Becton, Dickinson, & Co., Mountain View, CA). Cells were gated at 372 forward light scatter and 309 side light scatter, and fluorescence was monitored at 488 nm. 5,000 platelets were analyzed and the results were expressed on a logarithmic scale. MARK-1, a MAb against rat κ chain (Immunotech SA) was used in parallel to monitor the level of nonspecific fluorescence.

Fibrinogen binding assay. Human fibrinogen purified according to Kekwick et al. (35), was labeled with ^{125}I to a specific activity of 75,000–200,000 cpm/μg using the Iodogen procedure (Pierce Chemical Co., Rockford, IL). The binding of purified human ^{125}I-fibrinogen to ADP-stimulated platelets was performed according to the procedure described by Mustard et al. (36). The binding was measured in triplicate

2. Early biochemical studies on this patient were performed while Drs. Fournier and Nurden were members of Unité 150 INSERM, Hôpital Lariboisière, Paris, France.

samples 15 s after the addition of 5 μM ADP, with a 0.3 μM final concentration of fibrinogen, and a platelet concentration of 3×10^8/ml. Under these nonequilibrium conditions normal human platelets bind ~ 1.2 pmol fibrinogen per 10^8 platelets which represent 7,200 binding sites per platelet. When platelets are stimulated by a strong agonist such as thrombin, binding at 3 min represents ~ 35,000 sites per platelet.

Platelet fibrinogen content. Washed platelets were pelleted and resuspended in 38 mM Tris-HCl, 0.1 M glycine, pH 8.7, to a final concentration of 5×10^9/ml. One volume of 10% (vol/vol) Triton X-100 was added, and the mixture was agitated for 30 min at 4°C. Solubilized proteins were subjected to rocket immunoelectrophoresis according to the method of Laurell (37) using a polyclonal antibody to purified human fibrinogen (Centre Régional de Transfusion Sanguine, Strasbourg). The fibrinogen content was evaluated by comparison with an internal standard curve obtained using known amounts of purified human fibrinogen.

Platelet adhesion assay. Washed platelets (2×10^8/ml) in Tyrode-Hepes-albumin buffer were allowed to adhere for 30 min at room temperature in microtiter 96-well plates coated with BSA (Sigma Chemical Co.) or purified human fibrinogen, according to the procedure described by Cheresh et al. (38). Adherent platelets were fixed with 2% paraformaldehyde, and stained using the May-Grünwald Giemsa method. The number of adherent platelets was measured by microscopy using a combination of visual and image analysis counting (Biocom, Les Ulis, France). Six areas totaling 0.3 mm^2 were analyzed for each well and the number of adherent platelets per well was calculated. Specific adhesion of platelets was that obtained after substraction of adhesion on BSA.

PCR amplification of genomic DNA and platelet cDNA. DNA was extracted from peripheral blood lymphocytes as previously described (39). In brief, the mononuclear cells from 5 ml of EDTA-anticoagulated blood were isolated using Lymphoprep (Nycomed, Oslo, Norway). The cells were lysed for 1 h in 3.5 ml of 6 M guanidium chloride and 0.25 ml of 7.5 M ammonium acetate. After addition of 0.15 mg/ml of proteinase K and 1% (wt/vol) sarkosyl, the mixture was incubated for 1 h at 60°C, and the DNA was ethanol precipitated. Platelets corresponding to 50 ml of ACD anticoagulated blood were washed according to the standard procedure. The final platelet pellet was dissolved in 1 ml of a 4 M guanidium isothiocyanate, 3 M Na acetate, and 0.8% (vol/vol) 2-mercaptoethanol mixture, layered onto a 0.8-ml 5.7 M CsCl, 25 mM Na acetate, pH 6 cushion, and centrifuged for 3 h at 201,000 *g* in a TL-100 tabletop ultracentrifuge (Beckman Instruments, Inc., Palo Alto, CA). The RNA pellet was dissolved in 0.1 ml of 0.3 M Na acetate and ethanol precipitated. Platelet cDNA was synthesized using a commercially available kit (Boehringer, Mannheim, FRG). First-strand synthesis was performed at 37°C for 30 min using oligodT priming, and 400 U of MuMLV reverse transcriptase (Gibco BRL, Cergy-Pontoise, France). Sequences of the GPIIIa gene corresponding to exons B (*ii*), C (*iii*), and D (*iv*), and sequences covering the entire coding sequence of GPIIb and GPIIIa cDNAs were selected for PCR amplification. The list of the oligonucleotides used in these experiments is provided in Table I. The target sequences were amplified in a 0.1-ml reaction volume containing 500 ng of chromosomal DNA or 100 ng of platelet cDNA; 20 pmol of each oligonucleotide primer; 0.2 mM each dNTP; 1× reaction buffer (50 mM KCl, 10 mM Tris-HCl, 3 mM $MgCl_2$, 0.001% (wt/vol) gelatin, pH 10), and 2 U/ml Taq polymerase (Perkin Elmer-Cetus, St. Quentin, France). 30 cycles of PCR amplification were performed using a programmable thermal cycler (Perkin Elmer-Cetus). Each cycle consisted of 1 min of denaturation at 94°C, annealing for 1 min at 55°C (genomic DNA) or 45°C (platelet cDNA), and extension for 2 min at 72°C. After the PCR, the amplified samples were analyzed on a 2–3% agarose gel.

Subcloning and sequencing of the amplified fragments. The PCR amplified fragments were digested with Eco RI and Sal I restriction endonucleases (Xba I and Sal I for primers G and K), purified on a 2% low-melt agarose gel, and electroeluted from the gel. The purified fragments were subcloned in the M13 vector in both orientations, and were sequenced using the Sequenase kit procedure (United States Biochemical Corp., Cleveland, OH).

Results

Platelet function testing. The Glanzmann's thrombasthenia patient (M.S.) possessed platelets that failed to aggregate with a range of physiologic agonists whereas ristocetin-induced agglutination was normal (see case history). This platelet function defect was accompanied by a much reduced binding of ^{125}I-labeled fibrinogen to platelets stimulated with ADP (Table II). The patient had a subnormal clot retraction, and a decreased but detectable platelet fibrinogen content (36 ng/10^8 platelets

Table I. Sequences and Locations of Nucleotide Primers Used in the PCR Amplification of GPIIIa and GPIIb DNA

Primers	Sequence, (+) strand	Sequence, (−) strand	Location
			GPIIIa gene
A	5'GAGGTAGAGAGTCGCCATAGT3'	5'TCTCCCCATGGCAAAGAGTCC3'	Exon B
B	5'CCAAATCTGCTTATTCAATCT3'	5'GAACCAGGACTTGGACCTTCC3'	Exon C
C	5'CATGCTGCCTTTTCCATGAAG3'	5'GCCATTTTGATCTATGCCAGC3'	Exon D
			GPIIIa cDNA
D	5'GGGAGGCGGACGAGATGC3'	5'CCTGCCGCACTTGGATGG3'	1–417
E	5'CGGCTCCGGCCAGATGAT3'	5'TGGGATGAGCTCACTATA3'	369–1076
F	5'GTAGTCAATCTCTATCAG3'	5'CCCACAGCTGCACTGGCC3'	1038–1734
G	5'AAGGGGGAGATGTGCTCA3'	5'TGAGGATGACTGCTTATC3'	1692–2406
			GPIIb cDNA
H	5'GATGGCCAGAGCTTTGTG3'	5'GCCCACGGCCACCGAGTA3'	1–820
I	5'CCAGAGTACTTCGACGGC3'	5'CAGCAGCAGCACCCGCCG3'	776–1665
J	5'AGCTGGACCGGCAGAAGC3'	5'AGCTCATAGGTGTGCTCC3'	1620–2451
K	5'CAGAACAGCTTGGACAGC3'	5'TAGAATAGTGTAGGCTGC3'	2403–3143

The oligonucleotide primers for the GPIIIa gene exons corresponding to flanking intronic sequence are taken from Lanza et al. (14). The nucleotide locations for primers corresponding to GPIIb and GPIIIa cDNAs are numbered according to Poncz et al. (9) and Fitzgerald et al. (11), respectively. To facilitate further subcloning EcoR I sites were added to the (+) strand primers (with the exception of primers G and K where an Xba I site was added), and Sal I sites were added to the (−) strand primers.

Table II. Platelet Interaction with Soluble (^{125}I-Fibrinogen Binding) or Immobilized (Platelet Adhesion) Fibrinogen

	Control	Patient	Father	Mother
^{125}I-fibrinogen binding (*pmol/10^8 platelets*)	1.20±0.11	0.025±0.035	0.66±0.04	0.57±0.9
Platelet adhesion (*platelets per well* $\times 10^{-5}$)	3.42±0.14	0.15±0.07	ND	ND

^{125}I-fibrinogen binding was measured on ADP-stimulated platelets. Adhesion of platelets was performed on purified human fibrinogen coated onto plastic. After adhesion the platelets were fixed and stained, and the cell number was evaluated by counting under a microscope. Further details are given under Methods. Values are expressed as mean±SEM, n = 3. ND, not determined.

vs. a control value of 168 ng/10^8 platelets). The two parents had no bleeding tendency, a normal bleeding time, a normal platelet aggregation, and a normal platelet fibrinogen content. The binding of ^{125}I-labeled fibrinogen to the parent's platelets was ~ 50% of control (Table II).

Recent experiments have shown that resting platelets and nucleating cells expressing GPIIb–IIIa cannot bind soluble fibrinogen but can attach to immobilized fibrinogen (40). In order to further characterize the fibrinogen binding defect of the Strasbourg I variant, we sought to determine whether the patient's platelets could adhere to adhesive proteins immobilized on a plastic surface. Using the assay described by Cheresh et al. (38), we found that platelets from the patient had a reduced adhesion (4% of control) to immobilized fibrinogen (Table II).

SDS-PAGE. In order to test for the presence of GPIIb and GPIIIa the patient's platelets were surface labeled with ^{125}I and the proteins subjected to SDS-PAGE electrophoresis. Autoradiography of the nonreduced gel (Fig. 1) revealed the apparently normal presence of labeled bands corresponding to GPIIb (135 kD) and GPIIIa (90 kD) on the patient's pattern. Identity of these bands was further confirmed after disulfide reduction, when a characteristic increase in apparent molecular weight of GPIIIa (from 90 to 100 kD) and cleavage of GPIIb into a small (GPIIbβ) and a large (GPIIbα) subunit occurred normally. The usual migration of GPIIb/GPIIbα and GPIIIa showed that there was no major deletion in the patient's GPIIb and GPIIIa. In addition, the intensity of the ^{125}I-labeling suggested that the patient's GPIIb and GPIIIa were normally expressed at the platelet surface. Substantial amounts of GPIIb and GPIIIa, with a normal migration, were also identified in the patient and two parents by Western blotting using polyclonal antibodies specific for GPIIb and GPIIIa, respectively (data not shown).

CIE analysis. CIE experiments were performed in order to determine if GPIIb and GPIIIa were present as GPIIb–IIIa complexes in the patient's platelets. As illustrated in Fig. 2, a major immunoprecipitate with a migration pattern corresponding to the GPIIb–IIIa complex was revealed for the patient after CB-R250 staining. The identity of the GPIIb–IIIa arc was established by the specific binding of ^{125}I-labeled AP-2, a MAb specific for GPIIb–IIIa complexes (Fig. 2, *upper panel*). CIE experiments performed in the presence of divalent cations (Figs. 2 and 3, *upper panel*) failed to show residual, noncomplexed, GPIIb or GPIIIa. The amount of GPIIb–IIIa complexes in the patient was evaluated by planimetry of the GPIIb–IIIa arc and represented 64±9% of control values (mean±SEM from four separate experiments, range 47–71%).

Experiments were performed to test for the stability of the GPIIb–IIIa complexes to divalent cation chelation. Thus, ^{125}I-labeled platelets were incubated with EDTA at room temperature. CIE analysis revealed a complete dissociation of the GPIIb–IIIa complexes when the patient's platelets were treated with 5 mM EDTA in this way (Fig. 3, *lower panel*). This was shown by the loss of the arc corresponding to the GPIIb–IIIa complex (*arrow*), and appearance of an arc with a position corresponding to free GPIIIa. The arc corresponding to GPIIb was less apparent, this could be due to a lower reactivity of the antiplatelet rabbit sera against free GPIIb. CIE analysis of control platelets treated with EDTA at 22°C revealed only a partial dissociation of the GPIIb–IIIa complexes. Previous reports have described no or minimal dissociation of the GPIIb–IIIa complexes of normal platelets treated by EDTA at room temperature. The difference in our experiment was the continuous presence of EDTA throughout the solubilization procedure.

^{125}I-MAb binding. To quantify the number of GPIIb–IIIa complexes in M.S.'s platelets, the direct binding of ^{125}I-labeled

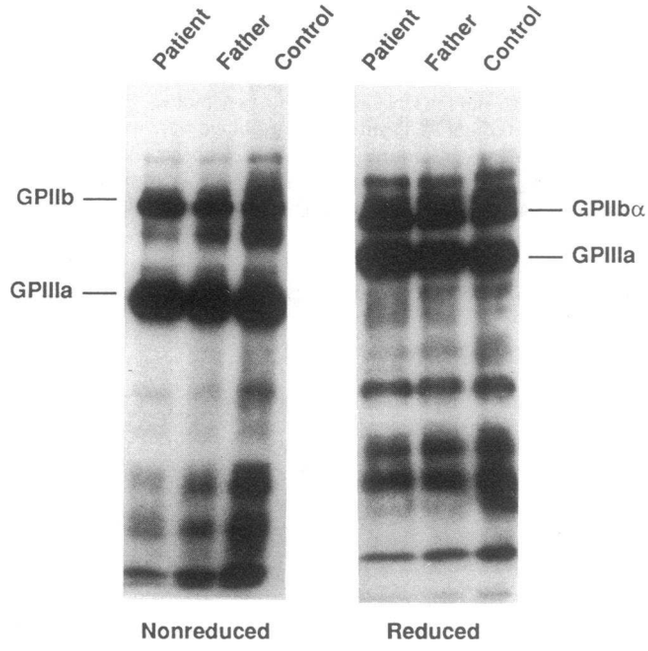

Figure 1. One-dimensional electrophoresis of patient M.S.'s, her father's, and control ^{125}I-labeled platelet glycoproteins. Surface proteins were labeled by the lactoperoxidase-catalyzed iodination procedure (see Methods). 40 μg of nonreduced or reduced proteins were separated on a 5–10% acrylamide slab gel. Typical autoradiograms are shown. The major labeled bands correspond to GPIIb and GPIIIa. Note the normal protein distribution and ^{125}I-labeling intensity of the patient's platelet proteins. Note also the characteristic increase in apparent molecular weight of GPIIIa upon reduction, and the parallel reduction of GPIIb with the appearance of a normal GPIIbα subunit.

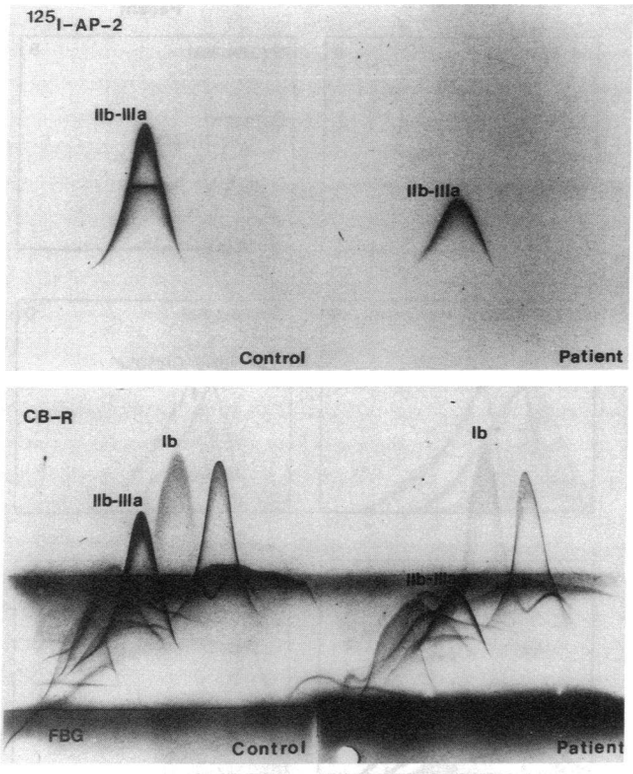

Figure 2. GPIIb–IIIa complexes in patient M.S.'s platelets as revealed by CIE. Washed, unlabeled platelets were solubilized with Triton X-100 and the soluble proteins were separated by electrophoresis in agarose. Second-dimension electrophoresis was performed across an intermediate gel containing trace amounts of ^{125}I-labeled AP-2, and into an upper gel containing a rabbit anti–human platelet antibody. A series of immunoprecipitate arcs were revealed by CB-R250 staining (*lower panel*). An immunoprecipitate in the position corresponding to GPIIb–IIIa can be visualized on the patient's gel. This arc was confirmed as being given by GPIIb–IIIa after autoradiography revealed the presence of the GPIIb–IIIa complex-specific ^{125}I-labeled AP-2 antibody (*upper panel*). Trace levels of platelet fibrinogen (*FBG*) can be visualized on the patient's CB-R250–stained gel. A control arc corresponding to GPIb is also indicated.

AP-2 was measured using washed platelets. Scatchard analysis revealed the presence of 19,400 AP-2 binding sites on the patient's platelets (Table III) compared with 36,130 binding sites for the control. Treatment of the patient's platelets with EDTA at 22°C resulted in a 98% decrease in ^{125}I-AP-2 binding sites from 19,400 to 300 binding sites per platelet. In comparison EDTA-treated control platelets bound the same amount of ^{125}I-AP-2 as in the absence of divalent cation chelation. Interestingly, the parents' platelets had a 38–49% decrease in ^{125}I-AP-2 binding upon EDTA treatment.

Flow cytometry analysis. A panel of GPIIb–IIIa complex-specific MAbs, including AP-2, P2, CS14, and D12A, was shown to bind to the patient's platelets by FACS analysis. Fig. 4 illustrates results obtained for P2. Binding to control platelets incubated with EDTA at room temperature was identical to that observed in the presence of Ca^{2+} (Fig. 4 *A*). However, binding to normal platelets fell to background levels when GPIIb–IIIa complexes were dissociated with 5 mM EDTA at 37°C, conditions where GPIIb–IIIa complexes of normal platelets do dissociate (see Discussion). In contrast, treatment of the patient's platelets with 2 mM EDTA at 22°C resulted in the dissociation of most of the patient's GPIIb–IIIa complexes as seen by a negative shift in the fluorescence (Fig. 4 *B*). In conclusion, these experiments firmly established an increased sensitivity for EDTA dissociation of the Strasbourg I variant GPIIb–IIIa.

Recently, antibodies have been described that preferentially react with GPIIb–IIIa after it binds RGD containing peptides. These have been referred to as LIBS antibodies (41). The binding of such a LIBS antibody, D3GP3, was evaluated by FACS analysis after incubation of control or patient's platelets with 1 mM GRGDSP for 30 min. A positive shift in fluorescence was seen in both the control (Fig. 4 *C*) and patient's platelet populations (Fig. 4 *D*) when compared to untreated platelets or platelets incubated with the control GRGESP peptide (data not shown). These results imply that the patient's

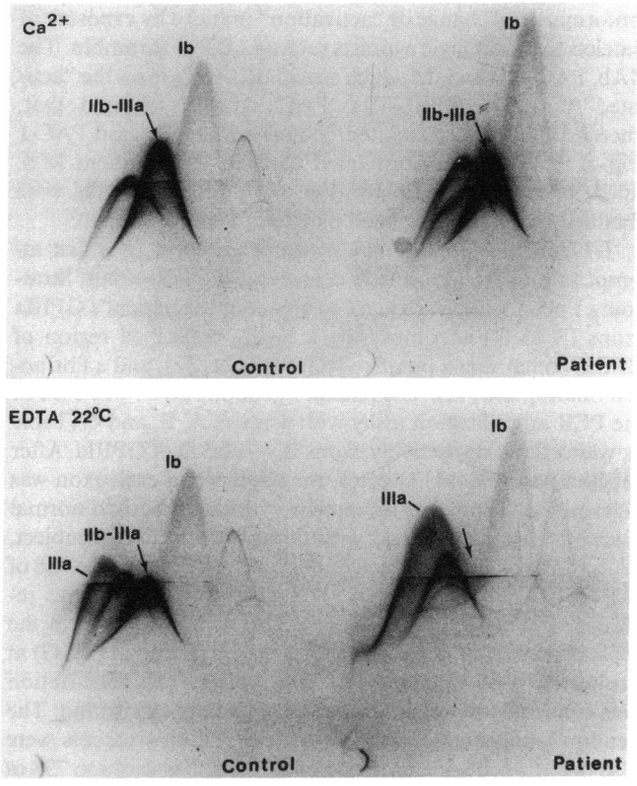

Figure 3. Increased sensitivity of patient M.S. platelet GPIIb–IIIa to EDTA dissociation as revealed by CIE. ^{125}I-labeled platelets were incubated for 30 min at 22°C in the presence of divalent cations (*upper panel*) or in the presence of 5 mM EDTA in a buffer where Ca^{2+} and Mg^{2+} were omitted (*lower panel*). Platelets were then processed for CIE as described in Fig. 2 with the exception that no ^{125}I-labeled antibody was present in the intermediate gel. Immunoprecipitates corresponding to surface-labeled proteins were revealed after autoradiography of the dried gel. In the presence of Ca^{2+}, a GPIIb–IIIa immunoprecipitate is present in the control and patient's CIE (*upper panel*). After treatment of the platelets with EDTA, there is a partial reduction of the GPIIb–IIIa immunoprecipitate in the control (*lower left panel*) with the appearance of a new immunoprecipitate corresponding to dissociated GPIIIa. Similar treatment with EDTA of the patient's platelets (*lower right panel*) induces the complete disappearance of the GPIIb–IIIa immunoprecipitate (*arrow*), which is fully dissociated into GPIIIa (*arrow*) and GPIIb (not visible on this gel).

Table III. MAb Binding

Antibody AP-2	Control	Patient	Father	Mother
	number of antibody binding sites per platelet			
With Ca^{2+}	36,130	19,400	25,430	29,130
With EDTA	33,610	300	15,930	15,020

The binding of increasing concentrations of ^{125}I-labeled antibodies was performed as described in Methods. Platelets were incubated at 22°C for 30 min in the presence of divalent cations or in the presence of 2 mM EDTA before the binding assay. The number of binding sites was estimated after Scatchard analysis of the data. The results represent the mean value of one experiment performed in triplicate.

GPIIb–IIIa bind the RGD-containing peptide which induces a conformational change in the complex.

Binding of soluble fibrinogen to GPIIb–IIIa necessitates a conformational change or "activation" induced by exposure of platelets to physiologic agonists such as ADP or thrombin. The MAb, PAC-1, is an IgM which specifically recognizes the "activated" form of GPIIb–IIIa. FACS analysis showed that, whereas thrombin-stimulated control platelets bound PAC-1 (Fig. 4 *E*), thrombin-stimulated platelets from patient M.S. failed to bind this antibody (Fig. 4 *F*). Similar results were obtained for ADP-stimulated platelets (data not shown).

GPIIIa and GPIIb DNA sequence analysis. In a first attempt to localize the genetic defect causing the variant Strasbourg I phenotype, we sought to sequence the patient's GPIIIa exons (B to D) encompassing a highly conserved region of GPIIIa containing a putative RGD (19, 21, 22), and a fibrinogen binding site (42). The patient's genomic DNA was used in the PCR amplification assay with primers A, B, and C (Table I), which flank respectively exons B, C, and D of GPIIIa. After subcloning in the M13 vector, the sequence of each exon was determined. There was agreement with the published normal nucleotide sequences, and with those from a control subject, except in exon D at the nucleotide corresponding to base 738 of the GPIIIa cDNA sequence (11), where a C residue was replaced by a T (Fig. 5). This substitution would result in the replacement of an arginine (CGG) by a tryptophan (TGG) at position 214 of the mature GPIIIa protein. This substitution was confirmed by sequencing the complementary strand. The genomic nucleotide sequences for the patient's parents were also determined and contained both C and T at position 738 of the GPIIIa cDNA (data not shown), suggesting heterozygosity for the mutation. Substitution of a C for a T at position 738 of the GPIIIa cDNA created an additional cleavage site for the restriction endonuclease Nla III (CATG/). Digestion of a control 235-bp amplified GPIIIa exon D–containing fragment with Nla III yielded the expected 120-, 60-, and 55-bp fragments (Fig. 6). Digestion of the patient's amplified fragment produced four fragments (80, 60, 55, and 40 bp) due to the presence of the additional Nla III site. Restriction enzyme analysis of the parents gave a mixed digestion pattern which agreed with their being heterozygous for the same substitution. A common polymorphism was ruled out by checking amplified fragments corresponding to exon D of GPIIIa in 100 unrelated chromosomes.

Primers were designed for the GPIIIa (primers D-G) and for the GPIIb (primers H-K) cDNA sequences in order to allow the

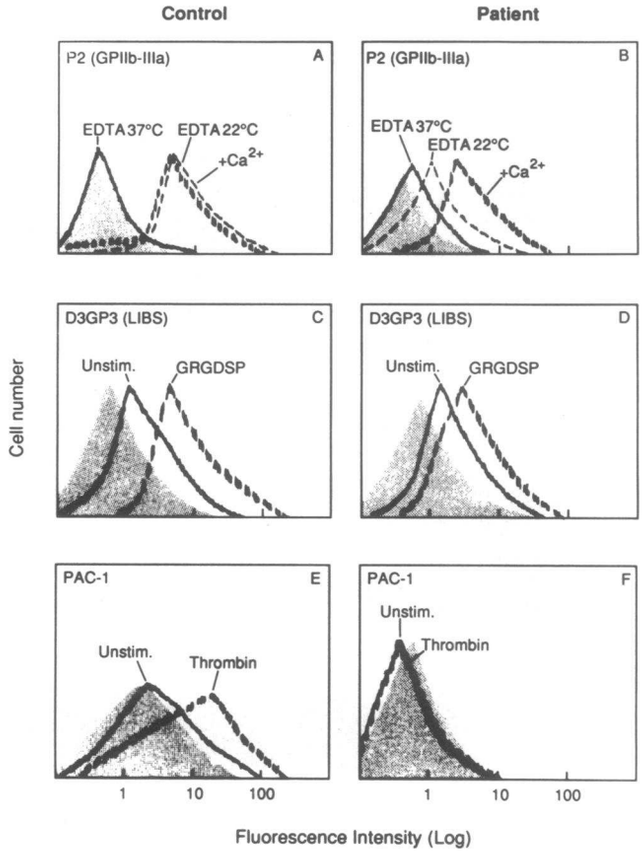

Figure 4. Flow cytometric analysis of the control and patient M.S. platelet GPIIb–IIIa. FACS analysis was performed on washed platelets (see Methods). Results are expressed as histograms of cell number on the ordinate versus fluorescence intensity (log scale) on the abscissa. The shaded area represents platelet autofluorescence in experiments performed with an irrelevant antibody. The presence of the GPIIb–IIIa complex on control and patient M.S.'s platelets is attested by the binding of the MAb P2 (*A* and *B*). Complete dissociation of surface GPIIb–IIIa occurs after treatment of both the control and patient's platelets with 5 mM EDTA at 37°C. Only the patient's platelets are dissociated after treatment with 2 mM EDTA at 22°C as noted by the decrease in P2 binding. D3GP3, an antibody revealing a LIBS on GPIIb–IIIa, binds poorly to unactivated platelets (control and the patient). In contrast, an increase in D3GP3 binding was seen when both control and M.S.'s platelets were incubated with the adhesive peptide GRGDSP (*C* and *D*). The activation-dependent epitope recognized by the PAC-1 antibody on GPIIb–IIIa is induced on normal platelets stimulated by 1 U/ml thrombin (*E*); thrombin-stimulated platelets of the patient did not bind PAC-1 (*F*).

amplification of the entire coding sequence for the two proteins (Table I). Sequence analysis confirmed the C to T substitution at position 738 of the patient's GPIIIa cDNA. In addition, three substitutions were found in the patient's GPIIIa cDNA (G to A, A to G, and T to C at positions 1553, 1565, and 1745, respectively), and two substitutions were found in the patient's GPIIb cDNA (T to G, and C to T at positions 2622, and 3064, respectively). None of them changed the amino acid composition, except for the T to G substitution at position 2622 of GPIIb which created a ^{843}Ile to ^{843}Ser transition. This mutation corresponds to a known polymorphism of GPIIb associated with the Bak^b (Lek^b) phenotype (43).

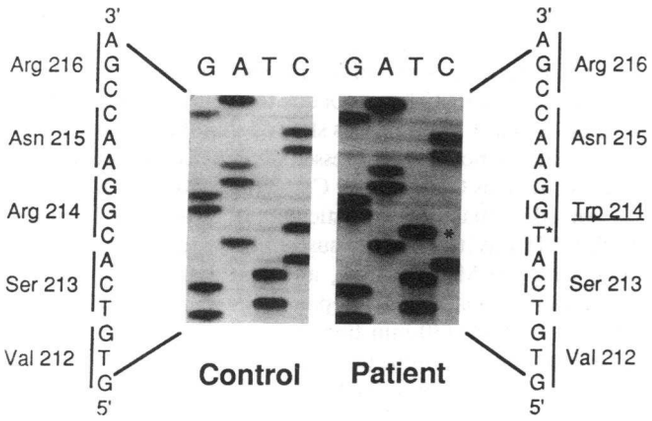

Figure 5. DNA sequence analysis of the amplified region of GPIIIa corresponding to nucleotides 729–753. In the gel for patient M.S., a band corresponding to T instead of C is indicated by an asterisk. This mutation changes the ^{214}Arg codon to a Trp. The tetranucleotide sequence (CATG) recognized by the restriction endonuclease Nla III is shown (*inside vertical dashed line*). Numbering was performed according to Fitzgerald et al. (11).

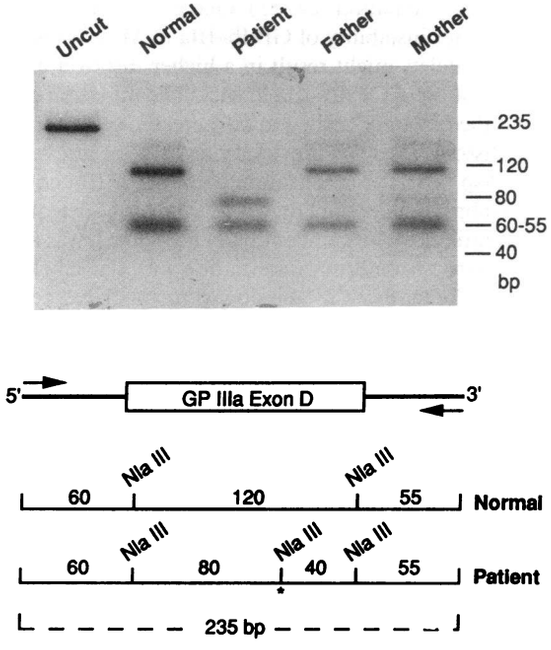

Figure 6. Restriction endonuclease digestion of PCR-amplified DNA. A 235-bp genomic DNA fragment containing exon D (*iv*) was amplified from a normal control, the patient, and the patient's father and mother using the oligonucleotide primer pair C (see Table I). The fragments were digested with Nla III and the resulting bands were separated on a 3% low-melt agarose gel. An uncut control fragment is presented at the left of the gel. Two Nla III sites are present in the normal fragment, generating a 120-bp band, and 60- and 55-bp bands which are indistinguishable on this gel. An additional Nla III site, indicated by an asterisk, is created by the C to T mutation at base 738 of the patient's DNA. This causes the cleavage of the 120-bp fragment into 80- and 40-bp fragments. The father and the mother exhibit both the normal and mutant pattern, indicating that they are heterozygous for the same mutation.

Discussion

The aim of the present study was to characterize the molecular and genetic defect of the Glanzmann's thrombasthenia variant Strasbourg I. The variant was characterized by the presence of subnormal amounts of platelet GPIIb–IIIa complexes with an increased sensitivity to EDTA dissociation. This defect was associated with an ^{214}Arg to ^{214}Trp substitution in GPIIIa. Several lines of evidence indicate that this single mutation is responsible for the variant Strasbourg I phenotype. First, this mutation was not a rare polymorphism inasmuch as it was not detected in 100 unrelated chromosomes. Second, the mutated ^{214}Arg residue is contained in a region of GPIIIa previously proposed to bind fibrinogen (42). Finally, no other amino acid substitution or deletion was found in the entire coding region of the patient's GPIIb and GPIIIa.

Including this study, four genetic defects associated with Glanzmann's thrombasthenia have been localized in GPIIIa. The two variants described so far, the Cam variant described by Loftus et al. (19) and the Strasbourg I variant reported here, are both caused by a single mutation within the GPIIIa coding sequence. The first example of GPIIIa gene defect in type I patients was reported by Bray and Shuman (18): the patient had no immunologically detectable GPIIb or GPIIIa but normal levels of the GPIIb mRNA. Southern analysis of the genomic DNA was consistent with a large (7 kb) insertion in the GPIIIa gene. Recent studies of Iraqi-Jewish patients by Newman et al. (17) provided a second example of GPIIIa gene defect in type I patients. Sequence analysis of amplified GPIIIa mRNA revealed a 11-base deletion within exon *xii* (L) of GPIIIa producing a frame-shift and protein termination before the transmembrane domain.

Two patients with defects of the GPIIb gene have also been described. Their platelets had a 1–5% level of immunologically detectable GPIIIa but no detectable GPIIb. Burk et al. (16) have described a patient with a 4-kb deletion between exons 2 and 9 of the GPIIb gene resulting in the transcription of a short mRNA that would encode for 32 out of 1,039 amino acids. The second GPIIb mutation was described in a Palestinian Arab population living in Israel (17). PCR analysis revealed a 13-base deletion at the splice acceptor site for exon 4 resulting in the in-frame deletion of amino acids 106–111. The defective GPIIb expression was speculated to result from the deletion of ^{107}Cys which may be critical for the proper folding of the protein.

Although integrins have common structural features and significant sequence homology, they have distinctive adhesive properties. For example, the platelet GPIIb–IIIa complex preferentially binds fibrinogen, but also binds vitronectin, fibronectin, and von Willebrand factor (vWF) (44, 45). In addition, the GPIIb–IIIa complex has the unique property among integrins in that it is dissociated into its individual α and β subunits upon removal of external Ca^{2+} by chelation (46–48). Another unique feature of GPIIb–IIIa, is that it requires platelet activation by physiologic agonists in order to bind soluble adhesive proteins (44, 45). The current challenge resides in the identification of the domains in GPIIb–IIIa responsible for its unique adhesive properties, for the assembly and stability of its Ca^{2+}-dependent heterodimeric structure, and for its activation-dependent function.

Three discrete sites have been identified so far on GPIIb–IIIa that are involved in the binding of its natural ligand, fibrin-

ogen. The RGD amino acid sequence is found twice in the fibrinogen α chain, and has been postulated to mediate at least part of the fibrinogen binding to GPIIb–IIIa (38). The binding of the RGD sequence can also "activate" the GPIIb–IIIa complex and render it competent to bind soluble adhesive proteins (49). Cross-linking experiments with RGDS peptides (21, 22), and analysis of the Glanzmann's thrombasthenia Cam variant (19) have pointed to a region in the amino-terminal portion of GPIIIa (residues 109–171) involved in the binding of RGDS peptides. A binding site for a fibrinogen γ chain dodecapeptide has been narrowed to a region of GPIIb corresponding to residues 296–306 by a combination of peptide cross-linking experiments (50), and competition assays with synthetic peptides corresponding to sequences of GPIIb (51). The γ chain dodecapeptide is unique to fibrinogen, and is not found on other adhesive proteins interacting with GPIIb–IIIa such as fibronectin, vWF, or vitronectin which appear to bind through RGD sequences (44, 45). A third fibrinogen binding site has been recently localized to residues 211–222 of GPIIIa based on the inhibition of fibrinogen binding to purified GPIIb–IIIa by synthetic peptides from GPIIIa and antibodies to these peptides (42). The mutation reported here affects the arginine at position 214 of GPIIIa, which is contained within the postulated fibrinogen binding 211–222 sequence of GPIIIa. Our result with variant Strasbourg I thus provides further evidence that this region of GPIIIa is taking part in the interaction with fibrinogen. The ^{214}Arg resides outside the previously identified RGD recognition sequence which was localized within residues 109–171 of GPIIIa (21) with ^{119}Asp being an essential residue (19). This suggests that the mutated GPIIb–IIIa ^{214}Trp would normally bind RGD-containing peptides. Indeed, incubation of the M.S. variant's platelets with GRGDSP resulted in the binding of the D3GP3 antibody which recognizes changes in conformation of GPIIb–IIIa upon RGDS or fibrinogen binding (30). This antibody also binds more avidly to dissociated GPIIb–IIIa, but the possible dissociation of GPIIb–IIIa upon GRGDSP treatment was ruled out by the normal binding of complex-specific antibodies such as AP-2 (data not shown). These data imply that GPIIb–IIIa complexes of M.S. platelets express a normal binding site for RGD. Such results are in agreement with the reported observation that the binding of the 211–222 GPIIIa peptide to fibrinogen was not inhibited by RGDS (42). Thus, patient M.S.'s GPIIb–IIIa seems to recognize short RGDS-containing peptides but does not appear to recognize this sequence within the intact adhesive proteins.

The antibody PAC-1 has been proposed to bind to, or in close proximity to, the proposed RGD binding region of GPIIb–IIIa. This conclusion was based on the inhibition of PAC-1 binding to activated platelets by RGD peptides (29), and on the presence of an RYD sequence within the third complementarity-determining region of the antibody (52). An absence of PAC-1 binding to Cam variant platelets has been reported (53), and is due to an abnormality in the RGDS binding site previously shown to be recognized by PAC-1. Our data showed an absence of PAC-1 binding to the patient's platelets activated by either ADP or thrombin. This was so even though, as discussed above, the platelets displayed an RGD binding capacity. Our result suggests that the binding of PAC-1 to activated platelets necessitates, like the binding of fibrinogen, additional sites on the GPIIb–IIIa complex outside of the RGD binding region on GPIIIa. Alternatively, RGDS being a small molecule may have access to the binding site, whereas that of a large molecule such as the IgM, PAC-1, may be limited as a result of conformational changes linked to the amino acid mutation at position 214 of GPIIIa. The inability of patient M.S.'s platelets to bind PAC-1 was not due to a general platelet refractoriness in that FACS analysis showed that the thrombin-activated platelets normally expressed other activation dependent antigens such as GMP-140 or CD63 (data not shown).

An additional characteristic of the Strasbourg I variant GPIIb–IIIa was its increased susceptibility to EDTA dissociation. CIE, ^{125}I-MAb binding, and FACS analysis showed a complete dissociation of the patient's GPIIb–IIIa complexes under conditions (30-min treatment with 2–5 mM EDTA at 22°C) leading to minimal dissociation of GPIIb–IIIa of normal platelets. In this respect, the Strasbourg I variant behaved like the variant (patient C.M.) previously described by Nurden et al. (23) whose GPIIb–IIIa complexes also displayed a similar sensitivity to dissociation with EDTA. However, the molecular defect of the Strasbourg I variant may be distinct from that of variant C.M. For example, differences include detectable amounts of platelet fibrinogen (21% of control) for M.S. versus no platelet fibrinogen for C.M., and half-normal number of platelet GPIIb–IIIa complexes; 19,400 AP-2 binding sites for M.S. versus 38,900 binding sites for C.M. The number of GPIIb–IIIa complexes detected in M.S. platelets by AP-2 binding fell within the lower range of the 22,400–58,600 AP-2 binding sites or 27,000–50,000 binding sites for MAb 10E5 found within the normal population (54, 55). One possible explanation is that a greater instability of GPIIb–IIIa in M.S. as compared to C.M. platelets might result in a higher turnover and therefore degradation of GPIIb–IIIa in M.S. The mutation described here is the first to be related to an increased susceptibility to EDTA dissociation. The previously described Cam variant with a ^{119}Asp to ^{119}Tyr substitution had GPIIb–IIIa complexes with an abnormal interaction with Ca^{2+} cations detected with a conformational dependent antibody PMI-1, but was not described as having an abnormal dissociation with Ca^{2+} chelating agents (20). Repetitive Ca^{2+}-binding domains have been assigned to GPIIb based on sequence homology with calmodulin or troponin C calcium binding motives (9, 56). Furthermore, there is now experimental evidence in support of the binding of divalent cations to GPIIb, inasmuch as fragments of GPIIb containing these segments and expressed in *Escherichia coli* were shown to directly bind ^{45}Ca (57). Because these sites could be involved in the Ca^{2+}-dependent instability of the M.S. GPIIb–IIIa complexes, we determined the entire sequence of the M.S. GPIIb cDNA, including the calcium binding sequences, and found no difference with the normal published sequences. The only mutation was the ^{214}Arg to ^{214}Trp substitution which resides in a region of GPIIIa which bears no apparent homology with other Ca^{2+} binding motives, and has not the proper spacing of anionic residues necessary to interact with divalent cations (56). To explain the role of ^{214}Arg in the Ca^{2+}-dependent instability of the GPIIb–IIIa complex, one can hypothesize that the transition to a tryptophan exerts a significant influence on the conformation of the resulting protein, and that this would render the Ca^{2+} sites more accessible to the chelating agents.

A certain number of questions can now be addressed through the in vitro expression in cultured cells of genetically engineered mutants incorporating the GPIIIa ^{214}Arg to ^{214}Trp mutation. GPIIb–IIIa is unique among the integrins in its sensitivity to EDTA dissociation, although all other integrins pos-

sess homologous Ca^{2+} binding motives in their α subunits. The $\alpha_v\beta_3$ vitronectin receptor is very closely related to GPIIb–IIIa (58, 59), contains a common β_3 subunit (GPIIIa), and has similar ligand binding specifities, but is not susceptible to EDTA dissociation (60). We have not been able to study the patient's $\alpha_v\beta_3$ in cells who highly express this receptor, such as the vascular endothelial cells. However, it will now be possible to examine by oligonucleotide-directed mutagenesis if a $\alpha_v\beta_3$ ^{214}Trp vitronectin receptor would lose its ligand binding capacity and also be rendered sensitive to EDTA dissociation. The ^{214}Arg to ^{214}Trp mutation resides in a region of GPIIIa, which has a high degree of sequence identity ($\sim$ 75%), when compared to that of other integrin β subunits, i.e., β_1, β_2, β_4, β_5 (61–66). The highest degree of homology is found between β_3 (GPIIIa) and β_5, and in the region corresponding to residues 211–222 of GPIIIa there is 100% sequence identity. Like β_3 (GPIIIa), β_5 associates with α_v to form a receptor for vitronectin and fibronectin. Thus, introduction of the Arg to Trp mutation could be applied to $\alpha_v\beta_5$ and help to further define the role of the region around ^{214}Arg in ligand and subunit–subunit interactions.

The two parents of M.S. were studied in parallel and displayed an heterozygous phenotype in terms of fibrinogen binding to ADP-stimulated platelets ($\sim$ 50% of control), and in their sensitivity to EDTA dissociation, i.e., treatment of their platelets at 22°C with EDTA decreased by half their platelet GPIIb–IIIa complex content. The heterozygous phenotype was extended at the molecular level by the finding that both parents were heterozygous for the same C to T mutation at base 738 of the GPIIIa cDNA. The inheritance of this mutation has not been explored in other members of the family but could now be easily studied by restriction enzyme analysis of amplified genomic DNA corresponding to the GPIIIa gene exon D. This mutation might have originated from a de novo mutation. The C to T sequence change reported here is a transition within a CG dinucleotide. The cytidine residue in CG dinucleotides is frequently methylated and may undergo deamination to yield a thymidine (67). Such regions are mutational hotspots in many genes; for example frequent mutations within hotspots have been described in the factor VIII (68), factor IX (69), and vWF genes (70, 71).

In conclusion, the ^{214}Arg to ^{214}Trp mutation of GPIIIa found in the Glanzmann's variant Strasbourg I points to an important domain of GPIIIa, distinct from the RGD binding region, involved in the binding of fibrinogen, and influencing the stability of the GPIIb–IIIa complex. This study shows that the molecular characterization of Glanzmann's variants is a very useful approach in order to identify functional domains in the GPIIb–IIIa complex, and should be of further aid in mapping sites involved in ligand and subunit–subunit interaction within the integrin family of adhesive receptors.

Acknowledgments

We thank Dominique Pidard for his help in preparing the CIE experiments; Hélène Pfitzinger from Codgene, Strasbourg, France who performed the HLA typing experiments; Corinne De la Salle and Jean-Louis Mandel for helpful discussions; Agnès Schwartz, Danila Gobaille, and Marie-Jeanne Baas for technical assistance; and Claudine Helbourg for secretarial assistance.

Note Added in proof. After the manuscript for this article was accepted for publication, a thrombasthenic variant with properties similar to the Strasbourg I variant and a ^{214}Arg to Gln mutation of GPIIIa was reported by Bajt et al. (72).

References

1. Caen, J. P., P. A. Castaldi, J. C. Leclerc, S. Inceman, M. J. Larrieu, M. Probst, and J. Bernard. 1966. Congenital bleeding disorders with long bleeding time and normal platelet count. *Am. J. Med.* 41:4–26.

2. Nurden, A. T., and J. P. Caen. 1974. An abnormal platelet glycoprotein pattern in three cases of Glanzmann's thrombasthenia. *Br. J. Haematol.* 28:253–260.

3. Phillips, D. R., and P. Poh Agin. 1977. Platelet membrane defects in Glanzmann's thrombasthenia: evidence for decreased amounts of two major glycoproteins. *J. Clin. Invest.* 60:535–545.

4. George, J. N., A. T. Nurden, and D. R. Philipps. 1984. Molecular defects of the interaction with platelets and the vessel wall. *N. Engl. J. Med.* 311:1084–1098.

5. George, J. N., J. P. Caen, and A. T. Nurden. 1990. Glanzmann thrombasthenia: the spectrum of clinical disease. *Blood.* 75:1383–1395.

6. Hynes, R. O. 1987. Integrins: a family of cell surface receptors. *Cell.* 48:549–554.

7. Phillips, D. R., I. F. Charo, L. V. Parise, and L. A. Fitzgerald. 1988. The platelet membrane glycoprotein IIb–IIIa complex. *Blood.* 71:831–843.

8. Ginsberg, M. H., J. C. Loftus, and E. F. Plow. 1988. Cytoadhesins, integrins, and platelets. *Thromb. Haemostasis.* 59:1–6.

9. Poncz, M., R. Eisman, R. Heidenreich, S. M. Silver, G. Vilaire, S. Surrey, E. Schwartz, and J. S. Bennett. 1987. Structure of the platelet membrane glycoprotein IIb: homology to the α subunit of the vitronectin and fibronectin receptors. *J. Biol. Chem.* 262:8476–8482.

10. Heidenreich, R., R. Eisman, K. Delgrosso, S. Surrey, J. S. Bennett, E. Schwartz, and M. Poncz. 1990. The organization of the gene for platelet glycoprotein IIb. *Biochemistry.* 29:1232–1244.

11. Fitzgerald, L. A., B. Steiner, S. C. Rall, S.-S. Lo, and D. R. Phillips. 1987. Protein sequence of endothelial glycoprotein IIIa derived from a cDNA clone: identity with platelet glycoprotein IIIa and similarity to integrin. *J. Biol. Chem.* 262:3936–3939.

12. Rosa, J.-P., P. F. Bray, O. Gayet, G. I. Johnston, R. G. Cook, M. A. Shuman, and R. P. McEver. 1988. Cloning of glycoprotein IIIa cDNA from human erythroleukemia cells and localization of the gene to chromosome 17. *Blood.* 72:593–600.

13. Zimrin, A. B., R. Eisman, G. Vilaire, E. Schwartz, J. S. Bennett, and M. Poncz. 1988. Structure of platelet glycoprotein IIIa: a common subunit for two different membrane receptors. *J. Clin. Invest.* 81:1470–1475.

14. Lanza, F., N. Kieffer, D. R. Phillips, and L. A. Fitzgerald. 1990. Characterization of the human platelet glycoprotein IIIa gene: comparison with the fibronectin receptor β-subunit gene. *J. Biol. Chem.* 265:18098–18103.

15. Zimrin, A. B., S. Gidwitz, S. Lord, E. Schwartz, J. S. Bennett, G. C. White II, and M. Poncz. 1990. The genomic organization of platelet glycoprotein IIIa. *J. Biol. Chem.* 265:8590–8595.

16. Burk, C. D., P. J. Newman, S. Lyman, J. Gill, B. S. Coller, and M. Poncz. 1991. A deletion in the gene for glycoprotein IIb associated with Glanzmann's thrombasthenia. *J. Clin. Invest.* 87:270–276.

17. Newman, P. J., U. Seligsohn, S. Lyman, and B. S. Coller. 1991. The molecular genetic basis of Glanzmann thrombasthenia in the Iraqi-Jewish and Arab population living in Israel. *Proc. Natl. Acad. Sci. USA.* 88:3160–3164.

18. Bray, P. F., and M. A. Shuman. 1990. Identification of an abnormal gene for the GPIIIa subunit of the platelet fibrinogen receptor resulting in Glanzmann's thrombasthenia. *Blood.* 75:881–888.

19. Loftus, J. C., T. E. O'Toole, E. F. Plow, A. Glass, A. L. Frelinger III, and M. H. Ginsberg. 1990. A β_3 integrin mutation abolishes ligand binding and alters divalent cation-dependent conformation. *Science (Wash. DC).* 249:915–918.

20. Ginsberg, M. H., A. Lightsey, T. J. Kunicki, A. Kaufmann, G. Marguerie, and E. F. Plow. 1986. Divalent cation regulation of the surface orientation of platelet membrane glycoprotein IIb. *J. Clin. Invest.* 78:1103–1111.

21. D'Souza, S. E., M. H. Ginsberg, T. A. Burke, S. C. T. Lam, and E. F. Plow. 1988. Localization of an Arg-Gly-Asp recognition site within an integrin adhesion receptor. *Science (Wash. DC).* 242:91–94.

22. Smith, J. W., and D. A. Cheresh. 1988. The Arg-Gly-Asp binding domain of the vitronectin receptor. *J. Biol. Chem.* 263:18726–18731.

23. Nurden, A. T., J.-P. Rosa, D. Fournier, C. Legrand, D. Didry, A. Parquet, and D. Pidard. 1987. A variant of Glanzmann's thrombasthenia with abnormal glycoprotein IIb-IIIa complexes in the platelet membrane. *J. Clin. Invest.* 79:962–969.

24. Legrand, C., V. Dubernard, A. T. Nurden. 1989. Studies on the mechanism of expression of secreted fibrinogen on the surface of activated human platelets. *Blood.* 73:1226–1234.

25. Fournier, D. J., A. Kabral, P. A. Castaldi, and M. C. Berndt. 1989. A variant of Glanzmann's thrombasthenia characterized by abnormal glycoprotein IIb/IIIa complex formation. *Thromb. Haemostasis.* 62:977–983.

26. Pidard, D., A. L. Frelinger III, C. Bouillot, and A. T. Nurden. 1991. Activation of the fibrinogen receptor on human platelets exposed to alpha-chymotrypsin: relation with a major proteolytic cleavage at the carboxyterminus of the membrane glycoprotein-IIb heavy chain. *Eur. J. Biochem.* 200:437–448.

27. Pidard, D., R. R. Montgomery, J. S. Bennett, and T. J. Kunicki. 1983.

Interaction of AP-2, a monoclonal antibody specific for the human platelet glycoprotein IIb–IIIa complex, with intact platelets. *J. Biol. Chem.* 258:12582–12586.

28. McEver, R. P., E. M. Bennett, and M. N. Martin. 1983. Identification of two structurally and functionally distinct sites on human platelet membrane glycoprotein IIb-IIIa using monoclonal antibodies. *J. Biol. Chem.* 258:5269–5275.

29. Shattil, S. J., M. Cunningham, and J. A. Hoxie. 1987. Detection of activated platelets in whole blood using activation-dependent monoclonal antibodies and flow cytometry. *Blood.* 70:307–315.

30. Kouns, W. C., C. D. Wall, M. M. White, C. F. Fox, and L. K. Jennings. 1990. A conformation-dependent epitope of human platelet glycoprotein IIIa. *J. Biol. Chem.* 265:20594–20601.

31. Cazenave, J.-P., S. Hemmendinger, A. Beretz, A. Sutter-Bay, and J. Launay. 1983. L'agrégation plaquettaire: outil d'investigation clinique et d'étude pharmacologique. Méthodologie. *Ann. Biol. Clin.* 41:167–179.

32. Kinlough-Rathbone, R. L., J. F. Mustard, M. A. Packham, D. W. Perry, H.-J. Reimers, and J.-P. Cazenave. 1977. Properties of washed human platelets. *Thromb. Haemostasis.* 37:291–308.

33. Nurden, A. T., D. Dupuis, T. J. Kunicki, and J. P. Caen. 1981. Analysis of the glycoprotein and protein composition of Bernard-Soulier platelets by single and two-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis. *J. Clin. Invest.* 67:431–440.

34. Kunicki, T. J., D. Pidard, J.-P. Rosa, and A. T. Nurden. 1981. The formation of Ca^{2+}-dependent complexes of platelet membrane glycoproteins IIb and IIIa in solution as determined by crossed immunoelectrophoresis. *Blood.* 58:268–278.

35. Kekwick, R. A., M. E. McKay, M. H. Nance, and B. R. Record. 1955. The purification of human fibrinogen. *Biochem. J.* 60:671–683.

36. Mustard, J. F., R. L. Kinlough-Rathbone, M. A. Packham, D. W. Perry, E. J. Harfenist, and K. R. M. Pai. 1979. Comparison of fibrinogen association with normal and thrombasthenic platelets on exposure to ADP or chymotrypsin. *Blood.* 54:987–993.

37. Laurell, C. B. 1966. Quantitative estimation of proteins by electrophoresis in agarose gel containing antibodies. *Anal. Biochem.* 15:45–52.

38. Cheresh, D. A., S. A. Berliner, V. Vicente, and Z. M. Ruggeri. 1989. Recognition of distinct adhesive sites on fibrinogen by related integrins on platelets and endothelial cells. *Cell.* 58:945–953.

39. De la Salle, C., M. J. Baas, L. Grunebaum, M. L. Wiesel, A. Bianco, R. Gialeraki, T. Mandalaki, and J.-P. Cazenave. 1989. Carrier detection in hemophilia using pedigree analysis coagulation tests and DNA probes. *Nouv. Rev. Fr. Hématol.* 31:193–202.

40. Kieffer, N., L. A. Fitzgerald, D. Wolf, D. A. Cheresh, and D. R. Phillips. 1991. Adhesive properties of the β_3 integrins: comparison of GP IIb-IIIa and the vitronectin receptor individually expressed in human melanoma cells. *J. Cell Biol.* 113:451–461.

41. Frelinger III, A. L., S. C.-T. Lam, E. F. Plow, M. A. Smith, J. C. Loftus, and M. H. Ginsberg. 1988. Occupancy of an adhesive glycoprotein receptor modulates expression of an antigenic site involved in cell adhesion. *J. Biol. Chem.* 263:12397–12402.

42. Charo, I. F., L. Nannizzi, D. R. Phillips, M. A. Hsu, and R. M. Scarborough. 1991. Inhibition of fibrinogen binding to GP IIb-IIIa by a GP IIIa peptide. *J. Biol. Chem.* 266:1415–1421.

43. Lyman, S., R. H. Aster, G. P. Visentin, and P. J. Newman. 1990. Polymorphism of human platelet glycoprotein IIb associated with the Bak^a/Bak^b alloantigen system. *Blood.* 75:2343–2348.

44. Plow, E. F., M. H. Ginsberg, and G. A. Marguerie. 1986. Expression and function of adhesive proteins on the platelet surface. *In* Biochemistry of Platelets. D. R. Phillips and M. A. Shuman, editors. Academic Press, Inc., New York. 225–256.

45. Kieffer, N., and D. R. Phillips. 1990. Platelet membrane glycoproteins: functions in cellular interactions. *Annu. Rev. Cell Biol.* 6:329–357.

46. Pidard, D., D. Didry, T. J. Kunicki, and A. T. Nurden. 1986. Temperature-dependent effects of EDTA on the membrane glycoprotein IIb-IIIa complex and platelet aggregability. *Blood.* 67:604–611.

47. Fitzgerald, L. A., and D. R. Phillips. 1985. Calcium regulation of the platelet membrane glycoprotein IIb-IIIa complex. *J. Biol. Chem.* 260:11366–11374.

48. Brass, L. F., S. J. Shattil, T. J. Kunicki, and J. S. Bennett. 1985. Effect of calcium on the stability of the platelet membrane glycoprotein IIb-IIIa complex. *J. Biol. Chem.* 260:7875–7881.

49. Du, X., E. F. Plow, A. L. Frelinger III, T. E. O'Toole, J. C. Loftus, and M. H. Ginsberg. 1991. Ligands "activate" integrin $\alpha_{IIb}\beta_3$ (platelet GP IIb-IIIa). *Cell.* 65:409–416.

50. D'Souza, S. E., M. H. Ginsberg, T. A. Burke, and E. F. Plow. 1990. The ligand binding site of the platelet integrin receptor GPIIb-IIIa is proximal to the second calcium binding domain of its α subunit. *J. Biol. Chem.* 265:3440–3446.

51. D'Souza, S. E., M. H. Ginsberg, G. R. Matsueda, and E. F. Plow. 1991. A discrete sequence in a platelet integrin is involved in ligand recognition. *Nature (Lond.).* 350:66–68.

52. Taub, R., R. J. Gould, V. M. Garsky, T. M. Ciccarone, J. Hoxie, P. A. Friedman, and S. J. Shattil. 1989. A monoclonal antibody against the platelet fibrinogen receptor contains a sequence that mimics a receptor recognition domain in fibrinogen. *J. Biol. Chem.* 264:259–265.

53. Ginsberg, M. H., A. L. Frelinger, S. C.-T. Lam, J. Forsyth, R. McMillan, E. F. Plow, and S. J. Shattil. 1990. Analysis of platelet aggregation disorders based on flow cytometric analysis of membrane glycoprotein IIb-IIIa with conformation-specific monoclonal antibodies. *Blood.* 76:2017–2023.

54. Nurden, A. T., D. Fournier, and D. Pidard. 1988. Pathological aspects of platelet aggregation: a consideration of the heterogeneity in Glanzmann's thrombasthenia. *In* Biochemistry and Physiopathology of Platelet Membrane. G. Marguerie and R. F. A. Zwall, editors. *Colloq. INSERM (Inst. Natl. Santé Rech. Méd.).* 158:95–105.

55. Coller, B. S., U. Seligsohn, A. Zivelin, E. Zwang, A. Lusky, and M. Modan. 1986. Immunologic and biochemical characterization of homozygous and heterozygous Glanzmann thrombasthenia in the Iraqi-Jewish and Arab populations of Israel: comparison of techniques for carrier detection. *Br. J. Hematol.* 62:723–735.

56. Strydnaka, N. C. J., and M. N. G. James. 1989. Crystal structures of the helix-loop-helix calcium-binding proteins. *Annu. Rev. Biochem.* 58:951–998.

57. Gulino, D., C. Boudignon, L. Zhang, E. Concord, and G. Marguerie. 1991. Analysis of the platelet GP IIb metal binding domain. *Thromb. Haemostasis.* 65:697. (Abstr.)

58. Fitzgerald, L. A., M. Poncz, B. Steiner, S. C. Rall, J. S. Bennett, and D. R. Phillips. 1987. Comparison of cDNA-derived protein sequences of the human fibronectin and vitronectin receptor α-subunits and platelet glycoprotein IIb. *Biochemistry.* 26:8158–8165.

59. Suzuki, S., W. S. Argraves, H. Arai, L. R. Languino, M. D. Pierschbacher, and E. Ruoslahti. 1987. Amino acid sequence of the vitronectin receptor α subunit and comparative expression of adhesion receptor mRNAs. *J. Biol. Chem.* 262:14080–14085.

60. Cheresh, D. A., and R. C. Spiro. 1987. Biosynthetic and functional properties of an Arg-Gly-Asp-directed receptor involved in human melanoma cell attachment to vitronectin, fibrinogen and von Willebrand factor. *J. Biol. Chem.* 262:17703–17711.

61. Argraves, W. S., S. Suzuki, H. Arai, K. Thompson, M. D. Pierschbacher, and E. Ruoslahti. 1987. Amino acid sequence of the human fibronectin receptor. *J. Cell Biol.* 105:1183–1190.

62. Kishimoto, T. K., K. O'Connor, A. Lee, T. M. Roberts, and T. A. Springer. 1987. Cloning of the β subunit of the leukocyte adhesion proteins: homology to an extracellular matrix receptor defines a novel supergene family. *Cell.* 48:681–690.

63. Hogervorst, F., I. Kuikman, A. E. G. Kr. von dem Borne, and A. Sonnenberg. 1990. Cloning and sequence analysis of beta-4 cDNA: an integrin subunit that contains a unique 118 kd cytoplasmic domain. *EMBO (Eur. Mol. Biol. Organ.) J.* 9:765–770.

64. Suzuki, S., and Y. Naitoh. 1990. Amino acid sequence of a novel integrin β_4 subunit and primary expression of the mRNA in epithelial cells. *EMBO (Eur. Mol. Biol. Organ.) J.* 9:757–763.

65. Ramaswamy, R., and M. E. Hemler. 1990. Cloning, primary structure and properties of a novel human integrin β subunit. *EMBO (Eur. Mol. Biol. Organ.) J.* 9:1561–1568.

66. McLean, J. W., D. J. Vestal, D. A. Cheresh, and S. C. Bodary. 1990. cDNA sequence of the human integrin β_5 subunit. *J. Biol. Chem.* 265:17126–17131.

67. Bird, A. P. 1980. DNA methylation and the frequency of CpG in animal DNA. *Nucleic Acids Res.* 8:1499–1504.

68. Youssoufian, H., H. H. Kazazian, Jr., D. G. Phillips, S. Aronis, G. Tsiftis, V. A. Brown, and S. E. Antonarakis. 1986. Recurrent mutations in haemophilia A give evidence for CpG mutation hotspots. *Nature (Lond.).* 324:380–382.

69. Giannelli, F., P. M. Green, K. A. High, J. N. Lozier, D. P. Lillicrap, M. Ludwig, K. Olek, P. H. Reitsma, M. Goossens, A. Yoshioka, et al. 1990. Haemophilia B: database of point mutations and short additions and deletions. *Nucleic Acids Res.* 18:4053–4059.

70. Randi, A. M., I. Rabinowitz, D. J. Mancuso, P. M. Mannucci, and J. E. Sadler. 1991. Molecular basis of von Willebrand disase type IIB: candidate mutations cluster in one disulfide loop between proposed platelet glycoprotein Ib binding sequences. *J. Clin. Invest.* 87:1220–1226.

71. Cooney, K. A., W. C. Nichols, M. E. Vruck, W. F. Bahou, A. D. Shapiro, E. J. W. Bowie, H. R. Gralnick, and D. Ginsburg. 1991. The molecular defect in type IIb von Willebrand disease: identification of four potential missense mutations within the putative GP Ib binding domain. *J. Clin. Invest.* 87:1227–1233.

72. Bajt, M. L., M. H. Ginsberg, A. L. Frelinger, M. C. Berndt, and J. C. Loftus. 1992. A spontaneous mutation of integrin $\alpha_{IIb}\beta_3$ (a platelet glycoprotein IIb-IIIa) helps define a ligand binding site. *J. Biol. Chem.* 267:3789–3794.