A Gln747→Pro Substitution in the α1Ibβ3 Subunit Is Responsible for a Moderate α1Ibβ3 Deficiency in Glanzmann Thrombasthenia

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To clarify a molecular defect responsible for moderate α1Ibβ3 deficiency, we examined two unrelated patients, MT and MS, suffering from type II and type I Glanzmann thrombasthenia (GT), respectively. Sequence analysis of polymerase chain reaction (PCR) fragments derived from platelet mRNA showed a single A→C substitution at nucleotide (nt) 2334 leading to a Gln747→Pro in α1Ib in both patients. Allele-specific restriction enzyme analysis (ASRA) of genomic DNA demonstrated that patient MT was homozygous for the Gln747→Pro substitution and patient MS was compound heterozygous for this substitution and for an RNA splice mutation at the consensus sequence of the splice acceptor site of exon 18 (AG→AA). Furthermore, ASRA showed that, among 17 unrelated Japanese GT patients, this Gln747→Pro substitution was detected in 4 patients, including MT and MS (homozygous, 2 patients; heterozygous, 2 patients). Cotransfection of Pro747α1Ib and β3 constructs into 293 cells resulted in moderate reduction in the amount of α1Ibβ3 within the transfected cells as well as on the cell surface. However, Pro747α1Ibβ3 bound the ligand mimetic monoclonal antibody (MoAb) PAC-1 after activation of α1Ibβ3 by the MoAb PT25-2, suggesting that the mutant α1Ibβ3 possesses the ligand-binding function. The association between the mutant proα1Ib and β3 was not disturbed. Surface labeling and pulse chase study showed that the Gin747→Pro substitution moderately impaired both intracellular transport of the α1Ibβ3 heterodimers to the Golgi apparatus and endoproteolytic cleavage of proα1Ib into heavy and light chains. By contrast, replacement of Gin747 with Ala by mutagenesis did not impair α1Ibβ3 expression on the cell surface. These results suggest that the presence of Pro, rather than the absence of Gin, at amino acid residue 747 on α1Ib is responsible for moderate α1Ibβ3 deficiency.

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INTTEGRIN α1Ibβ3 (platelet GPIIb-IIIa), a calcium-dependent heterodimeric complex, is a prototype integrin that functions as a physiologic receptor for fibrinogen and von Willebrand factor and plays a crucial role in normal hemostasis and platelet aggregation.1-3 The importance of this integrin has been documented by the clinical features of Glanzmann thrombasthenia (GT), a rare autosomal recessive bleeding disorder characterized by a quantitative or qualitative abnormality of α1Ibβ3.4 Analysis of cultured human leukemic and megakaryocytic cell lines has led to a better understanding of the key steps for α1Ibβ3 biosynthesis.5-7 The α1Ib subunit is synthesized as a single-chain precursor, proα1Ib, that associates with the β3 subunit within the endoplasmic reticulum of cells. The proα1Ibβ3 complex is then transported to the Golgi apparatus, where proα1Ib undergoes sugar modification and endoproteolytic cleavage into heavy and light chains. After these processing events within the Golgi apparatus, the mature α1Ibβ3 complex is rapidly transported to the cell surface. Classically, GT can be divided into three subgroups according to the amount of α1Ibβ3: type I has a severe α1Ibβ3 deficiency (<5% of normal), type II has a moderate α1Ibβ3 deficiency (10% to 20% of normal), and a variant has normal to near normal levels of a dysfunctional α1Ibβ3 (50% to 100% of normal).8 To date, more than 30 mutations in either the α1Ib or β3 gene responsible for the thrombasthenic phenotype have been identified.8,9 However, most of the reported mutations are responsible for severe α1Ibβ3 deficiency (type I GT). Among these, the single amino acid substitutions have been especially informative in defining precise structural domains of integrins that play a role in the biosynthesis and/or function. For example, Gly242→Asp (Gly242→Asp)10 and Gly418→Asp11 in α1Ib have been characterized in type I GT; these were highly conserved residues adjusted to the first calcium binding domain and flanking the fourth calcium binding domain of α1Ib, respectively. By contrast, the molecular basis for moderate α1Ibβ3 deficiency (type II GT) remains obscure. Four mutations have been reported: Leu183→Pro12 and Arg327→His13,14 in α1Ib; Leu117→Trp15 and Cys374→Thr16 in β3.

We have recently demonstrated that the amount of α1Ib in MT is much lower than that of β3 in a number of Japanese GT patients.17 Our data suggest that the molecular defect may exist more often in the α1Ib gene than in the β3 gene in Japanese GT patients. In this study, we describe a new single amino acid substitution (Gln747→Pro) in α1Ib responsible for moderate α1Ibβ3 deficiency in 4 unrelated GT patients. Among them, patient MT (type II) was homozygous for the Gln747→Pro substitution and patient MS (type I) was compound heterozygous for this substitution and a RNA splice mutation.

MATERIALS AND METHODS

Patients. Patient MT, the product of nonconsanguineous parents, was a 40-year-old Japanese woman who had a life-long history of moderate mucocutaneous bleeding. Hematological examinations showed a prolonged bleeding time and absence of platelet aggregation in

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response to ADP, epinephrine, and collagen, but a normal response to ristocetin. Clot retraction was normal. She was patient no. 7 in our previous report and was classified as type II GT.21 Patient MS, the product of nonconsanguineous parents, was a 44-year-old Japanese woman who was also diagnosed as a typical case of GT. Clot retraction was slightly impaired (38%; normal values, 48% to 68%). Patients MT and MS were unrelated.

Antibodies. Rabbit polyclonal antisera specific for αIIbβ3 and murine monoclonal antibodies (MoAbs) AP2 (αIIbβ3-specific MoAb) were generously provided by Dr Thomas J. Kunicki ( Scripps Research Institute, La Jolla, CA).18 AP3 (β3-specific MoAb) was a generous gift from Dr Peter Newman (The Blood Center of Southeastern Wisconsin, Milwaukee, WI).19 PAC-1 (a ligand mimetic MoAb) binds specifically to activated αIIbβ3, and was kindly provided by Dr Sanford Shattil (Scripps Research Institute).20 PT25-2 (αIIbβ3-specific MoAb) activates αIIbβ3, and was a kind gift from Drs Makoto Handa and Yasuo Ikeda (Keio University, Tokyo, Japan).21 TP80 (αIIbβ3-specific MoAb) and MOPC21 were purchased from Nichirei (Tokyo, Japan) and Sigma Chemical (St Louis, MO), respectively.

Synthetic ligand. FK633 (N-[N-(4-Aminophenyloxy)butyl]-a-L-aspartyl-L-valine), a peptidomimetic antigen specific for αIIbβ3, was generously provided by Dr Jiro Seki (Fujisawa Pharmaceutical Co, Osaka, Japan).22

Immunoblot assay and flow cytometry. Immunoblot assay using rabbit polyclonal antisera specific for αIIbβ3 and flow cytometric analysis using various MoAbs were performed as previously described.23,24 The amount of αIIb and β3 was semiquantified by densitometry using a CS 9000 dual-wavelength flying spot scanner (Shimadzu Corp, Kyoto, Japan).

Amplification and analysis of platelet RNA. Total cellular RNA of platelets was isolated from 30 mL of whole blood and αIIb or β3 mRNA was specifically amplified by reverse transcription-polymerase chain reaction (RT-PCR), as previously described.24 The primers for the amplification of αIIb mRNA and conditions for RT-PCR were described elsewhere.25 The following primers were constructed based on the published sequence of β3,25 and used for the first-round PCR of β3 mRNA: IIIa1, 5′-GGGCTACATTGTTGACGCTG-3′ (sense, nt 9324-9344), and IIIa8-31′, 5′-CAACTCTTCAGGGAGGTCACG-3′ (antisense, nt 10048-10027), mismatched sequences were underlined) and V ent Polymerase (Vent IIb gene), and IIbE24, 5′-GCTG-3′ (antisense, nt 1015-1034; mismatched sequence was underlined) and V ent Polymerase (Vent IIb gene). Then, second-round amplification was performed using 1 µL of the first-round PCR products as a template with nested primers IIb5A, 5′-CAGGTTACATTGTTGACGCTG-3′ (sense, nt 1032-1052; mismatched sequence was underlined) and V ent Polymerase (Vent I). The mutant clones were characterized by sequence analysis to verify the absence of any other substitutions and the proper insertion of the PCR cartridge into the vector.

To generate an Ala747αIIb construct, we performed the site-directed mutagenesis by PCR. We synthesized mismatched sense primer IIB747Ala 5′-CCAGATGAATCCGGATG-3′ (sense, nt 2185-2205), and IIB8Xhu I, 5′-CTTCTTAGAGATGATTGAGGCTCACC-3′ (antisense, nt 3148-3123; mismatched sequence was underlined) and Vent Polymerase (Vent I). The amplified fragments were digested with RsrII and XbaI, and the resulting 823-bp fragments (nt 2318-3140) were extracted using GeneClean II kit (Bio 101, La Jolla, CA). The 2.367-bp fragment extending from the beginning of the open reading frame to nt 2317 was obtained by digesting the full-length of αIIb cDNA with HindIII and RsrII. These two fragments were double-inserted into the pcDNA3 digested with HindIII and XbaI. Single clones that encode A or C at nt 2334 were selected by PCR followed by PvuII digestion. The selected clones were characterized by sequence analysis to verify the absence of any other substitutions and the proper insertion of the PCR cartridge into the vector.

The wild-type or mutant αIIb construct was cotransfected into 293 cells with wild-type β3 construct by the calcium phosphate method, as previously described.26

Surface labeling of the transfected cells. Surface proteins of the transfected cells were biotinylated, and immunoprecipitation using MoAbs was performed as previously described.23

RESULTS

Immunoblot analysis. We first analyzed platelet proteins from patients MT and MS in an immunoblot assay under nonreducing (not shown) and reducing conditions (Fig 1). Various amounts of platelet proteins obtained from three normal subjects were also examined to obtain a standard curve. In patient MT, the amounts of αIIb and β3 were 15% and 22% of

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GACTTAGCTTCTTTCG-3′ (sense, nt 9324-9344), and IIB818, 5′-GGGCTACATTGTTGACGCTG-3′ (antisense, nt 10048-10027), were reamplified using nested primers IIBE17A, 5′-ATGCCGAGCTG-CAGCTG-3′ (sense, nt 9501-9517), and IIB818, PCR products were digested with AvrII. The resulting fragments were electrophoresed in a 6% polyacrylamide gel.

Construction of αIIb expression vectors. The αIIb and β3 cDNA constructs were cloned into a mammalian expression vector pcDNA3 (Invitrogen Corp, San Diego, CA) and generously provided by Dr Peter Newman. To construct the expression vectors containing the 2334 A (wild-type [WT]) or 2335C (Pro747) form of αIIb cDNA, PCR-based cartridge mutagenesis was performed. The 1,184-bp region (nt 1988-3171) of platelet αIIb cDNA from patient MS, who was heterozygous for 2334A and 2335C, was amplified by RT-PCR using primers IIB5 and IIB8. Then, second-round amplification was performed using 1 µL of the first-round PCR products as a template with nested primers IIB5A, 5′-CCAGATGAATCCGGATG-3′ (sense, nt 2185-2205), and IIB8Xhu I, 5′-CTTCTTAGAGATGATTGAGGCTCACC-3′ (antisense, nt 3148-3123; mismatched sequence was underlined) and Vent Polymerase (Vent I). The amplified fragments were digested with RsrII and XbaI, and the resulting 823-bp fragments (nt 2318-3140) were extracted using GeneClean II kit (Bio 101, La Jolla, CA). The 2.367-bp fragment extending from the beginning of the open reading frame to nt 2317 was obtained by digesting the full-length of αIIb cDNA with HindIII and RsrII. These two fragments were double-inserted into the pcDNA3 digested with HindIII and XbaI. Single clones that encode A or C at nt 2334 were selected by PCR followed by PvuII digestion. The selected clones were characterized by sequence analysis to verify the absence of any other substitutions and the proper insertion of the PCR cartridge into the vector.

The wild-type or mutant αIIb construct was cotransfected into 293 cells with wild-type β3 construct by the calcium phosphate method, as previously described.26

Metabolic labeling with [35S] methionine and pulse chase. Metabolic labeling of transfected cell was performed 1 day after transfection, as previously described.27 The cells were incubated with 0.2 mCi/mL of [35S]-methionine for 30 minutes and the medium was then changed to DME/10% FCS with 50 µg/mL of nonradioactive methionine. Cells were equally divided into five dishes and chased after 0, 2, 4, 8, and 24 hours, respectively. Immunoprecipitation was performed as described previously.27

RESULTS

Immunoblot analysis. We first analyzed platelet proteins from patients MT and MS in an immunoblot assay under nonreducing (not shown) and reducing conditions (Fig 1).
control, respectively, whereas in patient MS, αIIb and β3 were 4% and 8%, respectively. Abnormal αIIb or β3, such as a premature form of αIIb, was not detected under nonreducing and reducing conditions in either patient. From these data, MT was classified as type II GT and MS as type I GT.

**Nucleotide sequence analysis of αIIb cDNA from MT and MS.**

To identify the molecular defect in patients MT and MS, platelet mRNA was isolated from these patients and normal controls. The whole coding regions of αIIb and β3 cDNA were amplified by RT-PCR. Examination of nucleotide sequences of the PCR fragments using an ABI 373A DNA sequencer (Applied Biosystems, Foster City, CA) showed a single A → C substitution at nt 2334 in αIIb cDNA that leads to a Gln747 → Pro substitution in exon 23 of αIIb (Fig 2). Patient MT appeared homozygous for the 2334 A → C substitution. The homozygosity of the substitution was confirmed by nucleotide sequence analysis of PCR fragments from genomic DNA (data not shown). No other nucleotide substitution was detected in either αIIb or β3 cDNA from patient MT. Patient MS was heterozygous for the 2334 A → C substitution.

Because patient MS was heterozygous with severe deficiency, another genetic defect in the αIIb gene was sought. Electrophoretic analysis of the first round of RT-PCR fragments using primers IIb3 and IIb4 in patient MS showed that two different-sized cDNAs were amplified: an expected size (1,031 bp) and a smaller size (~900 bp) (Fig 3). Each cDNA fragment was subcloned into pUC19, and nucleotide sequences were analyzed. Sequence analysis of the smaller-sized fragment showed that a 126-bp region corresponding to the whole nucleotide sequence of exon 18 was deleted (Fig 3). No abnormality existed in the nucleotide sequence of the expected-sized fragment. The flanking region of exon 18 of the αIIb gene was then amplified from genomic DNA of patient MS as well as control by PCR using primers IIbE16 and IIbI18. Nucleotide sequence showed an AG → AA substitution at the consensus splice acceptor site (−1) of exon 18 (Fig 3). No other nucleotide substitution was detected in either αIIb or β3 cDNA in patient MS. Thus, MS appeared to be heterozygous for the 2334 A → C substitution and the G → A substitution at the splice acceptor site of exon 18 in the αIIb gene.

**ASRA.** To confirm that patient MS was a compound heterozygote, exon 23 and exon 18 with their flanking regions were amplified by PCR, followed by digestion with PvuII and AvrII, respectively. A restriction site for PvuII and AvrII was created by the 2334 A → C substitution and a restriction site for AvrII was created by the 2334 A → C substitution and a restriction site for AvrII.
abolished by the G → A substitution. ASRA clearly indicated that the A → C substitution in exon 23 was derived from the patient’s father and that the G → A substitution at the splice acceptor site of exon 18 was derived from the mother (Fig 4). These data confirmed that patient MS was a compound heterozygote. ASRA further confirmed that patient MT was homozygous for the A → C substitution in exon 23 (data not shown).

Using ASRA, we examined the presence of the 2334 A → C substitution in 15 other unrelated Japanese GT patients (type I, 8 cases; type II, 7 cases) and 20 control subjects. This substitution was present in 2 type II GT patients who were homozygote and heterozygote, respectively (data not shown). None of control subjects had this substitution.

Effect of Gln747 → Pro substitution on αIIbβ3 expression.

To examine whether the 2334 A → C substitution leading to Gln747 → Pro substitution (Pro747) in αIIb might be responsible for type II GT, we constructed an expression vector that contained the wild-type or mutant Pro747 form of αIIb. Each vector was cotransfected with the wild-type β3 cDNA into 293 cells.

RNA blot analysis showed that the efficiency of transfection between the wild-type and the mutant Pro747 αIIb was essentially the same (data not shown). Flow cytometric analysis using the αIIb-specific MoAb, TP80; the β3-specific MoAb, AP3; and the αIIbβ3 complex-specific MoAb, AP2, showed that the level of mutant Pro747αIIbβ3 expression was moderately reduced compared with wild-type αIIbβ3 expression (Fig 5). Immunoprecipitation of surface-labeled transfected cells using AP2 MoAb also showed that the amount of Pro747αIIbβ3 complex was moderately reduced compared with wild-type and that the molecular weight of the mutant αIIb was the same as the wild-type (Fig 6A). Interestingly, in the mutant Pro747αIIbβ3 transfected cells, a significant amount of a premature form of αIIb (proαIIb) was precipitated by AP2 MoAb. These data indicate that proαIIb could be expressed and complexed with β3 on the surface of the mutant Pro747αIIbβ3 transfected cells.

Densitometric analysis showed approximately 20% of normal levels of αIIb (proαIIb) and approximately 29% of normal levels of β3 expressed on the surface of the Pro747αIIbβ3 transfectants (mean of 2 separate experiments). Employing
immunoblot assay using polyclonal antisera specific for \(\alpha_{IIb}\beta_3\), we also examined the amount of \(\alpha_{IIb}\beta_3\) in transfected cells. Again, the mature forms of Pro747\(\alpha_{IIb}\) and \(\beta_3\) in mutant transfected cells were moderately reduced compared with wild-type transfected cells (\(\approx 30\%\) of normal levels of \(\alpha_{IIb}\), \(\approx 48\%\) of normal levels of \(\beta_3\), \(n = 2\); Fig 6B). However, the amount of pro\(\alpha_{IIb}\) was not reduced in mutant transfected cells (\(\approx 100\%\) of normal levels of pro\(\alpha_{IIb}\), \(n = 2\); Fig 6B). These data indicate that the \(2334A \rightarrow C\) substitution leads to moderate reduction in the amount of \(\alpha_{IIb}\beta_3\) within the transfected cells as well as on the cell surface.

Effect of Pro747 mutant on \(\alpha_{IIb}\beta_3\) biosynthesis. To elucidate the mechanism of impaired expression of the mutant \(\alpha_{IIb}\beta_3\), we examined the association between the mutant Pro747pro\(\alpha_{IIb}\) and \(\beta_3\). Transfected cells were labeled with [\(^{35}\)S]-methionine for 2 hours; immunoprecipitation using TP80 MoAb or AP3 MoAb was then performed. Densitometric analysis of the immunoprecipitate showed that the \(\beta_3/\text{pro}\alpha_{IIb}\) + mature \(\alpha_{IIb}\) ratios were essentially the same between wild-type and the mutant transfected cells. They were 0.66 (wild-type) and 0.67 (mutant) using TP80 and 1.93 (wild-type) and 1.91 (mutant) using AP3 (\(n = 2\); Fig 7A). These results demonstrated that the association of the mutant pro\(\alpha_{IIb}\) with \(\beta_3\) was the same as that of wild-type pro\(\alpha_{IIb}\). The densitometric analysis also showed that wild-type and the mutant transfected 293 cells synthesized \(\beta_3\) in excess compared with pro\(\alpha_{IIb}\) and that approximately 70% of labeled \(\beta_3\) was still in the free form.

The fate of the recombinant proteins was further examined in pulse-chase experiments. First, we examined the stability of the mutant Pro747pro\(\alpha_{IIb}\). The \(\alpha_{IIb}\) transfected cells were pulsed with [\(^{35}\)S]-methionine for 30 minutes, chased with unlabeled methionine for various periods of time, and then immunoprecipi-
tated using TP80 MoAb. As shown in Fig 7B, Pro747 mutation did not affect the stability of the proαIIb subunit. Next, to examine the effect of this mutation on the kinetics of αIIbβ3 complex formation, wild-type or Pro747αIIb was cotransfected with wild-type β3 cDNA, and cells were labeled with 0.4 mCi/mL of [35S]-methionine for 30 minutes and chased with media containing 50 μg/mL of nonradioactive methionine for various periods of time, as indicated. Immunoprecipitation was performed using TP80. Precipitates were separated by 6% SDS-PAGE under reducing conditions. (C) Pulse-chase analysis of wild-type or the Pro747 mutant αβ3 in transfected cells. Wild-type or Pro747αβ3 transfected cells were labeled with 0.4 mCi/mL of [35S]-methionine for 30 minutes and chased with media containing 50 μg/mL of nonradioactive methionine for various periods of time, as indicated. Immunoprecipitation was performed using TP80. Precipitates were separated by 6% SDS-PAGE under reducing conditions. This figure shows a representative of six separate experiments. (D) Densitometric analysis of the kinetics of biosynthesis of αβ3 shown in (C). The bands corresponding to proαIIb (h), αIIb (e), and β3 (s) were analyzed by scanning densitometry. The results were normalized relative to dye-front band at each lane.

Expression of site-directed Ala747αβ3 mutant on 293 cells.

To further examine the role of the Gln residue at amino acid 747 of αβ3 on αβ3 expression, we introduced a Gln747 = Ala mutation (Ala747) by PCR-based site-directed mutagenesis. The mutant Ala747 form of αβ3 cDNA was cotransfected with wild-type β3 cDNA into 293 cells. Flow cytometric analysis using AP2 MoAb showed that the level of surface expression of Ala747αβ3 complex on the transfected cells was almost the same as wild-type αβ3 complex (mean fluorescence intensity: 81.9 for wild-type and 92.3 for Ala747αβ3, n = 2; Fig 8). These data indicate that the Gln747 → Ala mutation does not impair αβ3 expression.

PAC-1 binding to wild-type and mutant αβ3. Because the mutant Pro747αβ3 receptors were expressed at substantial levels on the surface of transfected cells, we then examined the binding of the ligand-mimetic MoAb PAC-1 in the presence of the activating MoAb PT25-2. Negative control for the PAC-1 binding was obtained using FK633, a peptidomimetic antagonist specific for αβ3. As shown in Fig 8, PAC-1 could bind to both Pro747αβ3 and Ala747αβ3 in the presence of PT25-2. The PAC-1 binding to activated αβ3 was dependent on the PT25-2 binding and the PAC-1/PT25-2 binding ratios were 1.28 and 0.97 for Pro747αβ3 and Ala747αβ3, respectively, which were normalized relative to the ratio for wild-type. These
data suggest that ligand binding function of Pro747α1β3 and Ala747α1β3 is not disturbed.

DISCUSSION

In this report, we described a new point mutation (C334A → C) leading to Gln747 → Pro amino acid substitution in αιβ that is responsible for moderate αιβ deficiency (type II phenotype) in 6 of the 34 possibly mutant chromosomes in 17 unrelated Japanese GT patients. In addition, a G → A mutation at the consensus sequence of the splice acceptor site of exon 18 of the αιb gene that is likely to be responsible for exon 18 skipping in αιb cDNA was also found. The exon 18 skipping leads to an in-frame deletion of 42 amino acids in the extracellular domain of αιb. Together with the Pro747 mutation, the deletion of exon 18 contributed to the severe reduction in αιβ expression (in type I GT patient MS).

We demonstrated that the Pro747 substitution in αιb was not a naturally occurring polymorphism of αιb. ASRA showed that none of 20 control subjects possessed this substitution. Mammalian expression vectors encoding the mutant Pro747 form of αιb were constructed and cotransfected with wild-type β3 cDNA into 293 cells. Both flow cytometric and immunoprecipitation analysis using anti-αιb MoAbs demonstrated that the Pro747 substitution directly leads to moderate reduction in αιβ expression on the cell surface (20% to 30% of wild-type). The impairment of reactivity with a panel of MoAbs was not due to disruption of their epitopes, because immunoblot analysis using polyclonal anti-αιβ antisera clearly showed reduction in the total amount of αιβ in transfected cells. Recently, it has been demonstrated that the Leu183 → Pro mutation in αιb leads to both quantitative and qualitative abnormalities in αιβ. However, the Gln747 → Pro mutation did not impair the ligand-binding function. These results demonstrate that the Pro747 mutation only leads to a quantitative abnormality.

Characterization of the two mutations (Asp242 and Asp418) flanking the calcium-binding domains of αιb responsible for type I GT indicates that these relatively well-conserved flanking sequences are not essential for assembly of the αιβ heterodimer; rather, they are critical for the proper folding of αιβ to be transported to the Golgi apparatus, where αιβ cleavage occurs. However, the Pro747 mutation was far from the calcium-binding domains. By means of protein analysis of the proteolytic fragments of isolated αιβ heterodimers, Calvete et al have demonstrated that three regions of the αιb heavy chain were involved in interaction with β3: amino acids 486-553, 696-734, and 780-814. Because amino acid 747 was close to the 696-734 region, we examined whether the Pro747 mutation might impair assembly of αιβ heterodimers. Immunoprecipitation analysis using metabolically labeled transfected cells clearly indicated that assembly of the mutant Pro747proαιb and β3 normally occurred. In contrast to the Asp242 and Asp418 mutations, pulse-chase studies demonstrated that some of the mutant Pro747proαιbβ3 complexes underwent endoproteolytic cleavage of proαιb into heavy and light chains. These data suggest that some αιβ heterodimers can be transported to the Golgi apparatus. Thus, the Pro747 mutation does not completely prevent intracellular transport of the heterodimers to the Golgi apparatus.

Recently, Kolodziej et al demonstrated that the endoproteolytic cleavage of proαιb occurs on the carboxyl side of dibasic Arg858-Arg859. They also demonstrated that the failure of cleavage does not prevent expression of αιβ on the cell surface. Immunoprecipitation using AP2 MoAb of biotin-labeled surface proteins showed that, in addition to mature αιb, a small amount of proαιb was expressed on the Pro747 mutant transfected cells. Pulse-chase studies demonstrated that the stability of the mutant Pro747proαιb was increased only when assembled with β3. These data suggest that the mutant proαιb
complexed with β3 also impairs cleavage of αIIbβ3 to some extent, probably due to the induction of a conformation in αIIbβ3 that is less favorable to protease activity. Because the cleavage of proαIIbβ3 into heavy and light chains is not critical for surface expression of αIIbβ3, our data suggest that the Pro747 mutation impairs intracellular transport of αIIbβ3 to the Golgi apparatus as well. Although our transfection experiments demonstrated that the mutation led to the expression of a significant amount of proαIIbβ3 on the transfected cells, proαIIbβ3 was not detected in platelets from patients MT and MS even in the immunoblot assay. This is probably due to differences between transfected cells and platelets that circulate for 10 days with no new mRNA being made. In contrast to our patients, Jung et al30 have reported a type I GT patient whose platelets contained no normal αIIbβ3, but did show a trace amount of a premature form of αIIbβ3 in an immunoblot assay.30 One of the genetic defects of their patient was an RNA splicing mutation leading to skipping of exon 26 that contains the endoproteolytic cleavage site of αIIbβ3. However, this abnormal αIIbβ3 could not be expressed on the platelet surface. Because exon 26 skipping led to loss of 42 amino acids as well as the cleavage site, it is likely that the mutation altered the conformation of αIIbβ3 heterodimer sufficient to prevent intracellular transport in their case.

We replaced Gln747 with Ala by mutagenesis to examine the role of Gln747 on αIIbβ3 expression. In contrast to the Pro747 mutation, Ala747 substitution did not impair αIIbβ3 expression. These data suggest that the presence of Pro747, rather than the absence of Gln747, is critical for moderately impaired αIIbβ3 expression. Pro is an uncharged amino acid known to disrupt secondary protein structures, and a number of naturally occurring mutations creating a Pro residue leading to impaired expression or function of proteins have been reported.12,32-34 In GT patients, it has been well documented that the Ser752 → Pro mutation in the cytoplasmic domain of β3 leads to a variant GT phenotype.32 Pro752 mutation makes αIIbβ3 incapable of being activated by intracellular signals and decreases its capacity to mediate cell spreading.35,36 However, replacement of Ser752 with Ala had no adverse effects on αIIbβ3-mediated cell spreading.37 Kahn et al38 have demonstrated that replacement of Gly242 residue with the nonpolar amino acids Ala or Val had no effect on αIIbβ3 expression, whereas replacement with the negatively charged Glu, positively charged Lys, or nonpolar Pro caused intracellular retention of αIIbβ3.

In summary, we have described a novel point mutation, Glu747 → Pro in αIIb responsible for type II GT. The mutation moderately impaired the intracellular transport of αIIbβ3 heterodimers to the Golgi apparatus and endoproteolytic cleavage of proαIIbβ3 into heavy and light chains. Our in vitro studies suggest that the impairment of αIIbβ3 is likely due to the presence of the Pro747 residue, rather than to the absence of Gln747. Molecular genetic examination of additional GT patients should provide further insight into the structural requirements for αIIbβ3 expression, as well as differences between type I and type II GT phenotypes.

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REFERENCES

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