Glycoprotein IIb Leu214Pro Mutation Produces Glanzmann Thrombasthenia With Both Quantitative and Qualitative Abnormalities in GPIIb/IIIa

By Christine M. Grimaldi, Fangping Chen, Changhong Wu, Harvey J. Weiss, Barry S. Coller, and Deborah L. French

Glanzmann thrombasthenia is an inherited bleeding disorder due to a functional reduction or absence of platelet GPIIb/IIIa (α<sub>b</sub>β<sub>3</sub>) integrin receptors. Based on a prolonged bleeding time and absence of platelet aggregation in response to physiological agonists, a 55-year-old white man was diagnosed as having Glanzmann thrombasthenia. The patient's platelet fibrinogen level was ~5% of normal. As judged by complex-dependent monoclonal antibody (MoAb) binding, surface expression of platelet GPIIb/IIIa receptors was less than 5.5% of normal, whereas the binding of an anti-GPIIIa specific MoAb (7H2) was ~12% of normal. Immunoblot analysis of the patient's platelet lysates showed ~35% of normal levels of GPIIIa, ~30% of normal levels of GPIIb, and an abnormally migrating fragment of GPIIb. Biotinylation of the surface proteins on the patient's platelets followed by immunoprecipitation and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis showed only GPIIb and GPIIIa subunits of normal size. Surface expression of platelet α<sub>b</sub>β<sub>3</sub> receptors was 192% of normal, suggesting that the patient's defect was in GPIIIa. Sequence analysis of the patient's GPIIIa cDNA identified a T to C transition at nucleotide 643, predicting a Leu214Pro substitution. Direct sequencing of GPIIb exon 6 indicated that the patient is homozygous for the mutation. The nature of the Leu214Pro mutation was analyzed by expression in Chinese hamster ovary (CHO) cells. As judged by subunit-specific MoAb binding, surface expression of mutant receptors was ~60% of normal, but these receptors were not recognized by the complex-dependent monoclonal antibodies, 10E5 and 7E3. In addition, mutant receptors pretreated with the ligand-induced binding site MoAb AP5 were not recognized by the activation-dependent MoAb PAC-1 and mutant expressing CHO cells did not adhere to immobilized fibrinogen. These data suggest that the Leu214Pro mutation in GPIIb disrupts the structural conformation, and either directly or indirectly, the ligand binding properties of the heterodimeric complex. This is in accord with studies from other integrins that have implicated a β-turn in a homologous region as important in ligand binding. Thus, the Leu214Pro mutation appears to produce the Glanzmann thrombasthenia phenotype by both qualitative and quantitative abnormalities. In addition, the mutation appears to confer susceptibility of the GPIIIa subunit to proteolysis.

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thrombin, or epinephrine as a result of the GPIIb/IIIa abnormality. A total of 27 GPIIb and 23 GPIIIa genetic defects responsible for Glanzmann thrombasthenia have been identified.\textsuperscript{11-23} This study characterizes the mutational defect in a Glanzmann thrombasthenia patient with a T to C transition in GPIIb exon 6 resulting in a Leu214Pro amino acid substitution. Decreased levels of the mutant receptor were expressed on the patient’s platelets, but this quantitative abnormality was not as severe as it is in many other patients with Glanzmann thrombasthenia, suggesting the possibility of an additional qualitative abnormality. To assess this possibility, the function of the mutant receptor was analyzed by expression in Chinese hamster ovary (CHO) cells. The proline substitution in GPIIb resulted in a disruption of the ligand-binding conformation of the receptor complex as shown by (1) the inability of GPIIb/IIIa complex-dependent monoclonal antibodies to bind to the receptor, (2) the inability of the mutant receptor to bind PAC-1, and (3) the inability of transfected CHO cells to adhere to immobilized fibrinogen.

**MATERIALS AND METHODS**

**Subject.** The patient (L.W.) is a 55-year-old product of a nonconsanguineous marriage who has been the subject of previous reports.\textsuperscript{24,25} He suffered from repeated bouts of epistaxis, excessive bleeding after dental extractions and lacerations, and episodes of pharyngeal and gastric bleeding. Several bleeding episodes required platelet transfusions. At age 39, he developed painful swelling of his ankles and x-rays obtained 2 years later showed destructive changes in both ankle joints with total loss of the entire tibial talar joint, eburnation of the cartilaginous tissues, and cystic changes. Bilateral ankle fusion was performed and histologic examination of the synovial tissue showed evidence of remote hemorrhage and no active inflammation. Studies for rheumatoid factor have been consistently negative.

Laboratory tests have demonstrated absent platelet aggregation in response to ADP (50 µmol/L), but a normal initial response to ristocetin. Clot retraction, observed at 37°C in citrated platelet-rich plasma (PRP) that had been clotted with thrombin and calcium, was absent at 1 hour and only partial at 24 hours.

**Surface expression of platelet GPIb, GPIIb/IIIa, and αβ3 receptors.** The preparation of PRP and the assessment of surface expression of platelet receptors based on the binding of radiolabeled monoclonal antibodies was performed as previously described.\textsuperscript{26} Platelet surface GPIb expression was assessed by the binding of murine monoclonal antibody (MoAb) 6D1\textsuperscript{27}; GPIIb/IIIa expression was assessed by the binding of the complex-dependent murine MoAb 10E5\textsuperscript{28} and the Fab fragment of the mouse/human chimeric antibody 7E3\textsuperscript{29} (which also reacts with αβ3); and GPIIIa expression was assessed by the binding of the murine MoAb 7H2.\textsuperscript{29} Surface expression of the αβ3 vitronectin receptor was assessed by the binding of murine monoclonal antibodies LM142,\textsuperscript{30} specific for human αv, and LM609,\textsuperscript{30} specific for the αβ3 complex (generously provided by Dr David Cheresi, Scripps Clinic, La Jolla, CA).

**Platelet fibrinogen levels and immunoblot analyses.** The preparation of platelets and the analysis of sodium dodecyl sulfate (SDS)-solubilized platelets for fibrinogen, GPIIb, and GPIIIa levels by SDS-polyacrylamide gel electrophoresis (PAGE) and immunoblot were performed as previously described.\textsuperscript{19} Platelet fibrinogen was quantified relative to myosin heavy chain by scanning densitometry as previously described.\textsuperscript{19} For immunoblot analyses, samples of SDS-solubilized untreated or surface-biotinylated platelet proteins (see below) were first separated by SDS-PAGE and then electrophoresed onto polyvinylidene difluoride (PVDF) membranes (Millipore, Burlington, MA). The membranes were analyzed with a murine MoAb specific for GPIIIa, 7H2,\textsuperscript{29} and a murine MoAb specific for the heavy chain of GPIIb, PM1-1 (a generous gift of Dr Mark Ginsberg, Scripps Clinic, La Jolla, CA)\textsuperscript{31,32,33} followed by an horseradish peroxidase (HRP)-conjugated rabbit antimouse kappa light chain specific antibody. For identification of bands in immunoprecipitates from lysates of surface-biotinylated platelets, HRP-streptavidin was used followed by detection using the avidin-ECL chemiluminescence detection system (Amersham, Arlington Heights, IL). To obtain a semiquantitative assessment of patient platelet GPIIIa and GPIIIa content, immunoblot band intensities of multiple dilutions of normal and patient samples were compared visually after normalizing for myosin heavy chain.

**Platelet surface biotinylation and immunoprecipitation analysis.** PRP from 30 mL of whole blood anticoagulated with EDTA (10 mmol/L) yielded a total of ≈4 × 10\textsuperscript{9} platelets. Platelets were washed three times in phosphate-buffered saline (PBS) (137 mmol/L NaCl; 2.7 mmol/L KCl; 4.3 mmol/L NaHPO\textsubscript{4}; 1.4 mmol/L KH\textsubscript{2}PO\textsubscript{4}; pH 7.4) containing EDTA (10 mmol/L), and the platelet pellet was put on ice for 5 minutes. A fresh solution of Sulfo-NHS-LC-biotin (5 mmol/L) (Pierce, Rockford, IL) in PBS was added to each pellet to yield a final platelet concentration of ≈2 × 10\textsuperscript{8} platelets/mL. The pellets were quickly resuspended and incubated on ice for 30 minutes with occasional mixing. The biotinylated platelets were added to tubes prechilled on ice containing 5 mmol/L glycine in 5 mL Tris-buffered saline (TBS) (10 mmol/L Tris-Cl, pH 7.4; 150 mmol/L NaCl; 0.05% NaN\textsubscript{3}) and EDTA (10 mmol/L) (TSE) and incubated on ice for 10 minutes. Tubes were centrifuged (900 g) for 10 minutes and platelets were washed two times with TSE containing glycine (5.0 mL each). Platelet pellets were solubilized in lysis buffer (TBS containing 0.5% NP-40 and 2 mmol/L phenylmethylsulfonyl fluoride) at a concentration of ≈4 × 10\textsuperscript{9}/mL, incubated on ice for 30 minutes with occasional mixing, and centrifuged (12,000 g) for 30 minutes at 4°C. Supernatants were added to fresh tubes containing 1% deoxycholate and 0.1% SDS, centrifuged as in the previous step, and transferred to fresh tubes for immunoprecipitation analysis.

**Platelet lysates (1.0 mL each) were precleared by adding 100 µL of a 1:1 protein G sepharose (Pharmacia, Piscataway, NJ) slurry that was washed and equilibrated in immunoprecipitation (IP) buffer (100 mmol/L Tris-Cl, pH 7.4, 150 mmol/L NaCl, 0.5% NP-40, 1% deoxycholate, 0.1% SDS). This step was repeated one time. Immunoprecipitation of platelet GPIIb/IIIa was accomplished with antibody 10E5, GPIIb with Tab\textsuperscript{14} (generously provided by Dr Roger McEver, Oklahoma City, OK), and GPIIIa with 7H2. Antibody (5 µg/mL) was added to 20 µL (normal control) or 300 µL (patient L.W.) aliquots, tubes were rotated for 1 hour at room temperature followed by addition of protein G Sepharose slurry (50-µL/Tube) and rotation of tubes for an additional 45 minutes at room temperature. After centrifugation (12,000 g for 10 seconds), beads were washed five times in IP buffer containing 600 mmol/L NaCl, 1% deoxycholate, 0.1% SDS (1.0 mL), and then proteins were eluted from the beads by heating to 95°C in sample buffer. Samples were then analyzed by SDS-PAGE in 6.5% gels under reduced and nonreduced conditions and transferred onto PVDF membranes (Millipore). Biotinylated proteins were identified using the avidin-ECL chemiluminescence detection system (Amersham), according to the manufacturer’s instructions.

**Identification of mutation by polymerase chain reaction (PCR) and sequencing.** Primers were synthesized (Operon Technologies, Inc, Alameda, CA), resuspended in ddH\textsubscript{2}O (1.0 mmol/L), and stored at −80°C. The sequences of the GPIIb specific primers that were used for reverse transcriptase (RT)-PCR were kindly provided by Dr Peter Newman (The Blood Center of SE Wisconsin, Milwaukee) and are listed in Table 1. The sequences of the GPIIb-specific sense 5′AGGCGAGTATGGGAGCAAAGAG and antisense 5′GAAAATAATCCGCAAC-TGGAGG3′ primers used for the amplification of exon 6 were previously described.\textsuperscript{15} The protocols for RT-PCR and PCR amplification reactions were performed as previously described.\textsuperscript{34} The primers were 5′ and 3′ specific and were labeled on the 5′ end with either [α-32P]dATP or [α-32P]dCTP (NEN, Boston, MA) with a terminal transferase (5 U/µL) (Promega, Madison, WI). After PCR amplification and purification (QIAquick PCR purification kit, QIAGEN, Chatsworth, CA), the DNA was visualized on a 2% agarose gel stained with ethidium bromide (0.5 µg/mL). Bands of the correct size were excised and purified from the gel using the QIAquick gel extraction kit (QIAGEN, Chatsworth, CA). The sequences of the amplified fragments were determined using the automated DNA sequencer ABI Prism 310 Genetic Analyzer (Applied Biosystems, Foster City, CA). The 10E5 antibody recognizes the following sequence: GAGTAGGGAGCAAAAG 3′, which is complementary to the sequence on the antisense strand of exon 6, 5′AGGCGAGTATGGGAGCAAAGAG. The DNA sequences were analyzed using the Primer (1.0) software (2000) (Perkin-Elmer Cetus, Norwalk, CT). 

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and for the sequence determinations of PCR amplified fragments, including cloned fragments, were performed as previously described. The identification of the mutant sequence was obtained from amplified PCR fragments that were subcloned into the PCR II vector according to the manufacturer’s protocol (Invitrogen Corp, San Diego, CA). Confirmation of the mutation was obtained from the patient’s DNA by direct sequencing of amplified fragments of GPIIb exon 6.

**Generation of GPIIb Lei214Pro mutant cDNA construct.** The GPIIb and GPIIIa cDNA constructs in the pcDNA3 mammalian cell expression vector (Invitrogen Corp) were kindly provided by Dr Peter Mannheim, Indianapolis, IN) and gel-purified by electroelution for the manufacture’s protocol (Invitrogen Corp, San Diego, CA). Confirmation of the mutation was obtained from the patient’s DNA by direct sequencing of amplified fragments of GPIIb exon 6.

**Table 1. GPIIb cDNA Oligonucleotide Primers**

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<tr>
<th>Primer</th>
<th>Sequence</th>
<th>NT No.*</th>
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<tr>
<td>A-sense</td>
<td>GGCAGAAGCTTTGCTCCAGTC</td>
<td>4-26</td>
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<tr>
<td>C-antisense</td>
<td>AAAGCAGCTTACCTAGGGCCTC</td>
<td>487-466</td>
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<tr>
<td>D-sense</td>
<td>CACATTCAAACCTTCAAGGC</td>
<td>338-360</td>
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<tr>
<td>B-antisense</td>
<td>CGTGTGGTCTCTGACTGAAGT</td>
<td>779-856</td>
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<tr>
<td>E-sense</td>
<td>CGTGCACACAGGGCGGAGGAC</td>
<td>613-635</td>
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<td>F-antisense</td>
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<td>1037-1015</td>
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<tr>
<td>G-sense</td>
<td>GGATTTCAATGACGCTAAAAAGT</td>
<td>904-1026</td>
</tr>
<tr>
<td>I-antisense</td>
<td>GCTGCAAGAGCCCTGGAGGAC</td>
<td>1320-1298</td>
</tr>
<tr>
<td>J-sense</td>
<td>GGTCAAGGACCATTGGAGCTC</td>
<td>1202-1224</td>
</tr>
<tr>
<td>K-antisense</td>
<td>TCGACAGCAGATTAGGAATG</td>
<td>1620-1598</td>
</tr>
<tr>
<td>L-sense</td>
<td>CTAATACGACGCTCAACTG</td>
<td>1604-1624</td>
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<tr>
<td>H-antisense</td>
<td>GGGGCGGCTGGTCTGGGTC</td>
<td>2026-2006</td>
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<tr>
<td>M-sense</td>
<td>GACGTGCAATGACGACGAC</td>
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<td>N-antisense</td>
<td>CCAACCCAAAGCTTGAGGAC</td>
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* Nucleotide (numbering according to Poncz et al.)*

**RESULTS**

**Expression of platelet surface receptors.** The binding of radiolabeled 6D1 antibody (anti-GPIIIa) to the patient’s platelets was 108% of the control value demonstrating that the patient’s GPIb receptor was expressed at normal levels (Table 2). The binding of antibody 10E5 (anti-GPIIb/IIIa complex), 7E3 (anti-GPIIb/IIa and α6β3), and Tab (anti-GPIIb) in 100 µL of DMEM on ice for 60 minutes, washed with PBS, and incubated with fluorescein isothiocyanate (FITC)-labeled donkey anti-mouse Fab(‘)2 fragments (Jackson Immunoresearch, West Grove, PA). To assess the ability of the receptors to bind the PAC-1MoAb, transfected CHO cells (<3 to 5 × 10⁶) were resuspended in TSBG buffer (50 µmol/L Tris, pH 7.4; 150 mmol/L NaCl; 5 mmol/L glucose) containing MgCl₂ (1.4 mmol/L) and 100 µg/mL of the ligand-induced binding site (LIBS) MoAb AP5 (generously provided by Dr Thomas Kunicki, Scripps Clinic, La Jolla, CA) or the GPIIIa-specific MoAb AP3 (generously provided by Dr Peter Newman and FITC-labeled PAC-1 (20 µL of 0.1 mg/mL) (Becton Dickinson Immunocytometry Systems, San Jose, CA) in a total volume of 100 µL. The samples were analyzed with a FACScan flow cytometer (Becton Dickinson, Mountain View, CA) using LYSIS II software.

**Adhesion of transfected CHO cells to immobilized fibrinogen.** Adhesion of transfected CHO cells to immobilized fibrinogen was performed as previously described. Briefly, CHO cells were transfected by electroporation and analyzed 48 hours later. The cells (1.5 × 10⁸ cells/mL in 2 mL) were 51Cr-labeled for 1 hour at 37°C and incubated with TBS alone and complex-dependent antibodies (50 µg/mL) 10E5 (anti-GPIIb/IIIa), 7E3 (anti-GPIIb/IIa and α6β3), and LM609 (anti-α6β3) for 30 minutes at room temperature. Cells (50 µL/well) were added to the fibrinogen-coated plates and incubated for 1 hour at room temperature, washed, and adherent cells were lysed in 2% SDS (100 µL/well) for 30 minutes at room temperature. Lysates were counted in a Packard Autogamma 5650 (Packard Instrument Company, Downers Grove, IL). Cells bound per well were calculated from the specific activity of a control aliquot (50 µL) of 51Cr-labeled cells.

**CHO cells (plated 24 hours before transfection at 2 × 10⁶ cells/100-mm tissue culture dish) and incubated at 37°C for 6 hours. The transfection efficiency of the GPIIb and GPIIIa expressing cDNA constructs in CHO cells was determined by cotransfection with another plasmid, pXGH5, that expresses human growth hormone (hGH) (Nichols Institute Diagnostics, San Juan Capistrano, CA). The secreted hGH was measured in the medium from each dish using the HGH-TGES kit (Nichols Institute Diagnostics, San Juan Capistrano, CA). Briefly, medium (100 µL) from each dish was incubated with 125I-labeled and biotin-labeled hGH antibodies and an avidin-coated bead for 90 minutes at room temperature. The samples were washed and counted in a Packard Autogamma 5650 (Packard Instrument Co, Downers Grove, IL).

**GPIIb/IIa surface expression was measured by flow cytometry approximately 48 hours after transfection. Cells (<3 to 5 × 10⁶) were transfected with 10 µg/mL of monoclonal antibodies 10E5 (anti-GPIIb/IIIa complex), 7E3 (anti-GPIIb/IIa and α6β3), and Tab (anti-GPIIb) in 100 µL of DMEM on ice for 60 minutes, washed with PBS, and incubated with fluorescein isothiocyanate (FITC)-labeled donkey anti-mouse Fab(‘)2 fragments (Jackson Immunoresearch, West Grove, PA). To assess the ability of the receptors to bind the PAC-1 MoAb, transfected CHO cells (<3 to 5 × 10⁶) were resuspended in TSBG buffer (50 µmol/L Tris, pH 7.4; 150 mmol/L NaCl; 5 mmol/L glucose) containing MgCl₂ (1.4 mmol/L) and 100 µg/mL of the ligand-induced binding site (LIBS) MoAb AP5 (generously provided by Dr Thomas Kunicki, Scripps Clinic, La Jolla, CA) or the GPIIIa-specific MoAb AP3 (generously provided by Dr Peter Newman and FITC-labeled PAC-1 (20 µL of 0.1 mg/mL) (Becton Dickinson Immunocytometry Systems, San Jose, CA) in a total volume of 100 µL. The samples were analyzed with a FACScan flow cytometer (Becton Dickinson, Mountain View, CA) using LYSIS II software.

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**RESULTS**

**Expression of platelet surface receptors.** The binding of radiolabeled 6D1 antibody (anti-GPIIIa) to the patient’s platelets was 108% of the control value demonstrating that the patient’s GPIb receptor was expressed at normal levels (Table 2). The binding of antibody 10E5 (anti-GPIIb/IIIa) was 2.6% of normal and the binding of c7E3 Fab (anti-GPIIb/IIIa + α6β3) was 5.4% of normal. In contrast, the patient’s platelets bound 12% of the normal amount of antibody 7H2 (anti-GPIIIa), which does not require GPIIIa complex formation. The discordance between the binding values for 7H2 versus those of 10E5 and 7E3, raised the possibility that there may be GPIIb/IIIa complexes on the platelet surface that are not recognized by 10E5 and 7E3 because of abnormalities in complex formation. Antibody LM609 (anti-α6β3) was tested at two different concentrations, and in both cases, the patient’s platelets bound more antibody molecules than did the normal platelets (~192% of normal). The increased level of surface α6β3 receptors indicates that
GPIIIa (β₃) is probably normal, making it likely that the patient’s abnormality is in GPIIb.

Platelet fibrinogen level and immunoblot analyses. Platelet α-granule fibrinogen levels depend on the expression of functional GPIIb/IIIa receptors and so we analyzed the patient’s platelet fibrinogen content. Based on scanning densitometry of SDS-PAGE gels, the patient’s platelet fibrinogen level was <5% of normal. This value is similar to the levels of fibrinogen (3% to 11% of normal) identified in the platelets of patients with Glanzmann thrombasthenia having no GPIIb/IIIa or only trace amounts of GPIIb/IIIa. This value is much less than the 36% of normal levels we found in a patient with only <10% of normal surface GPIIb/IIIa whose mutant GPIIb/IIIa receptors were able to support cell adhesion to fibrinogen.

To determine the levels of total GPIIb and GPIIIa in the patient’s platelets, immunoblot analyses were performed. Semiquantitative estimates of GPIIb and GPIIIa levels were determined by analysis of multiple dilutions of patient and control solubilized platelets using the antibodies 7H2 (anti-GPIIIa) and PMI-1 (anti-GPIIb). Under nonreduced conditions, GPIIIa migrates at an Mr of ≈95,000 (Fig 1A, arrow). The protein level of GPIIIa in the patient’s platelets was determined to be ≈35% of normal. This level is considerably higher than the low and undetectable levels of GPIIIa, respectively, in the platelets of two other patients with Glanzmann thrombasthenia who have mutations in GPIIb (Arab) and GPIIIa (Iraqi-Jewish). This level is also higher than the estimate of surface GPIIIa (Table 2), suggesting that a disproportionate amount of GPIIIa is intracellular. The GPIIb in the patient’s platelets was analyzed under nonreduced (data not shown) and reduced (Fig 1B) conditions to determine the levels of proGPIIb, which migrates at M₉ ≈140,000 (Fig 1B, closed arrow), and mature processed GPIIb heavy chain, which migrates at M₉ ≈120,000 (Fig 1B, open arrow). A semiquantitative estimate of mature GPIIb heavy chain in the patient’s platelets was ≈25% of normal. The total of proGPIIb and mature GPIIb was estimated to be ≈30% of normal, which is similar to the estimate of GPIIIa content (≈35%), but much higher than the <1% of normal GPIIb content we found in other patients with Glanzmann thrombasthenia. Of interest was the identification of an abnormally migrating immunoreactive GPIIb fragment of Mr ≈100,000 (Fig 1B, arrow head) in both reduced samples of the patient’s platelets obtained more than 5 years apart. Nonreduced samples of the patient’s platelets also showed an abnormal GPIIIb-immunoreactive fragment, but the Mr was 120,000 (data not shown). Because the epitope for the PMI-1 antibody is located near the carboxy-terminal end of the GPIIb heavy chain, these data suggest that the abnormal band may be due to proteolytic cleavage of a fragment from the amino-terminus of GPIIb.

Table 2. Surface Expression of Platelet Receptors

<table>
<thead>
<tr>
<th>Receptor/Subunit</th>
<th>125I-Antibody Concentration</th>
<th>Molecules of Antibody/Platelet Surface Expression (of normal)</th>
<th>Patient</th>
<th>Control</th>
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<tr>
<td>GPIIba</td>
<td>6D1 (20 µg/mL)</td>
<td>19,100</td>
<td>17,100</td>
<td>108.0</td>
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<tr>
<td></td>
<td></td>
<td>18,900</td>
<td>18,200</td>
<td></td>
</tr>
<tr>
<td>GPIIb/IIIa</td>
<td>10ES (20 µg/mL)</td>
<td>700</td>
<td>38,500</td>
<td>2.6</td>
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<tr>
<td></td>
<td></td>
<td>1,400</td>
<td>40,900</td>
<td></td>
</tr>
<tr>
<td>GPIIb/IIIa + αβ₃</td>
<td>c7E3 Fab (18 µg/mL)</td>
<td>2,800</td>
<td>49,200</td>
<td>5.4</td>
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<tr>
<td></td>
<td></td>
<td>2,800</td>
<td>54,900</td>
<td></td>
</tr>
<tr>
<td>GPIIIa</td>
<td>7H2 (20 µg/mL)</td>
<td>6,200</td>
<td>53,000</td>
<td>12.0</td>
</tr>
<tr>
<td>αβ₃</td>
<td>LM609 (36 ng/mL)</td>
<td>25</td>
<td>15</td>
<td>172.0</td>
</tr>
<tr>
<td></td>
<td>(162 ng/mL)</td>
<td>53</td>
<td>25</td>
<td>212.0</td>
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</table>

Fig 1. Immunoblot analyses of GPIIa (nonreduced) and GPIIb (reduced). SDS-solubilized platelets (1 µL of 5 x 10⁷ platelets/mL) were electrophoresed into a 7.5% polyacrylamide gel, electrotransferred onto PVDF membranes, and developed as previously described. (A) Control, patient, and two other GT patient samples with mutations in GPIIa (I-J: Iraqi-Jewish) and GPIIb (Arab) were run under nonreduced conditions. The membranes were incubated with the anti-GPIIa specific murine MoAb, H52. The arrow indicates the position of GPIIa. (B) Two patient samples prepared on different dates (January 1990 and February 1995) and a control sample were run under reduced conditions. The membranes were incubated with the anti-GPIIb heavy chain specific murine MoAb, PMI-1. The solid arrow indicates the position of proGPIIb, the open arrow, mature processed GPIIb, and the arrow head an abnormally migrating fragment.
Surface biotinylation and immunoprecipitation of platelet GPIIb/IIIa receptors. Because an abnormal fragment of GPIIb was identified in the patient’s platelet lysate, the molecular weights of the GPIIb and GPIIIa subunits expressed on the surface of the patient’s platelets were determined. Platelet surface proteins were biotinylated and the receptor was immunoprecipitated from platelet lysates using the GPIIIa specific MoAb, 7H2, the GPIIIa specific MoAb, Tab, and the complex-dependent MoAb, 10E5. To ensure that the anti-GPIIb MoAb /Tab recognized the abnormal fragment of GPIIb, immunoblots of the biotinylated platelet lysates using PMI-1 were performed with (Fig 2A, right) and without (Fig 2A, left) immunoprecipitation using Tab. As already shown in Fig 1B, the abnormally migrating fragment was demonstrated in the immunoblot using PMI-1 (Fig 2A, left) and this fragment was also identified in the sample after immunoprecipitation with Tab (Fig 2A, right). These data show that the GPIIIb-specific MoAb, Tab, recognized the abnormally migrating GPIIb fragment in the patient’s platelets.

We next assessed which glycoproteins were expressed on the platelet surface by first immunoprecipitating all of the immunoreactive proteins, but only detecting the ones that were surface-biotinylated by using an avidin-HRP conjugated reagent. In the nonreduced (Fig 2B) and reduced (Fig 2C) samples immunoprecipitated with 10E5, Tab, and 7H2; only GPIIb and GPIIIa subunits of normal Mr were detected by this technique. The surface GPIIb immunoprecipitated by both Tab and 7H2 only contained the band of normal mobility, indicating that the abnormal GPIIb molecules do not become expressed on the platelet surface. The 10E5 MoAb failed to immunoprecipitate the patient’s surface exposed GPIIb/IIIa receptors presumably because the abnormality affects the 10E5 epitope. These data are consistent with the surface expression studies in which 125I-10E5 antibody binding to the patient’s platelets was only 2.6% of normal (Table 2).

Identification of a GPIIb mutation. Based on increased expression of platelet surface αβ3 receptors and the presence of an abnormal fragment of GPIIb in the patient’s platelet lysates (Fig 2), sequence analysis of GPIIb RNA was performed. Using 10 pairs of primers (Table 1) that hybridize to sequences specific for GPIIb cDNA, PCR products were generated by RT-PCR from total RNA of the patient and control. The amplified products from patient and control samples were cloned and sequenced. The only abnormality that was identified was a T to C mutation (Fig 3A) at nucleotide 64336 that corresponds to a Leu214Pro substitution. The mutation was confirmed by performing another RT-PCR reaction using primers E and F (Table 1) and sequencing the cloned product. Because Leu 214 is encoded within exon 6 of the GPIIb gene,46 specific primers were also used to amplify this exon from high molecular weight DNA isolated from patient and control peripheral blood mononuclear cells. Direct sequence analysis of exon 6 PCR fragments showed only the T to C mutation in the patient’s sample indicating that the patient did not have any normal DNA (Fig 3).

Surface expression of GPIIb/IIIa on CHO cells. CHO cell expression studies were performed to assess the effect of the Leu214Pro mutation in GPIIb/IIIa receptor expression and function. A GPIIb cDNA construct containing the T to C transition at position 64337 was generated by RT-PCR amplifica-
binding of mock transfected cells. To assess the possibility that adhesion by wild-type cells is mediated exclusively by cells expressing the highest level of receptors, flow cytometry using the GPIIb-specific MoAb, Tab, was performed on wild-type cells after adhesion to immobilized fibrinogen. The profile of

Fig 2. Immunoblot and immunoprecipitation of solubilized, surface-biotinylated platelets. (A) Left panel, immunoblot of patient and control platelet lysates using the GPIIb-specific MoAb, PMI-1. This pattern is essentially identical to that in Fig 1 except that because the control sample was diluted 20-fold, the bands in the patient's sample are more intense than the bands in the control sample. Right panel, immunoprecipitation of lysates using the GPIIb-specific MoAb, Tab, and immunoblotting with PMI-1. Samples were electrophoresed under reduced conditions and arrows mark the positions of pro-GPIIb, mature GPIIb, and the GPIIb fragment. These data indicate that Tab recognizes all forms of the mutant GPIIb. (B) Patient and normal control surface-biotinylated platelet lysates were immunoprecipitated with the GPIIb/IIIa complex-specific MoAb, 10E5, and the GPIIb-specific MoAb, Tab, and the GPIIIa-specific MoAb, 7E3. Immunoprecipitates were electrophoresed under nonreduced conditions and blotted onto PVDF membranes. The membranes were treated with HRP-streptavidin and the bands developed. Arrows mark the positions of GPIIb and GPIIIa. Because only surface-labeled molecules are detected by the avidin reagent, the failure to identify the patient's abnormal GPIIb bands indicates that these were not present on the surface of the patient's platelets. (C) Experiment conducted as in (B) except immunoprecipitates were reduced before electrophoresis.

Fig 3. PCR-amplified fragments and sequence analyses of control and patient RNA and DNA samples. (A) RT-PCR amplification of RNA extracted from normal control (N) and patient (LW) platelets. Using primers A and C (Table 1), a 424-bp fragment was cloned and sequenced. Arrows indicate the T (normal) to C (patient) nucleotide base change. (B) PCR amplification of DNA extracted from peripheral mononuclear cells isolated from a normal control (N) and the patient (LW). Using primers specific for amplification of GPIIb exon 6, the 245-bp fragments were directly sequenced as described previously. Arrows indicate the T (normal) to C (patient) nucleotide base change. No normal sequence was identified in the patient's DNA.

Table 3. Flow Cytometric Analyses of Transfected CHO Cells

<table>
<thead>
<tr>
<th>No.</th>
<th>Antibody</th>
<th>Subunit/Receptor</th>
<th>MFI*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>WT</td>
</tr>
<tr>
<td>1</td>
<td>Tab</td>
<td>GPIIb</td>
<td>42.6†</td>
</tr>
<tr>
<td>10E5</td>
<td>GPIIb/IIIa</td>
<td>41.3</td>
<td>4.2</td>
</tr>
<tr>
<td>2</td>
<td>Tab</td>
<td>GPIIb</td>
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<tr>
<td>7E3</td>
<td>GPIIb/IIIa+αβ3</td>
<td>50.1</td>
<td>&lt;1.0</td>
</tr>
</tbody>
</table>

*Mean fluorescence intensity in arbitrary fluorescence units. †Each value represents the average MFI, minus background values from mock transfected cells, from 2 separate experiments. Values for mock transfected cells: experiment 1 Tab = 54.4 and 10E5 = 52.2, experiment 2 Tab = 120.5 and 7E3 = 115.6. Transfection efficiencies were determined by secreted hGH levels using 125I-anti-hGH antibody (expressed in cpm): experiment 1 WT = 4797 and LW = 5539, experiment 2 WT = 1913 and LW = 2148.
adherent wild-type–expressing cells was broad, encompassing cells with quite variable expression of GPIIb/IIIa receptors. This expression profile for adherent wild-type cells overlapped the profile of Tab binding to the entire population of mutant-expressing cells, showing that the mutant cells expressed sufficient levels of receptors for adhesion to immobilized fibrinogen (data not shown). In conclusion, these data indicate that the Leu214Pro mutation in GPIIb disrupts the ligand-binding properties of the GPIIb/IIIa receptor complex.

DISCUSSION

We have identified a patient with typical clinical and laboratory features of Glanzmann thrombasthenia who has a Leu214Pro mutation in the GPIIb subunit of the platelet GPIIb/IIIa receptor. One unusual aspect of the patient’s history is long-standing seronegative arthritis. Although histology of his joint lining indicated evidence of distant hemorrhage, it is unlikely that his platelet disorder was causal because he did not give a history suggestive of repeated hemarthroses and spontaneous hemorrhages are rare in Glanzmann thrombasthenia.

The leucine residue at position 214 in human GPIIb is conserved in rodent GPIIb and a number of human α-chain subunits including αv, α1, α2, and α5 suggesting that this residue may be located in a site important for the conforma-
antibodies, to be activated into a high-affinity ligand binding conformation, and to adhere to immobilized fibrinogen suggests that this structural alteration affects ligand-binding and that the Leu214Pro mutation has either an indirect or direct effect on this site.

In addition to disrupting the ligand-binding conformation of the GPIIb/IIIa receptor, the Leu214Pro mutation resulted in decreased surface expression of the receptor on the patient’s platelets (Table 2). Platelet surface receptor levels were determined to be \( \approx 12\% \) of normal using an anti-GPIIIa specific antibody. A total of 27 mutations have been identified in the gene encoding GPIIb (including the mutation in this study). \(^{21,22}\) The majority of patients have surface expression levels of less than 10% of normal and one patient was reported to have expression levels greater than 20% of normal. \(^{54}\) Three patients have been reported to have receptor surface expression levels similar to that of patient LW \(^{55-57}\) and transfection studies showed comparable receptor expression levels to those identified on the patient’s platelets. In characterizing the Leu214Pro mutation in CHO cells, an interesting finding was that surface expression levels of the receptor were \( \approx 60\% \) of normal compared with the \( \approx 12\% \) of normal levels on the surface of the patient’s platelets. The discrepancy between the receptor expression levels on the surface of platelets and CHO cells is likely due to differences in proteolysis of the mutant GPIIb subunit between the cell types. Thus, when both intracellular and surface GPIIb/IIIa were measured by immunoblot analysis, the patient’s total platelet GPIIb/IIIa content was actually \( \approx 30\% \) to 35% of normal, with the proteolytic fragment of GPIIb a significant component. In contrast, this fragment was not present in lysates of CHO cells expressing the mutant GPIIb/IIIa receptor (data not shown). There are several possible reasons for the mutant GPIIb fragment being detectable in the patient’s platelets, but not in transfected CHO cells: (1) proteolysis may occur over a period of time during platelet formation and survival—the CHO cell expression studies were performed over 48 hours using cells that were transiently transfected with normal and mutant cDNA constructs, whereas platelets are formed over 8 to 10 days and circulate for another 10 days; (2) the enzyme(s) in platelets responsible for the cleavage of the GPIIb subunit may not be expressed or activated in CHO cells; and (3) the conformation of the GPIIb subunit that may result in susceptibility to proteolytic cleavage may not be formed during biogenesis of the receptor complex in CHO cells. It is interesting to speculate that the absence of mutant GPIIb cleavage in CHO cells contributed to the higher surface expression than was found in the patient’s platelets.

By immunoblot with an antibody to the carboxy-terminus of GPIIb (residues 875-891), the patient’s GPIIb fragment was \( M_r \) 120,000 with nonreduced platelets (which is \( \approx \) 20,000 less than proGPIIb or nonreduced GPIIb). On reduction, a fragment of \( M_r \approx 100,000 \) was identified, which presumably is the same fragment identified under nonreducing conditions, but without the GPIIb light chain \( (M_r \approx 20,000) \). These data suggest that the GPIIb fragment is formed by proteolysis of the amino-terminal end of the GPIIb heavy chain. If the cleavage interferes with transport of GPIIb to the platelet surface, which seems plausible, as the signal sequence is likely to be included, this might explain the relatively large amount of intraplatelet GPIIb/IIIa compared with platelet surface-expressed receptors, as well as the failure to find the cleaved GPIIb fragment on the surface of the patient’s platelets. The \( M_r \) of the fragment suggests that cleavage occurs near amino acid 200, which is near to the mutation site, suggesting that the additional proline may expose a nearby site that is susceptible to proteolysis.

We could not detect any normal GPIIb sequence in our analyses of the patient’s RNA or DNA, and this raises interesting questions because both the patient and his mother deny that he is the product of a consanguineous relationship. We excluded the presence of a large deletion affecting a possible second GPIIb allele by Southern blot and fluorescent in situ hybridization (FISH) analyses (V. Najfeld, unpublished data, November 1996). Another Glanzmann thrombasthenia patient was recently reported in whom only a mutant GPIIIa could be identified even though consanguinity was denied. \(^{58}\) Haplootype analyses of chromosome 17 suggested that homozygosity was due to uniparental disomy from the patient’s mother. We would like to perform similar studies, but the patient’s father is dead and his mother has not yet been available to study; the patient denies having any children.

In conclusion, we have identified a new GPIIb mutation, Leu214Pro, that produces a Glanzmann thrombasthenia phenotype due to both qualitative and quantitative abnormalities. This mutation alters the conformation of the GPIIb/IIIa receptor and disrupts ligand-binding. It is located within the first 334 residues of GPIIb, a region that has been identified as contributing to the ligand-binding pocket of the GPIIb/IIIa receptor, \(^{59}\) and amino-terminal to a putative binding site for the fibrinogen \( \gamma \)-chain dodecapeptide sequence (residues 294-314). \(^{60,61}\) The presumed defect in ligand binding capacity of the patient’s GPIIb/IIIa receptor is supported by the finding that the patient’s platelets contained only \( \approx 5\% \) of normal levels of plasma fibrinogen even though they contained \( \approx 12\% \) of the normal number of receptors on the platelet surface. The platelet fibrinogen level in this patient is much lower than the \( \approx 36\% \) of normal that we identified in another patient with Glanzmann thrombasthenia whose platelets contained only \( \approx 10\% \) of the normal numbers of surface receptor. \(^{19}\) This latter patient has a Cys374Tyr mutation in GPIIIa that affects surface expression, but not the ability of the receptor to mediate adhesion to immobilized fibrinogen. Thus, even though these two patients had mutations resulting in comparable levels of surface GPIIb/IIIa receptors, the characterization of parameters such as platelet fibrinogen levels and receptor function in transfected cells provided important structure/function information on biosynthesis, ligand-binding, and protein trafficking functions of the receptor. Finally, our studies highlight the importance of rigidly classifying Glanzmann thrombasthenia mutations into quantitative versus qualitative abnormalities, as a single mutation may produce both effects.

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Glycoprotein IIb Leu214Pro Mutation Produces Glanzmann Thrombasthenia With Both Quantitative and Qualitative Abnormalities in GPIIb/IIIa

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