A naturally occurring Tyr143Hisα_{IIb} mutation abolishes $\alpha_{IIb}\beta_3$ function for soluble ligands but retains its ability for mediating cell adhesion and clot retraction: comparison with other mutations causing ligand-binding defects

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The molecular basis for the interaction between a prototypic non–I-domain integrin, $\alpha_{IIb}\beta_3$, and its ligands remains to be determined. In this study, we have characterized a novel missense mutation (Tyr143His) in α_{IIb} associated with a variant of Glanzmann thrombasthenia. Osaka-12 platelets expressed a substantial amount of $\alpha_{IIb}\beta_3$ (36%-41% of control) but failed to bind soluble ligands, including a high-affinity $\alpha_{IIb}\beta_3$-specific peptidomimetic antagonist. Sequence analysis revealed that Osaka-12 is a compound heterozygote for a single ^{521}T>C substitution leading to a Tyr143His substitution in α_{IIb} and for the null expression of α_{IIb} mRNA from the maternal allele. Given that Tyr143 is located in the W3 4-1 loop of the β-propeller domain of α_{IIb}, we examined the effects of Tyr143His or Tyr143Ala substitution on the expression and function of $\alpha_{IIb}\beta_3$ and compared them with KO (Arg-Thr insertion between 160 and 161 residues of α_{IIb}) and with the Asp163Ala mutation located in the same loop by using 293 cells. Each of them abolished the binding function of $\alpha_{IIb}\beta_3$ for soluble ligands without disturbing $\alpha_{IIb}\beta_3$ expression. Because immobilized fibrinogen and fibrin are higher affinity/avidity ligands for $\alpha_{IIb}\beta_3$, we performed cell adhesion and clot retraction assays. In sharp contrast to KO mutation and Asp163Ala$\alpha_{IIb}\beta_3$, Tyr143His$\alpha_{IIb}\beta_3$-expressing cells still had some ability for cell adhesion and clot retraction. Thus, the functional defect induced by Tyr143Hisα_{IIb} is likely caused by its allosteric effect rather than by a defect in the ligand-binding site itself. These detailed structure–function analyses provide better understanding of the ligand-binding sites in integrins. (Blood. 2003; 101:3485-3491)

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Introduction

Integrins are a family of noncovalently associated αβ heterodimeric adhesion receptors that mediate cellular attachment to the extracellular matrix and cell–cell cohesion.[1,2] Integrins are involved in a variety of physiological processes, including development, immune response, wound healing, and hemostasis.[1] They are also involved in pathologic processes, such as tumor metastasis, thrombosis, and atherosclerosis.[1]

Although its expression is restricted to the megakaryocyte/platelet lineage, $\alpha_{IIb}\beta_3$ (GPIIb-IIIa) is a prototypic integrin that functions as a physiologic receptor for fibrinogen and von Willebrand factor and that plays a crucial role in platelet aggregation, normal hemostasis, and pathologic thrombosis.[3] Indeed, Glanzmann thrombasthenia (GT) is an autosomal recessive bleeding disorder caused by a defect in the expression or the function of integrin $\alpha_{IIb}\beta_3$.[4] Recent clinical studies have shown the beneficial effects of $\alpha_{IIb}\beta_3$ antagonists in patients undergoing coronary angioplasty and in those with unstable angina.[5,6] Integrin α subunits are grouped into 2 classes based on the presence or absence of an inserted domain of approximately 200 amino acid residues (I- or A-domain); α_{IIb} and α_v subunits do not have the I-domain.[7-9] Recently, the crystal structures of the extracellular segment of the other β_3 integrin, $\alpha_v\beta_3$—in the presence or absence of its small ligand—have been described.[10,11] As predicted, the putative ligand-binding head is primarily formed by a 7-bladed β-propeller domain from α_v and a β I-domain from β_3.[10-12] The β-propeller domain contains 7 4-stranded β-sheets (W1-W7) arranged in a torus around a pseudosymmetry axis. However, the ligand-binding sites in the β-propeller domain of the α_{IIb} subunit remain to be determined.

The characterization of molecular defects in GT from dysfunctional $\alpha_{IIb}\beta_3$ (variant GT) has succeeded in pinpointing ligand-binding sites and functionally important domains.[13-15] We have demonstrated that 2-amino acid insertion (Arg-Thr) between amino acid residues 160 and 161 in α_{IIb} is responsible for the ligand-binding defect in a Japanese variant GT known as KO.[16] The insertion is located within the small loop (Cys146-Cys167) between W2 and W3 (W3 4-1 loop) located on the upper face of the β-propeller. Alanine substitutions further indicate that Asp163 within the Cys146-Cys167 loop is one of the critical residues for ligand binding.[16] In this context, 2 other naturally occurring missense mutations, Pro145Ala and Leu183Pro, which impair $\alpha_{IIb}\beta_3$ expression and its ligand-binding function, have been identified in the W3 4-1 loop and the W3 2-3 loop of the β-propeller, respectively.[17,18]

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In this study we demonstrate that a novel naturally occurring missense mutation (Tyr143His) within the W3 4-1 loop in α_{IIb} is responsible for a binding defect in $\alpha_{IIb}\beta_3$ for soluble ligands. Because Tyr143His, KO, and Asp163Ala mutations are located within the same loop in α_{IIb}, we further compared functions of these mutant $\alpha_{IIb}\beta_3$ by using cell adhesion and clot retraction assays. Compared with the KO mutation and Asp163Ala$\alpha_{IIb}\beta_3$, Tyr143His$\alpha_{IIb}\beta_3$-expressing cells still had some ability for cell adhesion and clot retraction. Our results indicate that the KO mutant and Asp163Ala$\alpha_{IIb}\beta_3$ impair ligand-binding function in $\alpha_{IIb}\beta_3$ more severely than Tyr143His$\alpha_{IIb}\beta_3$.

Patients, materials, and methods

Patient history

Patient Osaka-12, a product of nonconsanguineous parents, was a 21-year-old Japanese woman who had a history of moderate mucocutaneous bleeding in her childhood. Hematologic examinations revealed a prolonged bleeding time (more than 15 minutes) and an absence of platelet aggregation in response to adenosine diphosphate (ADP), epinephrine, and collagen but a normal response to ristocetin. Clot retraction using the MacFarlane method was 46% (normal range, 40%-70%).[19] Informed consent for analyzing their molecular genetic abnormalities was obtained from Osaka-12 and her parents.

Antibodies and synthetic ligands

AP1 (GPIb-specific monoclonal antibody [mAb]), AP2 ($\alpha_{IIb}\beta_3$-specific mAb), and AP5 (β_3-specific mAb) were generously provided by Dr Thomas J. Kunicki (Scripps Research Institute, La Jolla, CA).[20,21] AP3 (β_3-specific mAb) was a generous gift from Dr Peter J. Newman (Blood Center of Southeastern Wisconsin, Milwaukee).[22] OP-G2 is a ligand-mimetic $\alpha_{IIb}\beta_3$-specific mAb that binds to nonactivated and activated $\alpha_{IIb}\beta_3$.[23] PAC-1, a ligand-mimetic $\alpha_{IIb}\beta_3$-specific mAb that binds specifically to activated $\alpha_{IIb}\beta_3$, was kindly provided by Dr Sanford J. Shattil (Scripps Research Institute).[24] PT25-2 ($\alpha_{IIb}\beta_3$-specific mAb) activates $\alpha_{IIb}\beta_3$ and was a kind gift from Drs Makoto Handa and Yasuo Ikeda (Keio University, Tokyo, Japan).[25] PMI-1 (α_{IIb}-specific mAb), anti-LIBS1 (β_3-specific mAb), and anti-LIBS6 (β_3-specific mAb) were generously provided by Dr Mark H. Ginsberg (Scripps Research Institute).[26,27] PMI-1, AP5, anti-LIBS1, and anti-LIBS6 recognize ligand-induced conformational changes on $\alpha_{IIb}\beta_3$, termed ligand-induced binding sites (LIBS).[21,26,27] TP80 (α_{IIb}-specific mAb) and MOPC21 (mouse myeloma immunoglobulin G_1 [IgG_1]) were purchased from Nichirei (Tokyo, Japan) and Sigma Chemical (St Louis, MO), respectively. FK633 ($\alpha_{IIb}\beta_3$-specific peptidomimetic antagonist) was generously provided by Dr Jiro Seki (Fujisawa Pharmaceutical, Osaka Japan),[28] and cyclo(RGDfV) [cyclo(-Arg-D-Gly D-Asp-D-Phe-L-Val-D-)] peptide ($\alpha_v\beta_3$-specific antagonist) was a generous gift from Merck KGaA (Darmstadt, Germany).[29] Fibrinogen was purchased from Calbiochem-Novabiochem (San Diego, CA). MOPC21, AP1, AP2, TP80, AP3, PAC-1, and fibrinogen were labeled with fluorescein isothiocyanate (FITC), as previously described.[28]

Flow cytometry

Flow cytometric analysis using various mAbs was performed as previously described.[30] FITC-labeled mAbs were used to quantify the expression levels of $\alpha_{IIb}\beta_3$ on 293 cells and on platelets.

Analysis of platelet messenger RNA

Total cellular RNA of platelets was isolated from 30 mL whole blood, and α_{IIb} or β_3 messenger RNA (mRNA) was specifically amplified by reverse transcription–polymerase chain reaction (RT-PCR), as previously described.[30] Primers for the amplification of α_{IIb} or β_3 mRNA and conditions for RT-PCR were described elsewhere.[30,31] Nucleotide sequences of PCR products were determined by using Taq DyeDeoxy Terminator Cycle Sequencing kit and an ABI373A DNA sequencer (Applied Biosystems, Foster City, CA).

Allele-specific restriction enzyme analysis

Amplification of the region around exon 4 of the α_{IIb} gene was performed by using primers IIbE3, 5′-GTCGGTCGTCAGCTGGAGC-3′ (sense, nucleotide [nt] 3940-3958 in the α_{IIb} gene), and IIbE4, 5′-CAGGTCG-TAGCTGGCGCTTAC-3′ (antisense, nt 4192-4172) and by using 250 ng DNA as a template. First-round PCR products were reamplified using primers IIbg3947S, 5′-GTCAGCTGGAGCGACGTCATTGTG-3′ (sense, nt 3947-3970) and IIbE4 and then were digested with restriction enzyme *Sca*I. Resultant fragments were electrophoresed in a 1.5% agarose gel.

Construction of α_{IIb} expression vectors and cell transfection

Wild-type α_{IIb} and β_3 complementary DNAs (cDNAs) cloned into a mammalian expression vector pcDNA3 (Invitrogen, San Diego, CA) were generously provided by Dr Peter J. Newman. To construct the expression vector containing the ^{521}C (His143) form of α_{IIb}, PCR-based cartridge mutagenesis was performed as previously described.[16] Platelet α_{IIb} cDNA from Osaka-12 was amplified by RT-PCR using primers IIb1 and IIb3αAS, 5′-CCCACGATCAGGTCTGGGTATCCG-3′ (antisense, nt 1407-1384). Second-round amplification was then performed using 1 μL first-round PCR products as a template with nested primers IIb187S, 5′-GAGAGTG-GCCATCGTGGTGG-3′ (sense, nt 187-206), and IIb3αAS2C, 5′-CCGTTGTCATCGATGTCTACGGC-3′ (antisense, nt 1364-1386). To construct the expression vector for Tyr143Ala α_{IIb} substitution, we carried out the overlapping extension PCR as previously described.[16] Mismatched sense primer IIb143Ala-S, 5′-GAGCGGCCGCCGCGCCGAGGCCTC-CCCCTG-3′ (sense, nt 502-531, 2 bp mismatched), and antisense primer IIb143Ala-AS, 5′-CAGGGGGAGGCCTCGGCGCGGCGGCCGCTC-3′ (antisense, nt 531-502, 2 bp mismatched) were synthesized. PCR was performed by using α_{IIb} cDNA as a template and primers IIb1 and IIb143Ala-AS, or primers IIb143Ala-S and IIb3αAS. The 2 individually amplified PCR products were mixed and used as a template of PCR using primers IIb1 and IIb3αAS. Amplified fragments were digested with restriction enzyme *Sac*II and *Cla*I, and the resultant fragments were extracted using GeneClean II kit (Bio 101, La Jolla, CA). Fragments were introduced into the pcDNA3 that had been digested with *Sac*II and *Cla*I. Inserted fragments were characterized by sequence analysis to verify the absence of any other substitutions and the proper insertion of the PCR cartridge into the vector. Expression vectors containing the KO mutant (2-amino acid [Arg-Thr] insertion between residues 160 and 161) α_{IIb} cDNA and Asp163AlaαIIb cDNA were constructed as previously described.[16]

The wild-type or mutant α_{IIb} construct was cotransfected into 293 cells with the wild-type β_3 construct by the calcium phosphate method, as previously described.[16] The 293 cells transiently expressing mutant $\alpha_{IIb}\beta_3$ were obtained and analyzed 2 days after transfection. In selected experiments, CD36 expression vector was cotransfected with α_{IIb} and β_3 constructs to monitor transfection efficiency.[32] In addition, stable transfectants expressing wild-type, Tyr143His$\alpha_{IIb}\beta_3$, or KO mutant $\alpha_{IIb}\beta_3$ were selected for G418 resistance (Gibco BRL, Grand Island, NY) and were cultured in Dulbecco modified Eagle medium (DMEM) with 10% heat-inactivated fetal calf serum (Life Technologies, Gaithersburg, MD).

Adhesion assays

Adhesion assays were performed as described by Faull et al.[33] Wells from 96-well microtiter plates were coated with up to 0.5 μg fibrinogen per well in 100 μL phosphate-buffered saline (PBS) and were incubated at 4°C overnight. After washing with PBS, wells were blocked with PBS containing 1% bovine serum albumin (BSA) (Sigma) for 90 minutes at 22°C. To determine background adhesion, control wells were coated with 1% BSA. Then 293 cells transiently expressing wild-type or mutant $\alpha_{IIb}\beta_3$ were washed twice with PBS and resuspended in DMEM containing 0.1% BSA at a concentration of 1×10^6 cells/mL, and 100 μL aliquots cell

suspension were added to wells in triplicate. The plate was incubated in a humidified 37°C incubator for 60 minutes. After washing with PBS 3 times, the adherent cells were quantified by measuring endogenous cellular acid phosphate activity in an enzyme-linked immunosorbent assay.[34]

For morphologic analysis, 293 cells stably expressing wild-type or mutant $\alpha_{IIb}\beta_3$ were added on fibrinogen-coated glass coverslips for 60 minutes at 37°C. After they were washed with PBS, adherent cells were fixed in 3.7% formaldehyde for 10 minutes, permeabilized in 0.5% Triton X-100 in PBS for 5 minutes at room temperature, and washed twice with PBS. Cells on coverslips were stained with rhodamine-phalloidin (Sigma) and were analyzed under a fluorescence microscope (Olympus, Tokyo, Japan).[35]

Tyrosine phosphorylation of pp125^{FAK}

Adherent cells on fibrinogen were lysed in Triton X-100 buffer (1% Triton X-100, 25 mM Tris-HCl, 100 mM NaCl, pH 7.4, 0.1 mg/mL leupeptin, 4 μg/mL pepstatin A, 1 mM phenylmethylsulfonyl fluoride, and 10 mM benzamide) containing sodium vanadate and were scraped into microcentrifuge tubes. Lysates were incubated on ice for 30 minutes, and clarified supernatants were processed for immunoprecipitation. Focal adhesion kinase (pp125^{FAK}) was immunoprecipitated with 1 μg rabbit polyclonal antibody specific for FAK (Santa Cruz Biotechnology, CA), and protein-G Sepharose (Pharmacia, Uppsala, Sweden). Precipitates were separated on 7.5% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and were transferred to a polyvinylidene difluoride (PVDF) membrane. Phosphotyrosine was detected with mAb 4G10. To monitor the loading of gel lanes, the blots were stripped (2% SDS, 62.5 mM Tris, pH 6.7, 100 mM 2-mercaptoethanol for 30 minutes at 70°C) and reprobed with anti-FAK.[36]

Clot retraction

Clot retraction of stable cell lines was performed based on the method described by Katagiri et al.[37] In brief, 293 cells stably expressing $\alpha_{IIb}\beta_3$ in Tyrode/HEPES (N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid) buffer (2×10^6 cells/mL) were incubated with 10 mM tranexamic acid, 250 μg/mL fibrinogen, and 2 mM $CaCl_2$ at 37°C in a siliconized glass tube. To block the effect of $\alpha_v\beta_3$ composed of endogenous α_v and transfected β_3, these stable transfectants were preincubated with 50 μM cyclo(RGDfV) ($\alpha_v\beta_3$-specific antagonist) at room temperature for 30 minutes. Then 1 U thrombin was added to 1 mL cell suspension. Fibrin gels began to form immediately after the addition of thrombin, and the tubes were kept at 37°C. Clot retraction was monitored by taking photographs every 30 minutes using a digital camera. Quantification of retraction was performed by an assessment of the clot area using the NIH Image 1.67e software (Bethesda, MD). Data were expressed as follows: % clot retraction = [(area t_0 − area t)/area t_0] × 100.

Results

Platelets from thrombasthenic patient Osaka-12 show impaired ligand-binding function

We first examined the surface expression of $\alpha_{IIb}\beta_3$ on platelets from patient Osaka-12 and 3 control subjects using flow cytometry. Although FITC-AP1 (GPIb-specific mAb) bound to Osaka-12 platelets slightly higher than to control platelets (113% of control value [mean value obtained from 3 control subjects]), FITC-AP3 (β_3-specific mAb) and FITC-TP80 (α_{IIb}-specific mAb) revealed a significant reduction in the expression of $\alpha_{IIb}\beta_3$ on Osaka-12 platelets (AP3 binding, 41% of control value; TP80 binding, 36% of control value; n = 2) (Figure 1). Compared with AP3 and TP80 binding to Osaka-12 $\alpha_{IIb}\beta_3$, AP2 binding was markedly impaired (13% of control value; n = 2) probably because of a disturbance of AP2 epitope formation. Because Osaka-12 platelets expressed 36% to 41% of normal amounts of $\alpha_{IIb}\beta_3$ on their surfaces, we examined

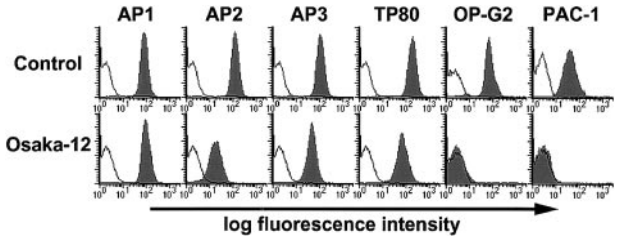

Figure 1. Flow cytometric analysis of the surface expression and ligand-binding function of $\alpha_{IIb}\beta_3$ Osaka-12 platelets. Washed platelets obtained from Osaka-12 or 3 control subjects were incubated with FITC-AP1 (GPIb-specific mAb), FITC-AP2 ($\alpha_{IIb}\beta_3$-specific mAb), FITC-AP3 (β3-specific mAb), and FITC-TP80 (α_{IIb}-specific mAb) at a concentration of 10 μg/mL for 30 minutes at room temperature. FITC-MOPC21 (mouse myeloma IgG_1) was used as a negative control. For OP-G2 binding (activation-independent ligand-mimetic $\alpha_{IIb}\beta_3$-specific mAb), bound antibodies were detected by FITC-conjugated goat $F(ab')_2$ antimouse IgG. Filled and open histograms represent the binding of specific and control antibodies, respectively. For PAC-1 binding, washed platelets were preincubated with 10 μg/mL PT25-2 (activating $\alpha_{IIb}\beta_3$-specific mAb) for 30 minutes, 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 ($\alpha_{IIb}\beta_3$-specific antagonist), respectively. Results are representative of 2 separate experiments.

their ligand-binding function. OP-G2 and PAC-1 mAbs are activation-independent and activation-dependent, ligand-mimetic mAbs specific for $\alpha_{IIb}\beta_3$, respectively. Neither OP-G2 nor PAC-1 in the presence of an activating PT25-2 mAb bound to Osaka-12 platelets (Figure 1). Thus, Osaka-12 $\alpha_{IIb}\beta_3$ seems to have a ligand-binding defect and a quantitative defect.

$\alpha_{IIb}\beta_3$ LIBS expression on Osaka-12 platelets by $\alpha_{IIb}\beta_3$-specific peptidomimetic antagonist FK633

PMI-1, AP5, anti-LIBS1, and anti-LIBS6 mAbs preferentially recognize LIBS on $\alpha_{IIb}\beta_3$, which are exposed following occupancy of the receptor by ligands. To further investigate the ligand-binding function of Osaka-12 $\alpha_{IIb}\beta_3$, we examined the effect of FK633, $\alpha_{IIb}\beta_3$-specific peptidomimetic antagonist on the expression of these LIBS. Compared with Arg-Gly-Asp-Trp peptide, FK633 has approximately 100-fold higher potency for LIBS induction.[28] Although we could not simply compare the binding of mAbs recognizing LIBS between Osaka-12 and control platelets because of the different expression levels of $\alpha_{IIb}\beta_3$, even 10 μM FK633 showed a negligible effect on the expression of LIBS1 of Osaka-12 $\alpha_{IIb}\beta_3$ (Table 1). On the other hand, Table 1 shows that Osaka-12 $\alpha_{IIb}\beta_3$ aberrantly expressed LIBS recognized by PMI-1, AP5, and anti-LIBS6. These data further suggested that the ligand-binding sites of Osaka-12 $\alpha_{IIb}\beta_3$ were markedly impaired.

Nucleotide sequence and allele-specific restriction enzyme analyses for Osaka-12 $\alpha_{IIb}\beta_3$

To identify the molecular genetic defect responsible for Osaka-12, all coding regions of α_{IIb} and β_3 cDNA amplified by RT-PCR from Osaka-12 and control platelet mRNA were analyzed. Direct sequence of the PCR fragments showed a single T>C substitution at nt 521 in α_{IIb} cDNA that would lead to a Tyr143His substitution in exon 4 of α_{IIb} (Figure 2A). No other nucleotide substitutions were detected in the coding regions of either α_{IIb} or β_3 cDNAs. The T>C substitution abolished a restriction site for *Sca*I, and allele-specific restriction enzyme analysis for the amplified α_{IIb} cDNA around exon 4 suggested that Osaka-12 might be homozygous for the substitution. However, allele-specific restriction enzyme analysis for the PCR fragments from genomic DNA revealed that Osaka-12 was heterozygous for the T>C substitution. The substitution was inherited from her father. The molecular genetic defect

Table 1. Analysis of LIBS expression in Osaka-12 $\alpha_{IIb}\beta_3$

	PMI-1		AP5		LIBS1		LIBS6	
	(−)	+FK633	(−)	+FK633	(−)	+FK633	(−)	+FK633
Control	1.53 ± 0.63	10.12 ± 4.14	0.95 ± 0.56	20.32 ± 7.78	16.59 ± 2.38	102.2 ± 14.66	2.24 ± 0.80	21.51 ± 1.53
Osaka-12	4.3 ± 0.37	3.82 ± 0.77	5.59 ± 0.57	7.61 ± 0.53	12.15 ± 1.64	14.49 ± 4.96	7.36 ± 1.12	9.44 ± 0.43

Washed platelets were incubated with or without 10 μM FK633 ($\alpha_{IIb}\beta_3$-specific antagonist), and then each anti-LIBS antibody was added. After washing, bound antibodies were detected by FITC-conjugated goat F(ab′)$_2$ antimouse IgG. Results are expressed as mean fluorescence intensity (MFI) ± SD of triplicate measurements. The MFI for MOPC21 used as a negative control was subtracted. Results are representative of 2 separate experiments.

inherited from her mother remains determined. However, only the allele having ^{521}C from her father was amplified by RT-PCR from Osaka-12 α_{IIb} mRNA, suggesting that the expression levels of maternal α_{IIb} mRNA seem to be extremely low in Osaka-12 platelets (Figure 2B-C).

Effect of Tyr143His substitution in α_{IIb} on $\alpha_{IIb}\beta_3$ expression and function

To confirm that the ^{521}T>C substitution leading to Tyr143His substitution in α_{IIb} is responsible for the functional defect, we constructed an expression vector that contained the wild-type or mutant His143 form of α_{IIb} and cotransfected it with wild-type β_3 vector into 293 cells. Effects of a Tyr143Ala substitution in α_{IIb} on $\alpha_{IIb}\beta_3$ expression and function were also examined. Because Tyr143 is located within the W3 4-1 loop of the β-propeller domain, close to the KO mutation (the Arg-Thr insertion between 160 and 161 residues of α_{IIb}) and the Asp163Ala α_{IIb} mutation, we compared the expression and function of these mutant $\alpha_{IIb}\beta_3$ in parallel.

Transfection efficiency monitored by the cotransfected CD36 expression vector was essentially the same between wild-type and mutant $\alpha_{IIb}\beta_3$ (Figure 3). Flow cytometric analysis using FITC-AP3 or FITC-TP80 mAb showed that the expression levels of Tyr143His$\alpha_{IIb}\beta_3$ were essentially the same as wild-type $\alpha_{IIb}\beta_3$ (AP3 binding, 91% ± 15% of wild-type; TP80 binding, 102% ± 2% of wild-type; mean ± SD; n = 3). In sharp contrast, Tyr143His$\alpha_{IIb}\beta_3$ failed to bind OP-G2 mAb, FITC-PAC-1 mAb, or FITC-fibrinogen in the presence of PT25-2 mAb (Figure 3). We also confirmed that the binding failure of these activation-dependent ligands was not caused by the binding failure of the activating mAb, PT25-2. In addition, FITC-AP2 binding was significantly impaired compared with TP80 binding (AP2 binding; 15% ± 3% of wild-type; mean ± SD; n = 3), which is consistent with the data obtained from Osaka-12 platelets (Figures 1 and 3). Given that the expression levels of abnormal α_{IIb} mRNA derived from her mother were so low that they were not detected in Osaka-12 platelets by RT-PCR, this maternal abnormality would reduce the expression of $\alpha_{IIb}\beta_3$ by 50%. As expected, the expression level of Tyr143His$\alpha_{IIb}\beta_3$ on 293 cells was roughly twice as much as that on Osaka-12 platelets. These data indicate that the Tyr143His mutation is responsible for the variant GT phenotype in Osaka-12. The Tyr143Ala substitution induced essentially the same effects on $\alpha_{IIb}\beta_3$ as the Tyr143His mutation (AP3 binding, 117% ± 14% of wild-type; TP80 binding, 113% ± 8% of wild-type; AP2 binding, 14% ± 1% of wild-type; mean ± SD; n = 3). Because Tyr143Ala abolished the ligand binding, it is likely that the presence of Tyr143 is critical for $\alpha_{IIb}\beta_3$ function. Interestingly, the phenotype of Asp163Ala$\alpha_{IIb}\beta_3$ appears the same as that of Tyr143His$\alpha_{IIb}\beta_3$. The phenotype of KO $\alpha_{IIb}\beta_3$ was essentially the same as wild-type $\alpha_{IIb}\beta_3$ except for the failure in OP-G2 and PAC-1 binding, as previously described.[15] Thus, none of these mutant $\alpha_{IIb}\beta_3$ showed a binding ability for soluble ligands (Figure 3).

Adhesion of the cells to immobilized fibrinogen

To further examine the functions of mutant $\alpha_{IIb}\beta_3$, we performed cell adhesion assay to immobilized fibrinogen. In contrast to parent cells, 293 cells transiently transfected with wild-type $\alpha_{IIb}\beta_3$ or mutant $\alpha_{IIb}\beta_3$ became adhesive to the immobilized fibrinogen. Given that endogenous α_v in 293 cells can form $\alpha_v\beta_3$ with the exogenous β_3 and may contribute to the adhesion, we preincubated the transfected cells with 50 μM cyclo(RGDfV) to block the $\alpha_v\beta_3$ function. Under these conditions, 293 cells transfected with

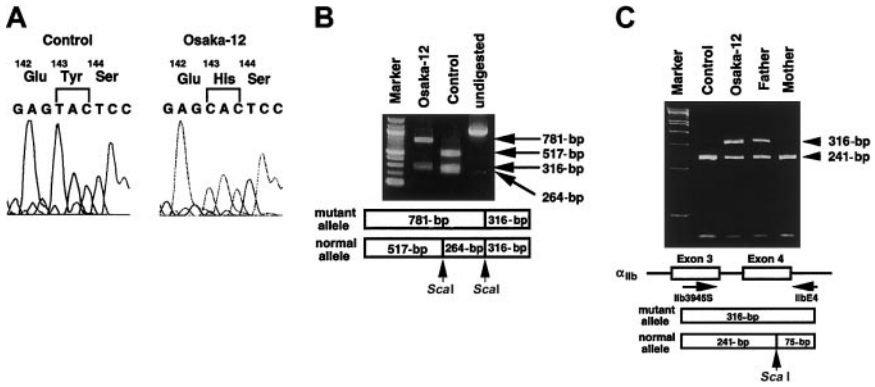

Figure 2. Identification of the genetic defect responsible for Osaka-12. (A) Nucleotide sequence analysis of α_{IIb} cDNA from Osaka-12. Platelet α_{IIb} mRNA was amplified by RT-PCR. Nucleotide sequence of the amplified fragments was determined by using Taq DyeDeoxy Terminator Cycle Sequencing kit and an ABI 373A DNA sequencer. (B) Allele-specific restriction enzyme analysis of α_{IIb} cDNA. The region around exon 4 of the α_{IIb} mRNA was amplified by PCR, followed by digestion with *Sca*I. *Sca*I digestion of the PCR products yielded 517-bp, 264-bp, and 316-bp fragments in the healthy allele. The T>C substitution abolished one of the restriction sites for *Sca*I. Resultant fragments were electrophoresed in a 1.5% agarose gel. Marker indicates 100-bp DNA ladder. (C) Allele-specific restriction enzyme analysis of α_{IIb} genomic DNA. The region around exon 4 of the α_{IIb} gene was amplified by PCR using primers IIb3945S and IIbE4, followed by digestion with *Sca*I. *Sca*I digestion of the PCR products yielded 241-bp and 75-bp fragments in the healthy allele. The T>C substitution abolished the restriction site for *Sca*I. Resultant fragments were electrophoresed in a 6% polyacrylamide gel. Marker indicates ϕX174 digested with *Hae*III.

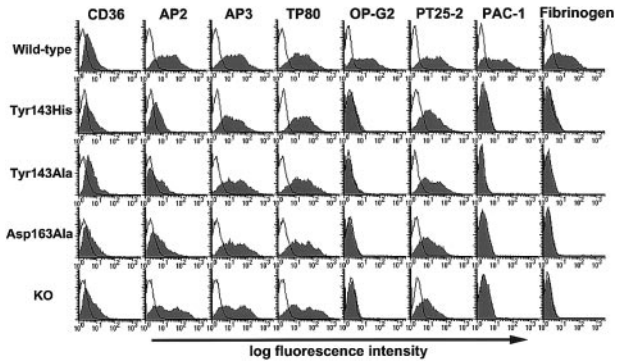

Figure 3. Effects of Tyr143Hisα_{IIb}, Tyr143Alaα_{IIb}, Asp163Alaα_{IIb}, or KO mutant on the expression and ligand-binding function of $\alpha_{IIb}\beta_3$ on 293 cells. Wild-type α_{IIb} or each mutant α_{IIb} cDNA was transiently cotransfected with wild-type β_3 cDNA into 293 cells. CD36 expression vector was cotransfected with α_{IIb} and β_3 constructs, and CD36 expression was measured by FITC–anti-CD36 mAb to monitor transfection efficiency. The binding of FITC-AP2, FITC-AP3, FITC-TP80, OP-G2, and PT25-2 was analyzed by flow cytometry 2 days after transfection (filled histograms). FITC-MOPC21 was used as a negative control (open histograms). For OP-G2 or PT25-2 binding, bound antibodies were detected by FITC-conjugated goat F(ab′)$_2$ antimouse IgG. For PAC-1 or fibrinogen binding, washed platelets were preincubated with 10 μg/mL PT25-2 (activating $\alpha_{IIb}\beta_3$-specific mAb) for 30 minutes, and then 10 μg/mL FITC-labeled PAC-1 or 150 μg/mL FITC-labeled fibrinogen was added. Closed and open histograms represent PAC-1 or fibrinogen binding in the absence and presence of 10 μM FK633 ($\alpha_{IIb}\beta_3$-specific antagonist), respectively. Results are representative of 3 separate experiments.

wild-type β_3 alone (data not shown), KO variant $\alpha_{IIb}\beta_3$, or Asp163Ala$\alpha_{IIb}\beta_3$ failed to adhere to immobilized fibrinogen (Figure 4). However, 293 cells expressing Tyr143His$\alpha_{IIb}\beta_3$ showed a significant adhesion to immobilized fibrinogen, and the amounts of adherent cells were approximately 50% compared with 293 cells expressing wild-type $\alpha_{IIb}\beta_3$. The adhesion of 293 cells expressing Tyr143His$\alpha_{IIb}\beta_3$ in the presence of 50 μM cyclo(RGDfV) was mediated solely by $\alpha_{IIb}\beta_3$ because 10 μM FK633 completely blocked the cell adhesion (data not shown).

We then obtained the cells stably expressing wild-type, Tyr143His, and KO mutant $\alpha_{IIb}\beta_3$ with G418 selection for morphologic analysis of the adherent cells. Adhesive functions of 293 cells stably expressing Tyr143His $\alpha_{IIb}\beta_3$ or KO $\alpha_{IIb}\beta_3$ were the same as those transiently expressing these mutant $\alpha_{IIb}\beta_3$ (data not shown). Although 293 cells stably expressing wild-type $\alpha_{IIb}\beta_3$ adhered firmly and showed complete spreading, 293 cells stably expressing KO mutant $\alpha_{IIb}\beta_3$ failed to adhere. In contrast to KO $\alpha_{IIb}\beta_3$, 293 cells stably expressing Tyr143His$\alpha_{IIb}\beta_3$ moderately impaired cell spreading and cell adhesion (Figure 5). Because FAK, a 125-kDa cytoplasmic tyrosine kinase, is a component of focal adhesions and is a well-established component of integrin signaling pathways,[38] the tyrosine phosphorylation of pp125^{FAK} in these adherent 293 cells stably expressing wild-type $\alpha_{IIb}\beta_3$ or Tyr143His$\alpha_{IIb}\beta_3$ was examined. As shown in Figure 6, pp125^{FAK} phosphorylation was observed but impaired in cells expressing Tyr143His$\alpha_{IIb}\beta_3$ compared with wild-type $\alpha_{IIb}\beta_3$ (Figure 6).

Clot retraction

We examined the capacity of Tyr143His$\alpha_{IIb}\beta_3$ and KO mutant $\alpha_{IIb}\beta_3$ to mediate fibrin clot retraction by using stable transfectants. This process reflects the capacity of $\alpha_{IIb}\beta_3$ to transduce mechanical force between the intracellular cytoskeleton and the extracellular fibrin strands. Parent 293 cells failed to retract fibrin clots. Given that $\alpha_v\beta_3$ is involved in mediating fibrin clot retraction,[37] we preincubated all stable transfectants with 50 μM cyclo(RGDfV) to block the $\alpha_v\beta_3$ function. Under these conditions, 293 cells stably transfected with wild-type β_3 alone failed to mediate fibrin clot retraction, whereas those with wild-type α_{IIb} and β_3 showed a marked retraction. Although 293 cells stably expressing Tyr143His$\alpha_{IIb}\beta_3$ showed some ability for fibrin clot retraction (Figure 7), 293 cells stably expressing KO mutant $\alpha_{IIb}\beta_3$ failed to mediate fibrin clot retraction.

Discussion

The molecular basis for the interaction between $\alpha_{IIb}\beta_3$ and its ligands remains to be determined. In this study, we have described a novel missense mutation (Tyr143His) in α_{IIb} associated with a ligand-binding defect in this receptor. Because the Tyr143His mutation is located in the W3 4-1 loop of the β-propeller domain of α_{IIb}, we compared the effects of Tyr143Hisα_{IIb} on the expression and function of $\alpha_{IIb}\beta_3$ with those of previously described KO and Asp163Ala mutations located in the same loop.[16] Although GT patient analysis demonstrated that the surface expression level of Tyr143His$\alpha_{IIb}\beta_3$ on platelets from heterozygous Osaka-12 was 36% to 41% of control, this reduction in the expression resulted mainly from the null expression of α_{IIb} mRNA from the maternal allele. Indeed, Tyr143Hisα_{IIb} mutation resulted in almost normal $\alpha_{IIb}\beta_3$ expression on 293 cells (91%-102% of wild-type $\alpha_{IIb}\beta_3$). In sharp contrast, this mutation abolished the interaction between $\alpha_{IIb}\beta_3$ and its macromolecular ligands OP-G2 mAb, PAC-1 mAb, and fibrinogen. The failure of LIBS expression by FK633 further suggested that Tyr143His$\alpha_{IIb}\beta_3$ has a binding defect for high-affinity $\alpha_{IIb}\beta_3$-specific small ligand. The effects of Tyr143Alaα_{IIb} on the expression and function of $\alpha_{IIb}\beta_3$ were essentially the same as Tyr143Hisα_{IIb}, suggesting that the presence of Tyr143, rather than the presence of a mutated residue such as His143, is critical for $\alpha_{IIb}\beta_3$ function. Interestingly, the defects for soluble ligands are essentially the same as those induced by KO or Asp163Ala mutation. However, cell adhesion and fibrin clot retraction assays revealed distinctive features among Tyr143His$\alpha_{IIb}\beta_3$, KO $\alpha_{IIb}\beta_3$, and Asp163Ala$\alpha_{IIb}\beta_3$. Thus, KO $\alpha_{IIb}\beta_3$ and Asp163Ala$\alpha_{IIb}\beta_3$ show

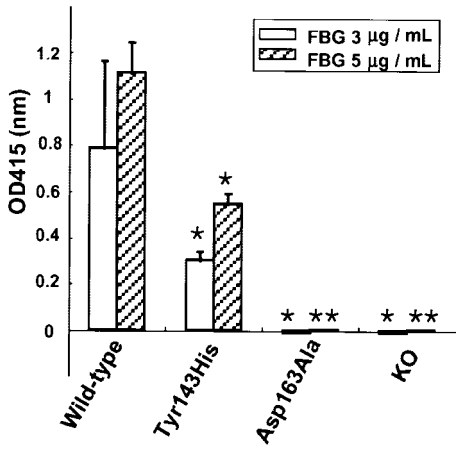

Figure 4. Adhesion of $\alpha_{IIb}\beta_3$ mutants to immobilized fibrinogen. Wild-type α_{IIb} or each mutant α_{IIb} cDNA was transiently cotransfected with wild-type β_3 cDNA into 293 cells. Two days after transfection, wild-type or mutant $\alpha_{IIb}\beta_3$-transfected cells (1×10^6 cells/well) were incubated with immobilized fibrinogen (FBG) in the presence of 50 μM cyclo(RGDfV) ($\alpha_v\beta_3$-specific antagonist) at 37°C. After washing with PBS, adherent cells were quantified with a colorimetric reaction using endogenous cellular acid phosphatase activity. Data represent the mean ± SD of triplicate measures of optical density at 415 nm. Statistical analysis (2-tailed *P* values for paired sample) was performed between 293 cells expressing wild-type $\alpha_{IIb}\beta_3$ and those expressing each mutant $\alpha_{IIb}\beta_3$ (**$P < .01$; *$P < .05$).

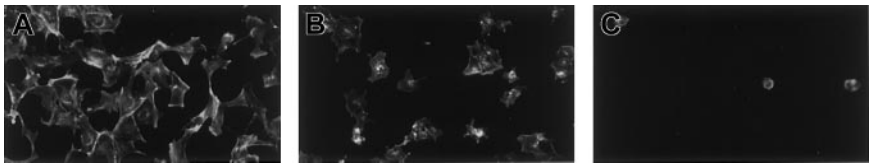

Figure 5. Morphologic analysis of the adherent cells stably expressing mutant $\alpha_{IIb}\beta_3$ to immobilized fibrinogen. 293 cells stably expressing (A) wild-type $\alpha_{IIb}\beta_3$, (B) Tyr143His$\alpha_{IIb}\beta_3$, or (C) KO mutant $\alpha_{IIb}\beta_3$ were incubated with immobilized fibrinogen (5 μg/mL) at 37°C for 60 minutes in the presence of 50 μM cyclo(RGDfV) ($\alpha_v\beta_3$-specific antagonist). After gentle washing with PBS, adherent cells were fixed in 3.7% formaldehyde and permeabilized in 0.5% Triton X-100. Cells were stained with rhodamine–phalloidin and analyzed under a fluorescence microscope. Original magnification, × 100.

a more profound binding defect for immobilized fibrinogen and fibrin strands than Tyr143His$\alpha_{IIb}\beta_3$, suggesting that Asp163 is located at or close to the ligand-binding sites in the β-propeller domain of α_{IIb}.

Recently described crystal structure of the extracellular segment of $\alpha_v\beta_3$ shows that the ligand-binding head is formed by a 7-bladed β-propeller domain from α_v and a β I-domain from β_3.[10] Moreover, there is mounting evidence that the W3 4-1 loop, W3 2-3 loop, and W4 4-1 loop of the β-propeller domain are important for ligand binding in non–I-domain α subunits.[39-41] In terms of the W3 4-1 loop of α_{IIb}, several residues critical for ligand binding have been suggested by alanine-scanning mutagenesis.[16,41] In addition, KO, Pro145Ala, and Tyr143His (this study) are naturally occurring mutations responsible for the ligand-binding defect in patients with variant GT. Compared with soluble ligands, immobilized fibrinogen and fibrin are activation-independent ligands for $\alpha_{IIb}\beta_3$. Immobilized fibrinogen binding to $\alpha_{IIb}\beta_3$ initiates outside-in signaling that leads to cellular responses such as cell spreading. In addition to outside-in signaling, fibrin clot retraction requires inside-out transmission of the contractile forces from intracellular cytoskeleton to fibrin strand. Much higher concentrations of $\alpha_{IIb}\beta_3$-specific antagonists are needed to block cell adhesion to fibrinogen and fibrin clot retraction.[42-44] Thus, these ligands show higher affinity/avidity for $\alpha_{IIb}\beta_3$ than soluble fibrinogen. Because some mutations in the W3 4-1 loop, such as Pro145Ala, impaired $\alpha_{IIb}\beta_3$ biosynthesis and its function,[17] such mutations are likely to affect the conformation of this receptor, including the W3 4-1 loop. Therefore, we used these higher affinity/avidity ligands to further examine the residual function of $\alpha_{IIb}\beta_3$ mutants. Neither the KO mutant nor Asp163Ala$\alpha_{IIb}\beta_3$-expressing 293 cells adhered to immobilized fibrinogen or showed fibrin clot retraction. In contrast, Tyr143His$\alpha_{IIb}\beta_3$-expressing 293 cells showed some ability for fibrin clot retraction and for cell adhesion to fibrinogen with a reduction in spreading. Consistent with these findings, the impaired tyrosine phosphorylation of pp125^{FAK} mediated through $\alpha_{IIb}\beta_3$ was observed in Tyr143His$\alpha_{IIb}\beta_3$-expressing 293 cells compared with wild-type $\alpha_{IIb}\beta_3$. These findings indicate that Tyr143His$\alpha_{IIb}\beta_3$ still has some ligand-binding function. Although a differential engagement of $\alpha_{IIb}\beta_3$ in fibrin clot retraction versus aggregation has been suggested,[45,46] KO mutant and Asp163Alaα_{IIb} abolished the interaction with soluble fibrinogen and immobilized fibrin(ogen), as shown in this study. These findings suggest that the integrin residues involved in these phenomena, at least in part, overlap. The close location of Tyr143 with Asp163 suggests that the defect in soluble ligand binding induced by the Tyr143Hisα_{IIb} mutation is likely the result of its allosteric effect rather than of a defect in the ligand-binding site itself.

More recently, the crystal structure of $\alpha_v\beta_3$ with cyclo(RGDfV) has been described.[11] The Arg and Asp side chains exclusively contact the propeller and β I-domain, respectively, confirming the critical roles of these domains in ligand binding. The Arg side chain inserts into a narrow groove at the top of the propeller domain, formed primarily by the W3 4-1 loop and the W4 4-1 loop, and the Arg guanidinium group is held in place by a salt bridge to Asp218 in the W4 4-1 loop and to Asp150 in the W3 4-1 loop.[11] However, our previous data showed that alanine-scanning mutagenesis in the W3 4-1 loop, including Asp150Ala, did not inhibit the ligand-binding function of $\alpha_v\beta_3$; this is not consistent with the crystal structure data.[34] The role of the W3 4-1 loop appears to be different between non–I-domain α subunits. The W3 4-1 loops in α_{IIb} and in α_3 (Thr162 and Gly163) appear critical for ligand binding, whereas those in α_4 and in α_v do not.[16,34,39,40] Although alanine-scanning mutagenesis suggested several residues within the W3 4-1 and W3 2-3 loops of α_{IIb} may be critical for ligand binding, the same procedure showed only Tyr178 in the W3 2-3 loop of α_v as a critical residue.[34,41] In

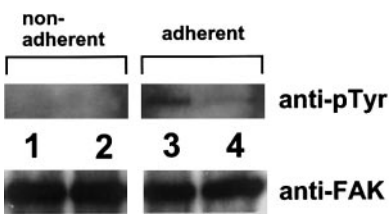

Figure 6. Tyrosine phosphorylation of pp125^{FAK}. Nonadherent or adherent 293 cells stably expressing wild-type $\alpha_{IIb}\beta_3$ (lanes 1 and 3) or Tyr143His$\alpha_{IIb}\beta_3$ (lanes 2 and 4) on fibrinogen were lysed in Triton X-100 buffer containing sodium vanadate. pp125^{FAK} was immunoprecipitated with 1 μg rabbit polyclonal antibody specific for FAK. Precipitates were separated on 7.5% SDS-PAGE and transferred to a PVDF membrane. Phosphotyrosine was detected with monoclonal antibody 4G10. To monitor the loading of gel lanes, the blots were reprobed with anti-FAK.

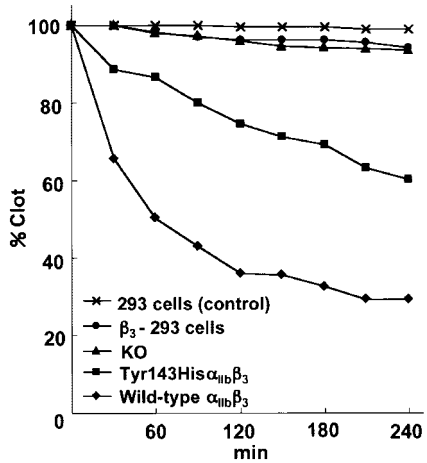

Figure 7. Fibrin clot retraction mediated by the cells stably expressing mutant $\alpha_{IIb}\beta_3$. 293 cells (control), 293 cells stably expressing wild-type β_3 alone, wild-type $\alpha_{IIb}\beta_3$, Tyr143His$\alpha_{IIb}\beta_3$, or KO mutant $\alpha_{IIb}\beta_3$ suspended in Tyrode/HEPES buffer (2×10^6 cells/mL) were incubated with 10 mM tranexamic acid, 250 μg/mL fibrinogen, and 2 mM $CaCl_2$ at 37°C in a siliconized glass tube in the presence of 50 μM cyclo(RGDfV) ($\alpha_v\beta_3$-specific antagonist). Then 1 U thrombin was added to 1 mL cell suspension. Clot retraction was monitored by digital photography every 30 minutes. Data were expressed as % clot retraction = [(area t_0 − area t)/area t_0] × 100. Results are representative of 3 separate experiments.

addition, our recent study suggests that the expression and function of $\alpha_{IIb}\beta_3$ are more strictly regulated than $\alpha_v\beta_3$.[47] Among non–I-domain α subunits, α_{IIb} has the longest W3 4-1 loop, and it is possible that differences in the structure between α_{IIb} and α_v may account for the distinctive role of the W3 4-1 loop in ligand binding between these β_3 integrins. Unique features of the W3 4-1 loop of $\alpha_{IIb}\beta_3$ are also likely to account for the discrepancy between the ability for soluble ligand binding and cell adhesion. Detailed structure–function analyses of β_3 integrins and of their crystal structure would provide a better understanding of integrin function and a new antagonist design for these integrins to prevent thrombosis.

Acknowledgments

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