A Two–Amino Acid Insertion in the Cys146–Cys167 Loop of the α_{IIb} Subunit Is Associated with a Variant of Glanzmann Thrombasthenia

Critical Role of Asp163 in Ligand Binding

Shigenori Honda,* Yoshiaki Tomiyama,* Masamichi Shiraga,* Seiji Tadokoro,* Junki Takamatsu,‡ Hidehiko Saito,§ Yoshiyuki Kurata,‖ and Yuji Matsuzawa*

**The Second Department of Internal Medicine, Osaka University Medical School, Suita 565-0871, Japan; ‡Department of Transfusion Medicine, Nagoya University Hospital, Nagoya 466-8550, Japan; §The First Department of Internal Medicine, Nagoya University School of Medicine, Nagoya 466-8550, Japan; and ‖Department of Blood Transfusion, Osaka University Hospital, Suita 565-0871, Japan*

Abstract

The ligand binding site(s) of the α subunit of integrin $\alpha_{IIb}\beta_3$ (GPIIb-IIIa), a prototypic non–I domain integrin, remains elusive. In this study, we have characterized a Japanese variant of Glanzmann thrombasthenia, KO, whose platelets express normal amounts of $\alpha_{IIb}\beta_3$. KO platelets failed to bind the activation-independent ligand-mimetic mAb OP-G2 and did not bind fibrinogen or the activation-dependent ligand-mimetic mAb PAC-1 following activation of $\alpha_{IIb}\beta_3$ under any condition examined. Sequence analysis of PCR fragments derived from KO platelet mRNA revealed a 6-bp insertion leading to a 2-amino-acid insertion (Arg-Thr) between residues 160 and 161 of the α_{IIb} subunit. Introduction of the insertion into wild-type recombinant $\alpha_{IIb}\beta_3$ expressed in 293 cells led to the normal expression of $\alpha_{IIb}\beta_3$ having the defect in ligand binding function. The insertion is located within the small loop (Cys146-Cys167) in the third NH_2-terminal repeat of the α_{IIb} subunit. Alanine substitution of each of the oxygenated residues within the loop (Thr150, Ser152, Glu157, Asp159, Ser161, and Asp163) did not significantly affect expression of $\alpha_{IIb}\beta_3$, and only Asp163Ala$\alpha_{IIb}\beta_3$ abolished the ligand binding function. In addition, Asp163-Ala$\alpha_{IIb}\beta_3$ as well as KO mutant $\alpha_{IIb}\beta_3$ constitutively expressed the PMI-1 epitope. Our present data suggest that Asp163 of the α_{IIb} subunit is one of the critical residues for ligand binding. (*J. Clin. Invest.* 1998. 102:1183–1192.) Key words: bleeding disorder • platelet • integrin • in vitro mutagenesis • β-propeller

Introduction

Integrins are a family of heterodimeric adhesion receptors that mediate cellular attachment to the extracellular matrix and cell cohesion. Integrins are involved in many physiologic processes, such as development, immune response, and hemostasis (1, 2). Generally, all integrins require divalent cations for ligand recognition, and both α and β subunits are involved in ligand binding (3). The NH_2-terminal region of integrin α subunits has seven repeats of homologous sequences of about 60 amino acid residues. In several integrin α subunits (e.g., α_2, α_L, α_M), an inserted domain of about 200 amino acid residues (the I domain) between the second and the third repeats in α subunit is critically involved in ligand binding (4, 5), and its crystal structure has been determined (6). However, the ligand binding region of non–I domain integrin α subunits has not been well characterized.

Platelet integrin $\alpha_{IIb}\beta_3$ (GPIIb-IIIa) is a prototypic non–I domain integrin and plays a crucial role in platelet aggregation, a key event of the normal hemostatic process and pathologic thrombosis, by the binding of adhesive macromolecular ligands, such as fibrinogen and von Willebrand factor (7). The interaction of these ligands with $\alpha_{IIb}\beta_3$ is mediated in part by an Arg-Gly-Asp (RGD)[1] sequence and/or the His-His-Leu-Gly-Gly-Ala-Lys-Gln-Ala-Gly-Asp-Val (HHLGGAKQA-GDV) sequence at the COOH terminus of the γ-chain of fibrinogen (7). Previous studies have implicated a highly conserved I domain-like structure in the β_3 subunit in ligand binding function (8–10). However, the ligand binding sites on the α_{IIb} subunit remain elusive. A cross-linking study demonstrated that HHLGGAKQAGDV peptides cross-linked residues 294–314 of α_{IIb}, which contain the second putative calcium binding domain (11). Synthetic peptides from this region were reported to bind to fibrinogen and inhibit platelet aggregation (12), and a recombinant α_{IIb} fragment comprising residues 171–464 bound to fibrinogen (13). The NH_2-terminal residues 1–334 of α_{IIb} was shown to regulate the ligand binding specificity of $\alpha_{IIb}\beta_3$ (14). However, a recombinant α_{IIb} fragment comprising residues 1–233, which has no calcium binding domains, could form a heterodimer with a recombinant β_3 fragment (residues 111–318) and binds to RGD-containing peptide (15). Recently, Kamata et al. demonstrated that residues 184–193 in the third repeat of α_{IIb} are critical in ligand binding because they employ a mutagenesis approach (16).

Glanzmann thrombasthenia (GT) is a hereditary bleeding disorder that is due to a quantitative and/or qualitative defect in $\alpha_{IIb}\beta_3$. Characterization of molecular defects in GT due to dysfunctional $\alpha_{IIb}\beta_3$ (variant GT) has succeeded in pinpointing ligand binding site(s) (Asp119 → Tyr, Arg214 → Gln, and Arg214 → Trp) and a functionally important cytoplasmic domain (Ser752 → Pro) of the β_3 subunit (17–20). Recently, an approach to identify GT variants created in vitro by a mutagen has been reported (21). However, all 16 mutations identified were located within the β_3 subunit.

Address correspondence to Yoshiaki Tomiyama, M.D., The Second Department of Internal Medicine, Osaka University Medical School, 2-2, Yamadaoka, Suita 565-0871, Japan. Phone: 81-6-879-3732; FAX: 81-6-879-3739; E-mail: yoshi@hp-blood.med.osaka-u.ac.jp

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1. *Abbreviations used in this paper:* ED_{50}, fifty percent effective dose; GT, Glanzmann thrombasthenia; HHLGGAKQAGDV, His-His-Leu-Gly-Gly-Ala-Lys-Gln-Ala-Gly-Asp-Val; IC_{50}, fifty percent inhibitory concentration; LIBS, ligand-induced binding sites; MIDAS, metal ion-dependent adhesion site; PGE_1, prostaglandin E_1; RGD, Arg-Gly-Asp; RGDW, Arg-Gly-Asp-Trp.

In this study, we demonstrate that a 2-amino-acid insertion between residues 160 and 161 in the third repeat of the α_{IIb} subunit leads to a variant GT whose platelets express normal amounts of $\alpha_{IIb}\beta_3$. The insertion is located within a small loop (Cys146-Cys167) (22), which is located on the upper face of the Springer's β-propeller model (23). Moreover, employing alanine-scan mutagenesis, we show that residue Asp163, one of the oxygenated residues within the loop of α_{IIb}, is critical for the ligand-binding function of $\alpha_{IIb}\beta_3$.

Methods

Case history. The patient, KO, a product of nonconsanguineous parents, is a 38-yr-old Japanese female. She has been suffering from easy bruising since birth, and severe nasal bleeding has occurred at irregular intervals. Red cell transfusions have been required several times. Menorrhagia has also been noticed since her first menstruation. Hematologic examinations consistently revealed a normal platelet count and a prolonged bleeding time (> 15 min). Routine tests showed no coagulation abnormalities. Platelet aggregation was absent in response to ADP, epinephrine, collagen, and thrombin. However, ristocetin-induced platelet aggregation was observed. Clot retraction was 40% (normal value, 40–70%). There is no family history of bleeding disorders. These findings led to the diagnosis of GT.

Antibodies. OP-G2, a murine mAb specific for $\alpha_{IIb}\beta_3$ complex, has been characterized in detail (24). PAC-1 (25), an mAb specific for $\alpha_{IIb}\beta_3$ complex (a gift of Dr. Sanford Shattil, The Scripps Research Institute, La Jolla, CA), binds specifically to activated $\alpha_{IIb}\beta_3$ (25). OP-G2 and PAC-1 are well characterized, ligand-mimetic antibodies to $\alpha_{IIb}\beta_3$. These antibodies inhibit the ligand binding to $\alpha_{IIb}\beta_3$, and their binding is abolished by RGD peptides or $\alpha_{IIb}\beta_3$-specific antagonists such as FK633 (see Fig. 5) (24, 26). These ligand-mimetic antibodies have RGD-like Arg-Tyr-Asp (RYD) sequences in their antigen binding sites and are believed to be the ligand binding sites in $\alpha_{IIb}\beta_3$ (27–29). Rabbit polyclonal antisera specific for $\alpha_{IIb}\beta_3$ and mAbs AP1 (specific for GPIbα), AP2 (specific for $\alpha_{IIb}\beta_3$ complex) (30), and AP5 (specific for β_3) (31) were gifts from Dr. Thomas Kunicki (The Scripps Research Institute), PMI-1 (specific for α_{IIb}) (32, 33) was from Dr. Mark Ginsberg (The Scripps Research Institute), AP3 (specific for β_3) (34) was from Dr. Peter Newman (The Blood Center of Southeastern Wisconsin, Milwaukee, WI), and Tab (specific for α_{IIb}) (35) was from Dr. Rodger McEver (The University of Oklahoma, Oklahoma City, OK). PT25-2, an mAb specific for $\alpha_{IIb}\beta_3$ complex, activates $\alpha_{IIb}\beta_3$ and was kindly provided by Dr. Makoto Handa and Dr. Yasuo Ikeda (Keio University, Tokyo, Japan) (36).

Synthetic ligands. FK633 (N-(N-{4-(4-Amidinophenoxy)butyl}-a-L-aspartyl-L-valine), a peptidomimetic antagonist specific for $\alpha_{IIb}\beta_3$, and Arg-Gly-Asp-Trp (RGDW) peptide were generously provided by Dr. Jiro Seki (Fujisawa Pharmaceutical Co., Osaka, Japan) (37).

Preparation of platelets. Washed platelets were prepared as previously described (24). In brief, platelet-rich plasma was obtained by differential centrifugation of acid-citrate-dextrose-anticoagulated blood, incubated with 20 ng/ml prostaglandin E_1 (PGE_1; Sigma Chemical Co., St. Louis, MO) for 15 min, and then washed three times with 0.05 M isotonic citrate buffer (0.05 M sodium citrate, 0.1 M NaCl, and 0.14 M glucose, pH 6.2) containing 20 ng/ml PGE_1. The platelets were resuspended in appropriate buffer.

Treatment of washed platelets with α-chymotrypsin or dithiothreitol. Washed platelets were treated with α-chymotrypsin as previously described (38). Platelets suspended in modified Tyrode-Hepes buffer (137 mM NaCl, 2.7 mM KCl, 2 mM $CaCl_2$, 0.55 mM glucose, 12 mM $NaHCO_3$, 0.3 mM NaH_2PO_4, and 5 mM Hepes, pH 7.4) were incubated with 30 U/ml α-chymotrypsin (Worthington Biochemical, Freehold, NJ) for 10 min at 37°C. The reaction was terminated by 1 mM PMSF (Sigma Chemical Co.), and chymotrypsin-treated platelets were washed once in isotonic citrate buffer containing PGE_1. DTT treatments of washed platelets were performed according to the method of Zucker and Masiello (39). Platelets in modified Tyrode-Hepes buffer were incubated with 10 mM DTT for 15 min at 37°C, and DTT-treated platelets were washed once in isotonic citrate buffer containing PGE_1. The treated platelets were then resuspended in modified Tyrode-Hepes buffer. Modified Tyrode-Hepes buffer was used in the assay to assess ligand binding function.

Immunoblot assay and flow cytometry. Immunoblot assay using rabbit polyclonal antisera specific for $\alpha_{IIb}\beta_3$ and flow cytometry was performed as described previously (40). For flow cytometric analysis, aliquots (50 μl) of washed platelets (1×10^8/ml) in Tris-buffered saline containing 2 mM $CaCl_2$ (pH 7.4, TBS-$CaCl_2$) were incubated with each mAb examined at a final concentration of 10 μg/ml for 30 min at 22°C. Platelets were washed once in isotonic citrate buffer containing PGE_1 and then incubated with FITC-labeled goat $F(ab')_2$ anti–mouse IgG (Sigma Chemical Co.). For fibrinogen or PAC-1 binding, PT25-2 (10 μg/ml)-, chymotrypsin-, or DTT-treated platelets in modified Tyrode-Hepes buffer (1×10^8/ml) were incubated with either FITC-labeled fibrinogen (300 μg/ml) or FITC-labeled PAC-1 (10 μg/ml) for 30 min at 22°C. For the expression of ligand-induced binding sites (LIBS) induced by a synthetic ligand, FK633, or RGDW, washed platelets (1×10^8/ml) in TBS-$CaCl_2$ were incubated with serial concentrations of FK633 or RGDW for 30 min at 22°C and then incubated with AP5 or PMI-1 at a final concentration of 5 μg/ml. After 30 min, the mixtures were incubated with FITC-labeled goat $F(ab')_2$ anti–mouse IgG for an additional 30 min. The samples were analyzed on a flow cytometer (FACScan; Becton Dickinson, Mountain View, CA).

Amplification and analysis of platelet RNA and genomic DNA. Total cellular RNA was obtained, and $\alpha_{IIb}\beta_3$ mRNAs were specifically amplified by reverse transcription PCR (RT-PCR) as previously described (41). The primers for the amplification of α_{IIb} mRNA and conditions for RT-PCR have been described elsewhere (41). The following primers were constructed based on the published sequence (42) and used for the first-round PCR of β_3 mRNA: IIIa1, 5′-CGGCCCCGGCCGCTCTGGGTGACTG-3′ (sense, nucleotides [nt] 15–10); IIIa2, 5′-CAACTCTTCAGGGAGGTCACG-3′ (antisense, nt 1147–1127); IIIa3, 5′-GAGCTCATCCCAGGGACCAC-3′ (sense, nt 1015–1034); and IIIa4, 5′-CACTGACTCAATCTCGTCACGGC-3′ (antisense, nt 1974–1952). IIIa7 and IIIa8 have been described elsewhere (41). The following nested primers were used for the second round PCR: IIIa1-SalI, 5′-CTGTCGACGCGCTGGGGGCGCTG-3′ (sense, nt 8–30, 4 bp mismatched); IIIa2 SphI, 5′-GGGCATGCACGCACTTCCAGCTC-3′ (antisense, nt 1137–1114); IIIa3-SalI, 5′-GGGTCGACAGTTGGGGTTCTGTC-3′ (sense, nt 1027–1049); and IIIa4-SphI, 5′-GACGCATGCTCGTCACGGCAGTAACG-3′ (antisense, nt 1945–1970). IIIa7-SalI and IIIa8-SphI have been described elsewhere (41). Mismatched sequences are underlined.

For genotypic analysis, genomic DNA was extracted by using a SepaGene kit (Sanko Junyaku Co. Ltd., Tokyo, Japan). The following primers were used to amplify the region encompassing exon 4 through exon 5 of α_{IIb} gene (43): for the first round, IIbg3947s, 5′-GTCAGCTGGAGCGACGTCATTGTG-3′ (sense, nt 3947–3970); and IIbg4700as, 5′-CTCTGGGTTGCTGGAGTC-3′ (antisense, nt 4683–4700); for the second round, IIbg4020s, 5′-CCTGGCAGCACTGGAACG-3′ (sense, nt 4020–4037); and IIbg4566as, 5′-CAAGCACCAGCTCTCCGG-3′ (antisense, nt 4549–4566). Nucleotide sequences of PCR products were directly determined by using a Taq DyeDeoxy Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA).

Detection of a 6-bp insertion of the α_{IIb} gene. To identify a 6-bp insertion in exon 5 of the α_{IIb} gene in the patient's parents, we performed PCR using primers constructed from sequences immediately encompassing the insertion. The following primers were used: for the first round, IIbg3947s and IIbg4700as; for the second round, IIbg4260s, 5′-GCTTTGGATCTGGCCTCGC-3′ (sense, nt 4260–4278); and IIb1μ, 5′-GAGTGACCACGGAGCTGAAGC-3′ (antisense, nt 4472–4451). The amplified products were separated on a 6% polyacrylamide gel and visualized by staining with ethidium bromide.

Construction of α_{IIb} expression vectors and cell transfection. The wild-type α_{IIb} and β_3 cDNAs cloned into a mammalian expression vector pcDNA3 (Invitrogen Corp., San Diego, CA) were generously provided by Dr. Peter Newman (The Blood Center of Southeastern Wisconsin, Milwaukee, WI). To construct the expression vectors containing KO mutant α_{IIb} cDNA, PCR-based cartridge mutagenesis was performed. The 1094-bp region (nt 4–1098) of platelet α_{IIb} cDNA from patient KO was amplified by RT-PCR using primers IIb1 and IIb2 (41). Then the second round amplification was performed using 1 μl of the first round PCR products as a template with nested primers IIb187S, 5′-GAGAGTGGCCATCGTGGTGG-3′ (sense, nt 187–206); IIb1b, 5′-CTGTAGTGTTGAGATCCC-3′ (antisense, nt 848–831) and Vent Polymerase (New England Biolabs, Beverly, MA). The amplified fragments were digested with SacII and AflIII, and the resulting fragments (nt 214–742) were extracted using a GeneClean II kit (Bio 101, La Jolla, CA). The 2030-bp fragments (nt 742–2772) digested with AflIII and Sse8387I were isolated from the full-length α_{IIb} cDNA. These two fragments were introduced together into the pcDNA3 that had been digested with SacII and Sse8387I. The nucleotide sequences of the amplified fragments inserted were confirmed by sequence analysis.

For the introduction of single alanine mutation, we carried out the overlap extension PCR (44). For example, to generate the Asp163 → Ala (Asp163Ala) α_{IIb} mutant, we synthesized mismatched sense primer IIb163Ala-s, 5′-GATTTTAGCTGGGCTAAGCGTTACTGTGAAG-3′ (sense, nt 569–599, 2 bp mismatched) and antisense primer IIb163Ala-as, 5′-CTTCACAGTAACGCTTAGCCCAGCTAAAATC-3′ (antisense, nt 599–569, 2 bp mismatched). PCR was performed by using α_{IIb} cDNA as a template and primers IIb187S and IIb163Ala-as, or primers IIb163Ala-s and IIb1b. The two individually amplified PCR products were mixed and used as a template of PCR using primers IIb187S and IIb1b. The amplified PCR products were digested with SacII and AflIII, and then the fragments (nt 214–742) were introduced into pcDNA3 as described above. The nucleotide sequences of the fragments inserted were confirmed by sequence analysis.

The wild-type or mutant α_{IIb} construct was cotransfected into 293 cells with wild-type β_3 construct by the calcium phosphate method as previously described (45). The cells were cultured in DMEM with 10% heat inactivated FCS and analyzed 2 d after transfection. For LIBS expression study using transfectants, we performed two-color flow cytometry. In a preliminary study using platelets treated with 5 mM EDTA at 22°C, rabbit polyclonal antisera specific for $\alpha_{IIb}\beta_3$ at 1:10,000 dilution showed only modest inhibition (∼ 5%) of PMI-1 binding. Transfectants in TBS containing either 2 mM $CaCl_2$ or 5 mM EDTA were incubated with both PMI-1 (5 μg/ml) and rabbit polyclonal antisera specific for $\alpha_{IIb}\beta_3$ (1:10,000 dilution) for 30 min at 22°C. After washing, cells were incubated with biotinylated goat anti–rabbit IgG (1:1,000 dilution) for 30 min on ice, and then washed. The cells were incubated with both FITC-labeled goat $F(ab')_2$ anti–mouse IgG (1:320 dilution) and phycoerythrin-labeled streptavidin (1:5 dilution) for an additional 30 min on ice. PMI-1 binding (FITC) was analyzed only on a gated subset of cells highly positive for $\alpha_{IIb}\beta_3$ expression (phycoerythrin).

Results

KO $\alpha_{IIb}\beta_3$ exhibits a defect in ligand binding function. Platelet proteins obtained from patient KO or controls were analyzed

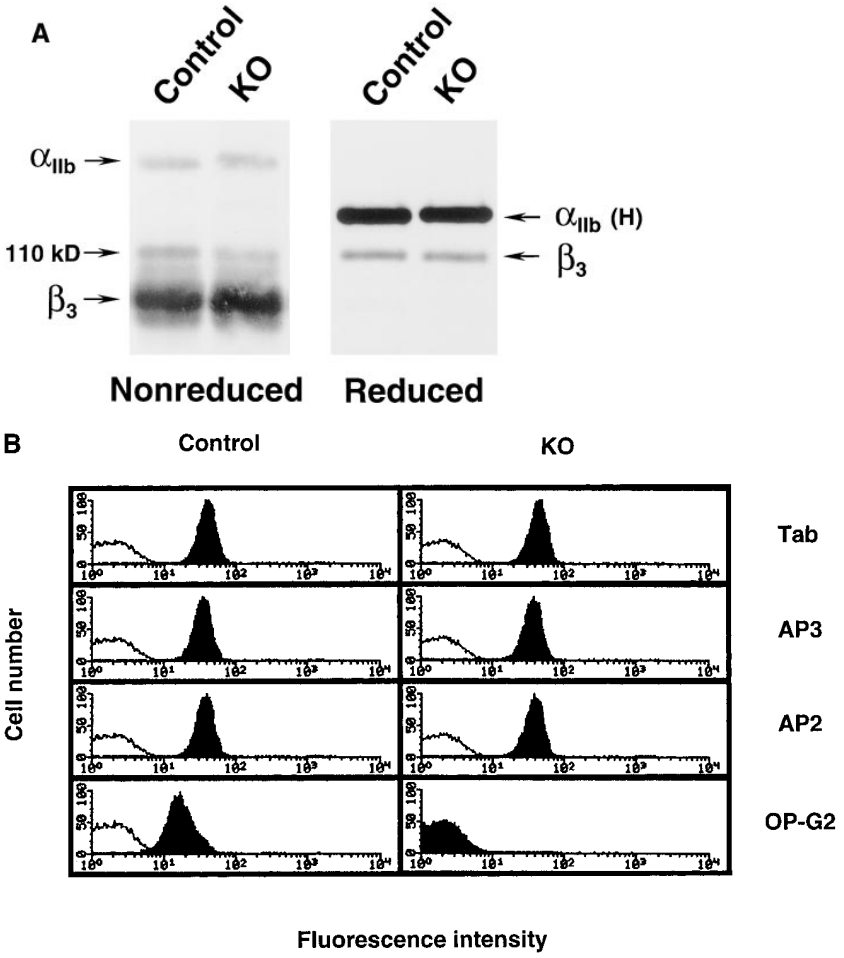

Figure 1. KO platelets possess normal amounts of $\alpha_{IIb}\beta_3$. (*A*) Immunoblot assay showing total cellular $\alpha_{IIb}\beta_3$. 5 μg of patient KO and control platelet proteins were separated on a 6% SDS polyacrylamide gel under nonreduced or reduced conditions, transferred to a polyvinylidine difluoride (PVDF) membrane, and detected with a 1:10,000 dilution of rabbit polyclonal antisera specific for $\alpha_{IIb}\beta_3$. Platelet proteins obtained from three normal subjects were pooled and used as a control. An additional 110 kD protein, which is immunologically related to β_3, was detected under nonreduced conditions (40). (*B*) Flow cytometry showing the surface expression and ligand binding function of $\alpha_{IIb}\beta_3$. Patient KO and control platelets were incubated with 10 μg/ml Tab (specific for α_{IIb}), 10 μg/ml AP3 (specific for β_3), 10 μg/ml AP2 (specific for $\alpha_{IIb}\beta_3$-complex), or 10 μg/ml OP-G2 (ligand-mimetic mAb) for 30 min at 22°C. After washing, bound antibodies were detected by FITC-labeled goat F(ab′) anti–mouse IgG. MOPC21 (mouse myeloma IgG_1) was used as a control antibody. Closed and open histograms represent the binding of specific and control antibodies, respectively. Results are representative of two separate experiments.

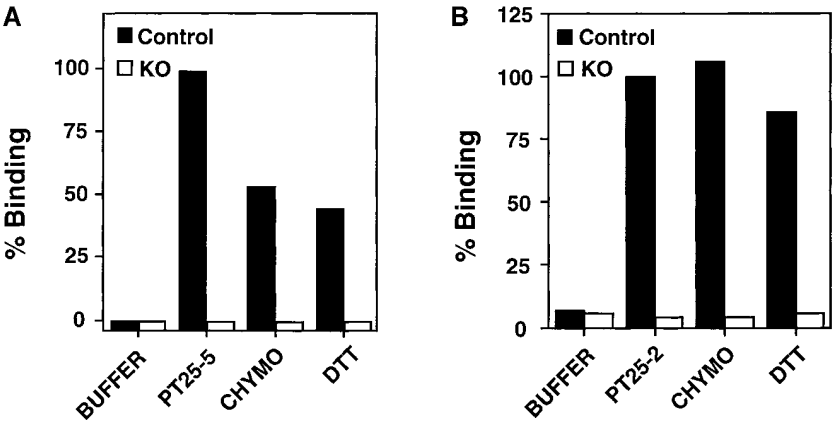

Figure 2. Analysis of ligand binding function of patient KO platelets. The binding of another ligand-mimetic monoclonal antibody, PAC-1 (*A*), and fibrinogen (*B*) were examined after activation of platelet $\alpha_{IIb}\beta_3$ with 10 μg/ml PT25-2 (activating mAb), 30 U/ml α-chymotrypsin, or 10 mM DTT. The treated platelets were incubated with FITC-labeled PAC-1 (10 μg/ml) or FITC-labeled fibrinogen (300 μg/ml) for 30 min at 22°C and then analyzed by flow cytometry. Relative amounts of ligand binding were normalized to a 100% value for PAC-1 (*A*) or fibrinogen (*B*) binding to control platelets treated with PT25-2.

in an immunoblot assay using rabbit polyclonal antisera specific for $\alpha_{IIb}\beta_3$ under both nonreduced and reduced conditions. Figure 1 *A* shows that the patient's platelets contain normal amounts of α_{IIb} and β_3 with apparently normal electrophoretic mobilities. The surface expression of $\alpha_{IIb}\beta_3$ on KO and control platelets was then examined by flow cytometry. The α_{IIb}-specific mAb Tab as well as the β_3-specific mAb AP3 reacted equivalently with KO and control platelets, confirming that KO platelets express normal levels of $\alpha_{IIb}\beta_3$ (Fig. 1 *B*). The $\alpha_{IIb}\beta_3$-specific mAb AP2 also reacted equivalently with KO and control platelets, and the addition of 5 mM EDTA at 22°C did not affect the AP2 binding, suggesting that premature dissociation of the subunits was not induced on KO platelets (data not shown). We next examined the binding of the ligand-mimetic, $\alpha_{IIb}\beta_3$-specific mAb, OP-G2, to KO platelets. OP-G2 recognizes at or near the ligand binding sites on $\alpha_{IIb}\beta_3$ in an activation-independent manner (24). Interestingly, OP-G2 failed to bind to KO platelets, suggesting that KO $\alpha_{IIb}\beta_3$ may have a defect in ligand binding domains (Fig. 1 *B*).

Activation of $\alpha_{IIb}\beta_3$ is a prerequisite for the binding of fibrinogen and another ligand mimetic mAb, PAC-1 (25). To examine further the ligand binding defect in KO $\alpha_{IIb}\beta_3$, platelets were treated with three different reagents, PT25-2, chymotrypsin, and DTT, each of which activates $\alpha_{IIb}\beta_3$. PT25-2, an $\alpha_{IIb}\beta_3$-specific mAb, as well as DTT, fully activates $\alpha_{IIb}\beta_3$ by a direct alteration of $\alpha_{IIb}\beta_3$ conformation (16, 36, 39). α-chymotrypsin activates $\alpha_{IIb}\beta_3$ without platelet activation, probably via a Na^+/Ca^{2+} exchanger (38). Fibrinogen and PAC-1 bound well to control platelets treated with these reagents, while neither fibrinogen nor PAC-1 bound to KO platelets (Fig. 2). Since PT25-2 and DTT activate $\alpha_{IIb}\beta_3$ without inside-out signaling, these findings confirm that KO has a variant form of GT in which a defect exists in ligand binding sites.

Aberrant expression of LIBS on KO $\alpha_{IIb}\beta_3$. AP5 and PMI-1 preferentially recognize LIBS on $\alpha_{IIb}\beta_3$, which are exposed following occupancy of the receptor by ligands or EDTA treatment (46). AP5 and PMI-1 epitopes are located at residues 1–6 on the β_3 subunit and residues 844–859 on the α_{IIb} heavy chain, respectively (31, 33). As shown in Fig. 3, KO $\alpha_{IIb}\beta_3$ aberrantly expresses LIBS, especially PMI-1 epitope, even in the presence of 2 mM $CaCl_2$. Interestingly, addition of 5 mM EDTA to KO platelets failed to induce additional expression of PMI-1 epitope. We then examined the dose-dependent induction of AP5 epitope by RGDW peptide or a peptidomimetic FK633. FK633 is a high affinity, $\alpha_{IIb}\beta_3$-selective, RGD mimetic, which, like RGDW, binds to nonactivated $\alpha_{IIb}\beta_3$ and inhibits platelet

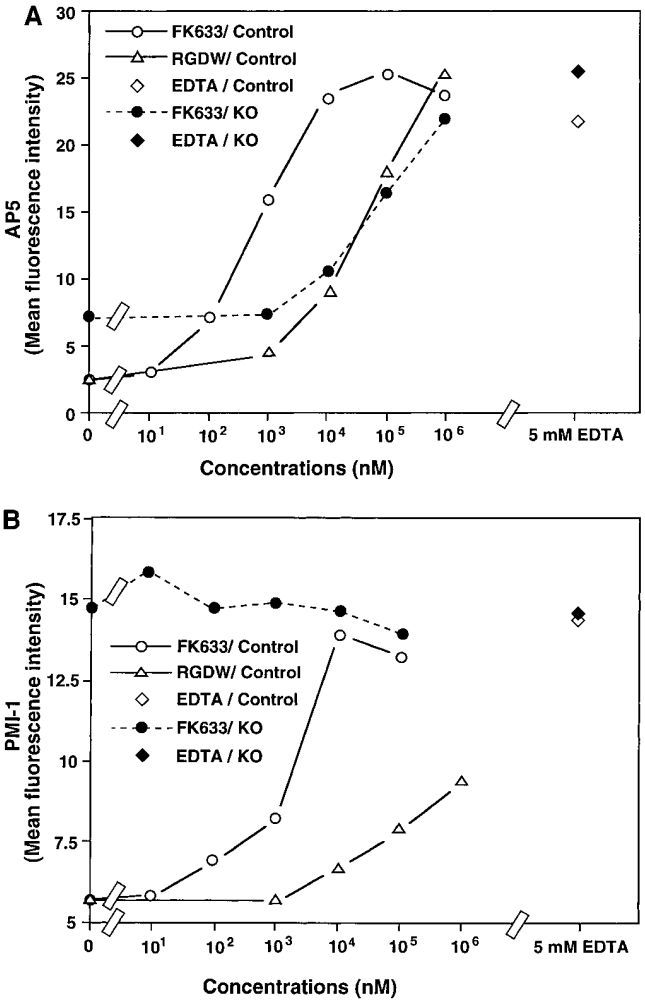

Figure 3. Analysis of LIBS expression on KO $\alpha_{IIb}\beta_3$. Two different mAbs, (*A*) AP5 (specific for β_3 LIBS) and (*B*) PMI-1 (specific for α_{IIb} LIBS), were employed to assess the LIBS expression. Platelets were incubated in the presence of serial concentrations of FK633 ($\alpha_{IIb}\beta_3$ selective antagonist), RGDW, or 5 mM EDTA for 30 min at 22°C, and then incubated with AP5 or PMI-1 (5 μg/ml) for 30 min at 22°C. The mixtures were further incubated with FITC-labeled goat F(ab′) anti–mouse IgG. Bound antibodies were analyzed by flow cytometry. Results are representative of two separate experiments.

aggregation with a 50% inhibitory concentration (IC_{50}) of 110 nM (~ 160-fold more potent than RGDW) (37). As shown in Fig. 3 *A*, FK633 is also approximately 100-fold more potent than RGDW in the induction of AP5 epitope on control platelets. However, the effect of FK633 on AP5 epitope on KO platelets was comparable to that of RGDW on control platelets. Indeed, 50% effective doses (ED_{50}) of FK633 for the induction of AP5 epitope on KO and control platelets were approximately 50 and 0.5 μM, respectively (means of two separate experiments) (Fig. 3). These data suggest that the affinity of KO $\alpha_{IIb}\beta_3$ for FK633 is ~ 100-fold less than normal $\alpha_{IIb}\beta_3$.

Sequence analysis of $\alpha_{IIb}\beta_3$ RNA and genomic DNA. To clarify the molecular genetic defect responsible for the KO variant GT, platelet mRNA was isolated from patient KO and normal controls. α_{IIb} and β_3 cDNA fragments, which encompass the whole coding regions of α_{IIb} and β_3, were generated by RT-PCR using either α_{IIb}- or β_3-specific nucleotide primers. Direct sequence of these cDNAs revealed a 6-bp insertion (5′-GGACAA-3′) in KO α_{IIb} cDNA, which would lead to a 2-amino-acid insertion (Arg and Thr) between Phe160 and Ser161 of α_{IIb} (Fig. 4, *A* and *D*). Patient KO appeared homozygous for the 6-bp insertion. No other nucleotide substitution was detected in either α_{IIb} or β_3 cDNA from patient KO. The region encompassing exon 4 through exon 5 in the KO α_{IIb} gene was then amplified by PCR from genomic DNA. Direct sequence of the PCR fragments revealed that the 6-bp insertion occurred at the 5′ end of exon 5 following an AG acceptor site (Fig. 4 *B*). Analysis of the α_{IIb} gene in the patient's parents, who were nonconsaguineous, further confirmed that patient KO is homozygous for the 6-bp insertion (Fig. 4 *C*). The PCR products derived from a normal control provided a 175-bp

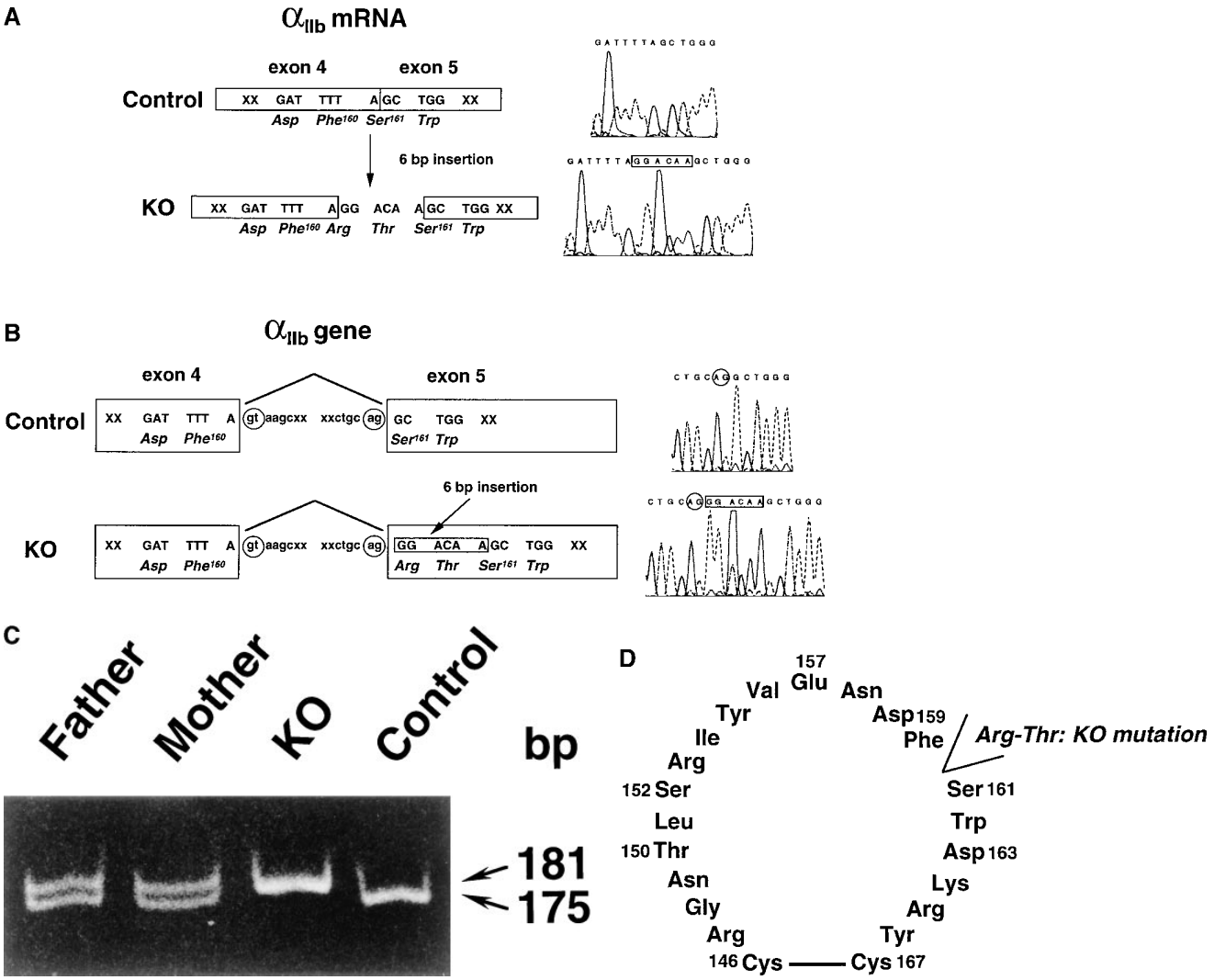

Figure 4. Identification of the genetic defect responsible for patient KO. (*A*) Sequence analysis of α_{IIb} mRNA. Platelet α_{IIb} mRNA was amplified by RT-PCR. Direct sequence of the PCR products revealed a 6-bp insertion in α_{IIb} cDNA, and this mutation would lead to an insertion of Arg and Thr between Phe160 and Ser161 of α_{IIb}. (*B*) Sequence analysis of α_{IIb} gene. Nucleotides encompassing exon 4 through exon 5 in KO α_{IIb} gene were amplified by PCR. Direct sequence of the PCR products revealed the 6-bp insertion at the 5′ end of exon 5. (*C*) Detection of the 6-bp insertion in the KO parents. The fragments amplified by PCR from the α_{IIb} gene were separated on a 6% polyacrylamide gel and visualized by staining with ethidium bromide. A normal control and KO exhibit a single 175- and a single 181-bp band, respectively. The father and mother exhibit both 181- and 175-bp bands. (*D*) Amino acid sequence of the Cys146-Cys167 small loop containing the KO mutation. The numbering residues except Cys represent oxygenated residues, which were substituted individually by Ala.

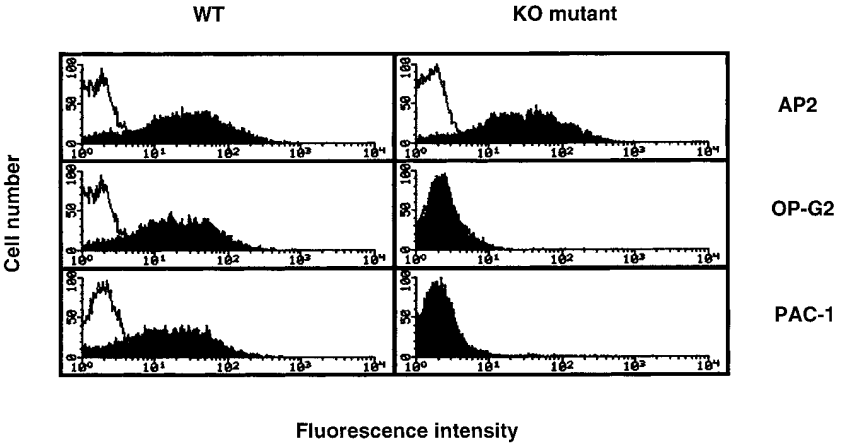

Figure 5. Surface expression and ligand binding function of recombinant $\alpha_{IIb}\beta_3$ containing the 2-amino-acid insertion in α_{IIb}. KO α_{IIb} (Phe160-Arg-Thr-Ser161) or wild-type α_{IIb} was cotransfected in 293 cells with wild-type β_3. After 2 d of culture, transfectants were analyzed by flow cytometry using AP2 (complex specific mAb), OP-G2 (activation-independent ligand-mimetic mAb), or PAC-1 (activation-dependent ligand-mimetic mAb). For PAC-1 binding, transfectants were preincubated with 10 μg/ml PT25-2 (activating mAb) for 30 min and then incubated with 10 μg/ml FITC-labeled PAC-1 for 30 min. Closed and open histograms represent the PAC-1 binding in the absence and presence of 10 μM FK633 ($\alpha_{IIb}\beta_3$-selective antagonist), respectively. For AP2 or OP-G2 binding, transfectants were incubated with AP2 or OP-G2 (5 μg/ml) for 30 min on ice, and then washed once. Bound antibodies were detected by FITC-labeled goat F(ab′) anti–mouse IgG. Open histograms represent the binding of control antibodies. Results are representative of three separate experiments.

band. PCR products derived from KO provided only an abnormal 181-bp band because of the 6-bp insertion, while PCR products derived from her father and mother provided both 181- and 175-bp bands.

Effect of recombinant $\alpha_{IIb}\beta_3$ containing the 2-amino-acid insertion in α_{IIb} on the ligand binding function. To confirm that the 2-amino-acid insertion (Arg and Thr between Phe160 and Ser161 of α_{IIb}) is responsible for the KO variant phenotype, we introduced the mutation into a wild-type α_{IIb} cDNA and then expressed mutant α_{IIb} with wild-type β_3 in 293 cells. As shown in Fig. 5, the KO mutant and wild-type $\alpha_{IIb}\beta_3$ expressed on 293 cells equally bound the $\alpha_{IIb}\beta_3$-specific mAb AP2, the α_{IIb}-specific mAb Tab, and the β_3-specific mAb AP3 (not shown), indicating that the 2-amino-acid insertion had no effect on the surface expression of $\alpha_{IIb}\beta_3$ or assembly of heterodimer. However, the KO mutant failed to bind the activation-independent ligand-mimetic mAb OP-G2. We also examined the PAC-1 binding in the presence of the activating mAb PT25-2. The PAC-1 binding to activated wild-type $\alpha_{IIb}\beta_3$ was dependent on the PT25-2 binding, and PAC-1 binding was specifically blocked with FK633. Although the KO mutant and wild-type $\alpha_{IIb}\beta_3$ equally bound PT25-2 (data not shown), the KO mutant $\alpha_{IIb}\beta_3$ failed to bind PAC-1 (Fig. 5). These data indicate that the 2-amino-acid insertion is responsible for the ligand binding defect in the KO variant.

Asp163 of α_{IIb} is critical for ligand-binding function. The disulfide bond between Cys146-Cys167 has been chemically defined (22). The 2-amino-acid insertion was located within the Cys146-Cys167 small loop consisting of 22 residues within a predicted loop between the second and third amino-terminal repeat of α_{IIb} (Fig. 4 *D*) (23). To investigate further the potential role of this loop for the ligand binding function, we individually substituted oxygenated residues within the loop (Thr150, Ser152, Glu157, Asp159, Ser161, and Asp163) by alanine and then expressed each mutant α_{IIb} with wild-type β_3 in 293 cells. Fig. 6 shows that each mutation did not markedly affect the surface expression of $\alpha_{IIb}\beta_3$. We also examined the reactivity of both ligand-mimetic mAbs, OP-G2 and PAC-1, with PT25-2 in a more quantitative fashion. Since, based on Tab binding, there was a twofold range of $\alpha_{IIb}\beta_3$ expression, OP-G2 and PAC-1 binding were normalized using Tab and PT25-2 binding, respectively, as the internal control for $\alpha_{IIb}\beta_3$ expression (Table I). It is notable that α_{IIb} (Asp163Ala) mutation abolished the binding of both OP-G2 and PAC-1, although it bound the activating mAb PT25-2 (Fig. 6 and Table I). Other mutants did not dramatically affect the binding of OP-G2 and PAC-1. These data indicate that the residue Asp163 within the loop of α_{IIb} is crucial for ligand binding function.

Table I. Reactivity of mAbs to $\alpha_{IIb}\beta_3$ Mutants

mAb	293 cell	WT	Thr150Ala	Ser152Ala	Glu157Ala	Asp159Ala	Ser161Ala	Asp163Ala
Tab (α_{IIb})	1.3	65.0 (100)	32 (100)	34.2 (100)	53.8 (100)	72.5 (100)	70.0 (100)	35.8 (100)
AP2 ($\alpha_{IIb}\beta_3$/blocking)	0.9	53.6 (82.5)	23.5 (73.4)	15.6 (45.6)	38.4 (71.4)	60.1 (83.0)	66.5 (95.0)	24.4 (68.2)
PT25-2 ($\alpha_{IIb}\beta_3$/activating)	0	49.0 (75.4)	22.9 (71.6)	14.2 (41.5)	42.2 (78.4)	51.1 (70.5)	48.4 (69.1)	30.3 (84.6)
OP-G2 ($\alpha_{IIb}\beta_3$/ligand-mimetic)	−0.4	24.1 (37.1)	10.5 (32.8)	7.84 (22.9)	22.5 (41.8)	17.9 (24.7)	20.8 (29.7)	0.6 (1.7)
PAC-1 ($\alpha_{IIb}\beta_3$/ligand-mimetic)	−0.7	20.0	11.2	9.8	11.0	10.4	18.1	0.4
PAC-1/PT25-2 ratio (%)		40.8	48.9	69.0	26.0	20.4	37.4	1.3

The reactivity of mAbs to $\alpha_{IIb}\beta_3$ mutants was examined by flow cytometry. Data are shown as mean fluorescence intensity with the subtracted background binding given by a control antibody. Values in parentheses represent relative binding to each antibody versus Tab (specific for α_{IIb}). An evaluation of PAC-1 binding is made by percent binding to PAC-1 versus PT25-2 (activating mAb), because the PAC-1 binding is dependent on the binding of PT25-2.

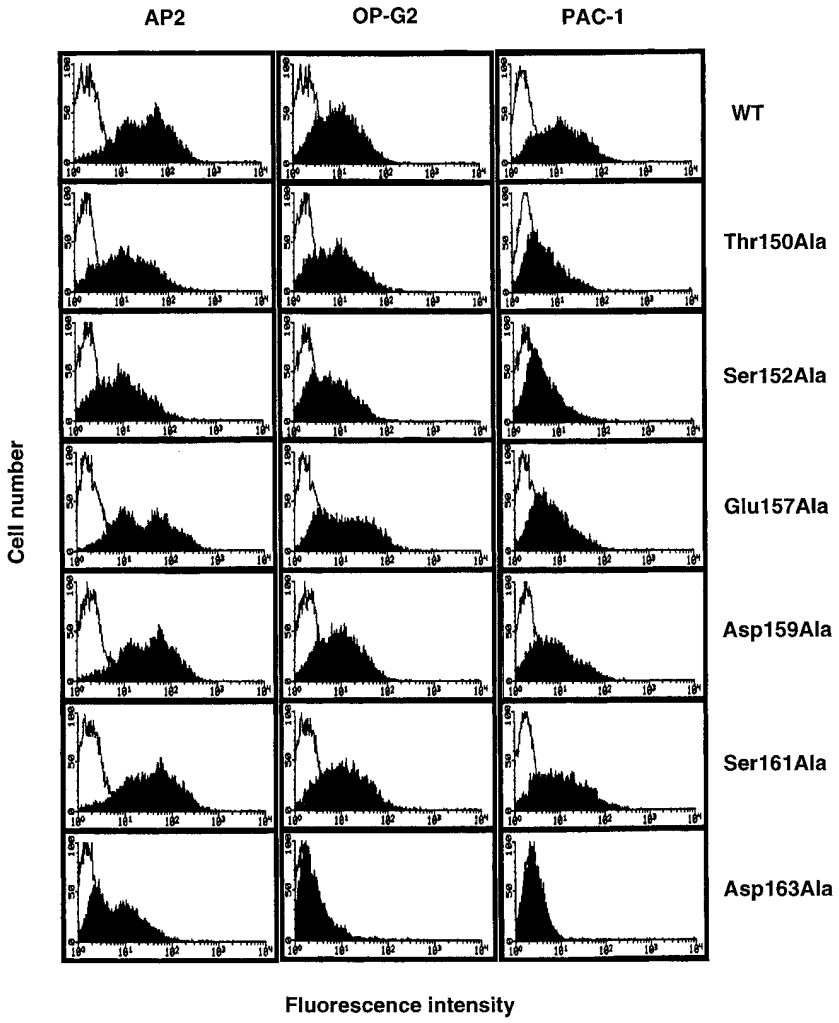

Figure 6. Effects of mutations in Cys146-Cys167 small loop on binding of ligand-mimetic monoclonal antibodies. Each mutant α_{IIb} or wild-type α_{IIb} was cotransfected in 293 cells with wild-type β_3. After 2 d of culture, the binding of AP2 (complex-specific mAb), OP-G2 (activation-independent ligand-mimetic mAb), or PAC-1 (activation dependent ligand-mimetic mAb) to transfectants were analyzed by flow cytometry. For PAC-1 binding, transfectants were preincubated with 10 μg/ml PT25-2 (activating mAb) for 30 min, and then 10 μg/ml FITC-labeled PAC-1 was added. Closed and open histograms represent the PAC-1 binding in the absence and presence of 10 μM FK633 ($\alpha_{IIb}\beta_3$-selective antagonist), respectively. For AP2 or OP-G2 binding, transfectants were incubated with AP2 or OP-G2 (5 μg/ml) for 30 min on ice. After washing, bound antibodies were detected by FITC-labeled goat F(ab′) anti–mouse IgG. Results are representative of three separate experiments.

Aberrant expression of PMI-1 LIBS on Asp163Ala$\alpha_{IIb}\beta_3$. $\alpha_{IIb}\beta_3$ on KO platelets fully expressed the PMI-1 epitope even in the presence of 2 mM $CaCl_2$ as shown in Fig. 3. This result may associate with ligand binding function. To address this question, the binding of PMI-1 to mutant $\alpha_{IIb}\beta_3$ was analyzed by flow cytometry (Fig. 7). The addition of 5 mM EDTA induced the PMI-1 epitope on wild-type $\alpha_{IIb}\beta_3$ and Thr150-Ala$\alpha_{IIb}\beta_3$, which has no defect in ligand binding. In contrast, EDTA treatment failed to induce the additional expression of the PMI-1 epitope on Asp163Ala$\alpha_{IIb}\beta_3$ as well as KO $\alpha_{IIb}\beta_3$. These data suggest that Asp163Ala$\alpha_{IIb}\beta_3$ and KO $\alpha_{IIb}\beta_3$ induce a change in α_{IIb} folding that disrupts ligand binding function.

Discussion

The interaction between fibrinogen and the α_{IIb} subunit, a non–I domain integrin, remains obscure. In this study, we have examined a variant GT patient, KO, whose platelets express normal amounts of $\alpha_{IIb}\beta_3$. KO $\alpha_{IIb}\beta_3$, which showed a defect in ligand binding function, bore a 6-bp insertion leading to a 2-amino-acid insertion (Arg-Thr) between residues 160 and 161 of the α_{IIb} subunit. Introduction of the insertion into wild-type recombinant $\alpha_{IIb}\beta_3$ expressed in 293 cells led to the normal expression of $\alpha_{IIb}\beta_3$ having the defect in ligand binding function. The insertion is located within the small loop (Cys146-Cys167) in the third NH_2-terminal repeat of the α_{IIb} subunit (22, 23). To pinpoint further the ligand binding site(s), alanine-scan mutagenesis of oxygenated residues within the Cys146-Cys167 loop was performed. Alanine substitution of each of the oxygenated residues (Thr150, Ser152, Glu157, Asp159, Ser161, and Asp163) did not significantly affect expression of $\alpha_{IIb}\beta_3$ and demonstrated that Asp163 of the α_{IIb} subunit is critical for ligand binding function of $\alpha_{IIb}\beta_3$.

Three mutations (Asp119 → Tyr, Arg214 → Gln, and Arg214 → Trp) that disrupt ligand binding on the β_3 subunit have been identified in variant GT patients (17–19). Asp119 appears to be directly involved in the ligand–receptor interaction. However, DTT treatment of platelets that bear the Arg214 → Trp mutation resulted in the formation of the fibrinogen binding site, suggesting that Arg214 may be involved in the formation and maintenance of the correct tertiary structure of the ligand binding site rather than in the direct interaction between ligand and $\alpha_{IIb}\beta_3$ (47). In contrast, none of the DTT, activating mAb PT25-2, or chymotrypsin treatment of KO platelets induced the fibrinogen binding site, suggesting that the 2-amino-acid insertion within the Cys146-Cys167 loop may be directly involved in the interaction. Affinity of KO $\alpha_{IIb}\beta_3$ for the activation-independent ligands such as FK633

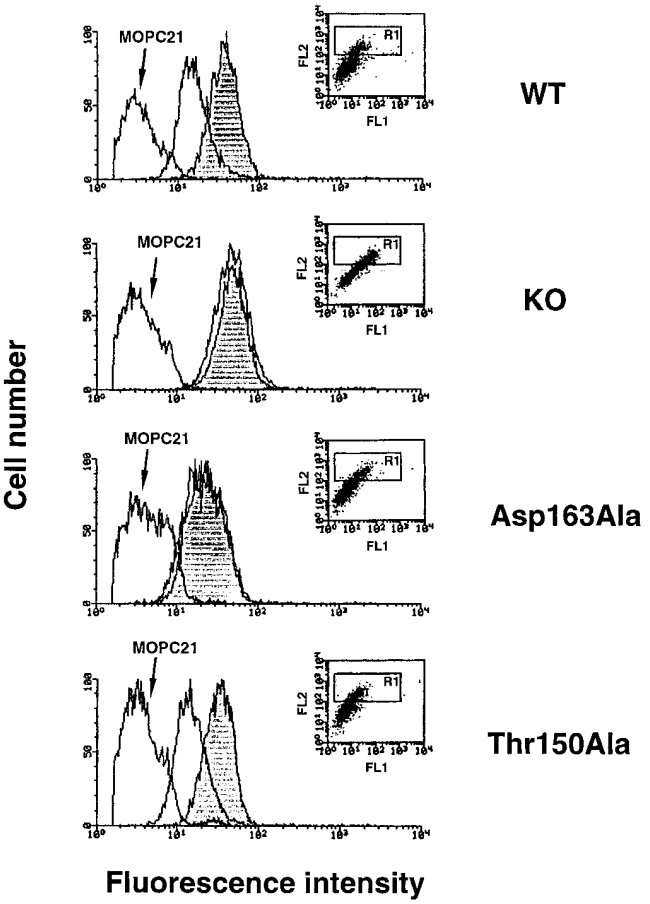

Figure 7. Effects of α_{IIb} (Asp163Ala) mutation on the PMI-1 epitope expression. 2 d after transfection, cells were analyzed by flow cytometry. In this assay, cells were double stained with rabbit polyclonal antisera specific for $\alpha_{IIb}\beta_3$ (fluorescence 2: FL2) and with PMI-1 (fluorescence 1: FL1) to analyze the cells expressing high levels of recombinant $\alpha_{IIb}\beta_3$. Transfectants were incubated with both rabbit polyclonal antisera specific for $\alpha_{IIb}\beta_3$ (1:10,000 dilution) and PMI-1 (5 μg/ml) in the presence of either 2 mM $CaCl_2$ or 5 mM EDTA for 30 min at 22°C, and washed once. The cells were incubated with biotinylated anti–rabbit IgG (1:1,000) for an additional 30 min on ice. After washing, bound antibodies were detected by phycoerythrin-labeled streptavidin (1:5) and FITC-labeled goat $F(ab')_2$ anti–mouse IgG (1:320). Open and shaded histograms represent PMI-1 binding in the presence of 2 mM $CaCl_2$ and 5 mM EDTA at 22°C, respectively. MOPC21 is a nonimmune control IgG_1. Dot plots in the histograms show the gated regions. Results are representative of three separate experiments.

seemed roughly 100-fold less than that of normal $\alpha_{IIb}\beta_3$. Although her parents showed no apparent consanguinity, genetic analysis demonstrated that patient KO is homozygous for the 6-bp insertion at the 5′ end of exon 5 of the α_{IIb} gene.

Employing alanine-scanning mutagenesis, we newly identified Asp163 within the Cys146-Cys167 loop as one of the critical residues of the α_{IIb} subunit for ligand binding. Irie et al. determined that Tyr187, Trp188, and Gly190 within the predicted β-turn structure (residues 181–190) in the third NH_2-terminal repeats of α_4, another non–I domain integrin, are critical residues for ligand binding to $\alpha_4\beta_1$ (48). The corresponding regions of the α_5 subunit (residues 181–190) and the α_{IIb} subunit (residues 184–193) have also been shown to be critical for ligand binding to $\alpha_5\beta_1$ and $\alpha_{IIb}\beta_3$, respectively (16, 48). However, Asp163 is separated from residues 184–193 in the primary structure of the α_{IIb} subunit. Recently, Springer proposed that the seven NH_2-terminal sequence repeats of integrin α subunits are folded into a β-propeller domain. The proposed domains contain seven four-stranded β-sheets (W1–W7) arranged in a torus around a pseudosymmetry axis (23). Integrin ligands are predicted to bind to the upper face of the β-propeller, while the putative Ca^{2+} binding domains are on the lower face of the β-propeller. Indeed, recent data reported by Irie et al. using swapping mutagenesis between the α_4 and α_5 subunits were consistent with this model (49). The critical regions of the α_{IIb} subunit for ligand binding identified by Kamata et al. and in this study are located within the 2-3 loop and the 4-1 loop of W3, respectively (16). The β-propeller model predicts that the 4-1 loop and the 2-3 loop of W3 are adjacent in the structure. In this context, our present data do not conflict with those reported by Kamata et al. (16). In addition, Pro145 → Ala and Leu183 → Pro substitution of the α_{IIb} subunit, which are located in the 4-1 and the 2-3 loop of W3, respectively, have been recently shown to be responsible for GT patients whose platelets show both qualitative and quantitative defects (50, 51). By contrast, in the α_4 subunit, the 4-1 loop of W3 may not be critical for ligand binding (48, 49). It is possible that the difference in the role of the 4-1 loop of W3 may reflect the difference in the ligand specificity between the α_{IIb} and α_4 subunits. The cysteines in this predicted loop are conserved among α_4, α_5, α_v, and α_{IIb} subunits. Since the α_{IIb} subunit has the longest Cys-Cys loop, it is also possible that the structural difference may account for the difference in the role of this loop in ligand binding.

It has been well documented that a divalent cation is required for ligand binding to integrins. In the I domain of the α subunit, a unique divalent cation coordination sphere has been demonstrated and designated the metal ion-dependent adhesion site (MIDAS) (6). Alanine mutagenesis of divalent cation–coordinating residues in the MIDAS abolished divalent cation binding and ligand binding (5). In the I domain-like structure of the β_3 subunit, the Asp119 → Tyr substitution abrogates ligand binding and produces an apparent divalent cation binding defect (17). In addition, D'Souza et al. demonstrated that residues 118–131 of the β_3 subunit possess both ligand and cation binding properties (52). These data suggest that a divalent cation is directly involved in ligand binding. In this study, we demonstrated that the PMI-1 epitope was fully expressed on Asp163Ala$\alpha_{IIb}\beta_3$ as well as KO mutant $\alpha_{IIb}\beta_3$ even in the presence of $CaCl_2$, since EDTA treatment failed to induce additional expression of the PMI-1 epitope. Our data suggest that KO $\alpha_{IIb}\beta_3$ and Asp163Ala$\alpha_{IIb}\beta_3$ produce an apparent divalent cation binding defect that is similar to that of the Asp119 → Tyr mutation of the β_3 (17). It is likely that the Arg-Thr insertion between residues 160–161 in the KO variant affects the orientation of the critical residue, Asp163. It remains obscure whether Asp163 of the α_{IIb} subunit may be directly involved in divalent cation binding or not.

Inhibitors for $\alpha_{IIb}\beta_3$ are likely to be the first anti-integrins (53). In this study, we show that Asp163 in the Cys146-Cys167 loop of the α_{IIb} subunit is one of the critical residues for ligand binding to $\alpha_{IIb}\beta_3$. Our present data provide a better understanding of the interaction between ligand and the non–I domain α_{IIb} subunit and a new design of antagonists for this integrin.

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