Truncation of the Cytoplasmic Domain of β_3 in a Variant Form of Glanzmann Thrombasthenia Abrogates Signaling through the Integrin $\alpha_{IIb}\beta_3$ Complex

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Abstract

Glanzmann thrombasthenia is an inherited bleeding disorder characterized by absence or dysfunction of the platelet integrin $\alpha_{IIb}\beta_3$. Patient RM is a thrombasthenic variant whose platelets fail to aggregate in response to physiological agonists, despite the fact that they express abundant levels of $\alpha_{IIb}\beta_3$ on their surface. Binding of soluble fibrinogen or fibrinogen mimetic antibodies to RM platelets did not occur, except in the presence of ligand-induced binding site (LIBS) antibodies that transformed the RM integrin complex into an active conformation from outside the cell. Sequence analysis of PCR-amplified genomic DNA and platelet mRNA revealed a C2268T nucleotide substitution in the gene encoding the integrin β_3 subunit that resulted in an Arg724Ter mutation, producing a truncated protein containing only the first eight of the 47 amino acids normally present in the cytoplasmic domain. Functional analysis of both RM platelets and CHO cells stably expressing this truncated integrin revealed that the $\alpha_{IIb}\beta_3$Arg724Ter complex is able to mediate binding to immobilized fibrinogen, though downstream events, including cytoskeletally-mediated cell spreading and tyrosine phosphorylation of focal adhesion kinase, pp125^{FAK}, fail to occur. These studies establish the importance of the membrane-distal portion of the integrin β_3 cytoplasmic domain in bidirectional transmembrane signaling in human platelets, and the role of integrin signaling in maintaining normal hemostasis in vivo. (*J. Clin. Invest.* 1997. 100:2393–2403.) Key words: inherited platelet defects • integrin signal transduction • Glanzmann thrombasthenia • pp125^{FAK}

Introduction

Integrins are a widely-distributed family of αβ heterodimeric adhesion receptors that mediate the interactions of cells with each other and with the extracellular matrix (1, 2). Both α and β integrin subunits are comprised of a large extracellular region, a single-pass transmembrane domain, and a relatively short cytoplasmic tail (3). $\alpha_{IIb}\beta_3$ (also known as the glycoprotein IIb-IIIa complex [4]) is a platelet-specific member of the integrin family that is abundantly expressed on the platelet surface at ~ 80,000 copies per cell (5), where it exists in a nonadhesive, resting conformational state (6).

In a poorly understood process known as inside-out signal transduction, platelet stimulation by agonists such as ADP, thrombin, or epinephrine, results in the generation of signals that are thought to affect one or both cytoplasmic domains of the $\alpha_{IIb}\beta_3$ complex (7, 8). This information is then relayed through the plasma membrane to the extracellular domain of the integrin complex, resulting in rapid conformational changes in the receptor that permit the binding of adhesive plasma proteins such as fibrinogen and vWf (6, 9) and the subsequent formation of a platelet plug. Integrins are also capable of transmitting signals into the cell following ligand- or antibody-induced oligomerization—a process termed outside-in signal transduction (10, 11). Cellular responses initiated by integrin clustering include changes in cytoplasmic pH (12, 13), elevation of intracellular free calcium (14–16), modified patterns of gene expression (10, 17–19), cytoskeletal reorganization (20–23), and enhanced tyrosine phosphorylation of a myriad of cytosolic proteins (24–26), including the focal adhesion kinase (FAK),[1] pp125^{FAK} (25, 27, 28). As in inside-out integrin-mediated signal transduction, integrin cytoplasmic domains have been implicated in mediating postligand binding events (29–31).

The ability of integrin cytoplasmic domains to mediate bidirectional transmembrane signaling has, to date, necessarily been examined in cultured transformed tumor cell lines transfected with recombinant integrin subunits (8, 30, 32–37). Though these studies have served to establish fundamental concepts of integrin-mediated signal transduction, there has been little opportunity to study the contribution of integrin cytoplasmic domains to this process within the context of a normal human cell. Given the well-known influence of cell type–specific cytosolic proteins in governing both outside-in and inside-out signal transduction (8, 32, 38), studies of naturally occurring integrin variants have the potential to provide important new insights into the role of these adhesion and signaling receptors in maintaining normal cellular function.

In this report, we describe the adhesive and signaling properties of an $\alpha_{IIb}\beta_3$ variant expressed on the surface of platelets derived from an individual suffering from the inherited integ-

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1. *Abbreviations used in this paper:* CHO, Chinese hamster ovary; FAK, focal adhesion kinase = pp125^{FAK}; LIBS, ligand-induced binding site; PMSF, phenylmethylsulfonyl fluoride; RIPA, radioimmunoprecipitation assay; TRAP, thrombin receptor–activating peptide.

rin defect, Glanzmann thrombasthenia (for review, see references 39 and 40). Functional analysis of both human platelets and transfected cells expressing a truncated form of β_3 revealed that both inside-out and outside-in integrin signaling are all critically dependent upon the membrane-distal portion of the β_3 cytoplasmic domain. These findings establish the importance of bidirectional transmembrane signaling in human platelet function, and in maintaining normal hemostasis in vivo.

Methods

Antibodies and peptides. The $\alpha_{IIb}\beta_3$ complex–specific murine mAb AP2 (41), β_3-specific antibody, AP3 (42), and GPIb-specific antibody, AP1, were produced at the Blood Research Institute (BRI) Hybridoma Core Lab (Milwaukee, WI) and purified by affinity chromatography. Tab (anti-α_{IIb}) (43) and S12 (anti–P-selectin) (44) were kindly provided by Dr. Rodger McEver (University of Oklahoma, Oklahoma City, OK). The ligand-induced binding site (LIBS) antibodies D3 (45) and anti-LIBS6 (46) were provided by Dr. Lisa Jennings (University of Memphis, Memphis, TN) and Dr. Mark Ginsberg (The Scripps Research Institute, La Jolla, CA), respectively. LIBS antibodies can induce and/or recognize the active conformational state of $\alpha_{IIb}\beta_3$ after ligand occupancy of the receptor (47). Two fibrinogen-mimetic antibodies, PAC-1 (48, 49), and Pl-55 (50) (a gift of Dr. Beat Steiner, Hoffmann-La Roche LTD, Basel, Switzerland) were used to report exposure of the macromolecular ligand-binding domain of the $\alpha_{IIb}\beta_3$ complex, and were used directly conjugated to FITC (51). The HRP-conjugated phosphotyrosine-specific monoclonal antibody, PY20, was purchased from Zymed Laboratories, Inc. (South San Francisco, CA). Rabbit polyclonal antiserum and a monoclonal antibody (2A7) to pp125^{FAK} were provided by Dr. Thomas Parsons (University of Virginia, Charlottesville, VA). RGDW, RGEW, and TRAP (a thrombin receptor-activating peptide having the sequence SFLLRN) peptides were synthesized and HPLC-purified in the BRI Protein Core Laboratory. The mass of all peptides was verified by mass spectrometry before use.

Patient information and platelet function studies. Patient RM is a black 10-yr-old child with normal platelet counts, but who has experienced severe bleeding since birth (bleeding time $>$ 20 min). Though ristocetin-induced agglutination was normal, RM platelets failed to aggregate (0% aggregation response) in response to all physiological agonists tested, including epinephrine, collagen, ADP, thrombin, and arachidonic acid, leading to the diagnosis of Glanzmann thrombasthenia. RM's parents are unrelated. Platelets from patient RM, his mother, or healthy drug-free, male volunteers were prepared from platelet-rich plasma (PRP) anticoagulated with acid-citrate-dextrose, pH 6.5, washed twice in Ringer's Citrate Dextrose (RCD) buffer (108 mM NaCl, 38 mM KCl, 1.1 mM $MgCl_2$, 1.8 mM $NaHCO_3$, 21.2 mM Na-citrate, 27.8 mM glucose, pH 6.8), and finally resuspended in the same buffer at pH 7.4. Platelet aggregation was initiated by the addition of 7 μM TRAP to 1.5×10^8 platelets in the presence of 0.25 mg/ml of human Peak I fibrinogen (kindly provided by Dr. Michael Mosesson, Mount Sinai Medical Center, Milwaukee, WI) in a final volume of 0.5 ml. Aggregometry was performed at 37°C for 3 min using a stirring rate of 1,000 rpm, and stopped by adding an equal volume of 2× radioimmunoprecipitation assay (RIPA) buffer (2% Triton X-100, 300 mM NaCl, 20 mM Tris, 2 mM EGTA, 2 mM Na_3VO_4, 0.2% SDS, 2% sodium deoxycholate, 1 mM phenylmethylsulfonyl fluoride [PMSF], and 10 μg/ml each of aprotinin and leupeptin).

Immunoblot blot analysis of platelet $\alpha_{IIb}\beta_3$ and pp125^{FAK}. Washed platelets were solubilized in 20 mM Tris, 100 mM NaCl, 1% Triton X-100, 10 mM *N*-ethylmaleimide, 2 mM PMSF, and 100 g/ml leupeptin at 4°C as described previously (52). After centrifugation at 15,000 *g* for 30 min, solubilized proteins were resolved by 7% SDS-PAGE under reducing or nonreducing conditions, and subjected to Western blot analysis using rabbit polyclonal antibodies specific for α_{IIb}, β_3, or PECAM-1 (53). Bound antibodies were detected using goat anti–rabbit IgG conjugated with alkaline phosphatase (Bio-Rad Laboratories, Richmond, CA), followed by color development using the nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate substrate pair (Sigma Chemical Co., St. Louis, MO).

The phosphorylation state of pp125^{FAK} was determined by solubilizing platelets in RIPA buffer, incubating 500 μg of platelet protein with a rabbit polyclonal antibody specific for FAK (54), and harvesting immunoprecipitates with protein A Sepharose beads. Immunoprecipitated pp125^{FAK} was resolved by 7.5% SDS-PAGE under reducing conditions, transferred to a polvinylidene difluoride (PVDF) membrane, and probed with either an anti-FAK polyclonal antibody (to assess antigen load) or a 1:5000 dilution of HRP-conjugated PY20. Membranes were then washed and developed using enhanced chemiluminescence (Amersham Corp., Arlington Heights, IL).

Flow cytometry. Surface expression of $\alpha_{IIb}\beta_3$ on washed platelets (5×10^6/ml) was evaluated by incubating washed platelets with 20 μg/ml AP3, Tab, or AP2, followed by FITC-conjugated goat anti–mouse IgG (Jackson Immunoresearch Labs, Inc., West Grove, PA) for 30 min. Samples were then diluted and subjected to flow cytometric analysis. For some experiments, 20 μl of washed platelets (2×10^9/ml) were resuspended in RCD containing 2 mM calcium, and stimulated with 7 μM TRAP for 1–2 min or 200 μg/ml of the LIBS antibody, D3, at room temperature for 30 min. Samples were then diluted into 500 μl of RCD, pH 7.4, incubated with 40 μg/ml FITC–PAC-1 or FITC-S12, and analyzed by flow cytometry. LIBS antibody binding was evaluated by measuring the binding of D3, anti-LIBS6, or AP3, as described above, in the presence or absence of 1 mM RGDW peptides.

Surface expression of $\alpha_{IIb}\beta_3$ on transfected Chinese hamster ovary (CHO) cells was evaluated by incubating $3–5 \times 10^5$ cells in PBS containing 0.5% (wt/vol) BSA and 0.01% (wt/vol) NaN_3 with 20 μg/ml AP3, Tab, AP2 or isotype control antibodies for 1 h at room temperature. The cells then were washed twice with PBS, incubated with FITC-conjugated $F(ab')_2$ fragment of goat anti–mouse IgG for 30 min, washed, resuspended into PBS, and assayed by flow cytometry. LIBS expression in the presence or absence of RGD peptides was evaluated as described above.

Nucleotide sequence analysis of α_{IIb} and β_3 from genomic DNA and platelet mRNA. PCR amplification of genomic DNA isolated from whole blood, or mRNA isolated from human platelets, was performed as described previously (55) using paired sets of primers designed to amplify all 30 exons and immediate flanking sequences of the α_{IIb} gene, and all 14 exons and immediate flanking sequences of β_3 based on published genomic DNA sequences (56, 57). Amplified DNA fragments were purified and subjected to direct cycle sequence analysis using an Applied Biosystems Automated sequencer according to the manufacturer's directions.

Site-directed mutagenesis of β_3 cDNA. A single nucleotide substitution corresponding to that found in patient RM's β_3 gene was introduced, together with two single nucleotide mismatched primers, into full length β_3 cDNA in the vector pGEM-7zf(+) (Promega Corp., Madison, WI) using the transformer site-directed mutagenesis kit (Clontech, Palo Alto, CA). One nucleotide mismatched primer (5′-ATGCTCCTCTCGACTCGAGG-3′), which abolishes a unique XbaI site on pGEM-7zf(+), was used for positive selection of mutated clones. The other mismatched primer corresponding to the β_3 cDNA sequence from base 2,255 to 2,281 (5′-CACCATCCACGAC*T*GAAAAGAATTTGC-3′) was used to construct the β_3Arg724Ter form of β_3. Mutant clones were verified by DNA sequence analysis. β_3 cDNA inserts were excised from pGEM-7zf(+) with EcoRI and ligated into the EcoRI cloning site of two different mammalian expression vectors; EMC-3, which contains a methotrexate resistance gene (gift of Dr. Glenn Larsen, Genetics Institute, Boston, MA), and pcDNA3, which contains a neomycin resistance gene (Invitrogen Corp., San Diego, CA).

Cell culture and transfection. COS-7 cells were maintained in DME containing 10% FCS, 2 mM glutamine, and 50 μg/ml Gentamicin at

37°C in 5% CO_2. COS-7 cells were transiently transfected with α_{IIb} and β_3 cDNAs using the DEAE-dextran method (55). 72 h after transfection, surface-expressed $\alpha_{IIb}\beta_3$ was labeled with biotin and analyzed by immunoprecipitation (see below). For the production of stable transfectants, CHO cells were cultured in alpha minimum essential media with ribo- and deoxyribo-nucleotides containing 10% FCS, and transfected using the calcium phosphate method according to standard procedures (58). 48 h after transfection, cells were subjected to drug selection in media containing 500–600 μg/ml G418 (geneticin; GIBCO BRL, Gaithersburg, MD) or alpha minimum essential media without ribo- or deoxyribo-nucleotides, depending on the vector used, for at least 2 wk. Resistant clones were isolated using cloning cylinders and assayed for expression of $\alpha_{IIb}\beta_3$ by flow cytometry using anti-$\alpha_{IIb}\beta_3$ monoclonal antibodies. To obtain homogeneous populations, cells were sorted in a FACStar (Becton Dickinson, San Jose, CA) using AP2, and maintained in selection media. In some cases, limiting dilution was performed to further select clonal cell lines.

Immunoprecipitation analysis of cell lysates from transfected COS-7 and CHO cells. Transfected cells were surface-labeled with 5 mM NHS-LC-biotin (Pierce Chemical Co., Rockford, IL) in PBS for 30 min at room temperature, and solubilized for 30 min on ice in 20 mM Tris, 100 mM NaCl, 1% Triton X-100, 2 mM PMSF, and 100 μg/ml leupeptin. After centrifugation at 15,000 *g* for 30 min, at 4°C, supernatants were incubated overnight at 4°C with AP3, AP2 or normal mouse IgG. Rabbit anti–mouse IgG was then added, and the immune complexes recovered using protein A Sepharose beads (Pharmacia Fine Chemicals, Uppsala, Sweden). The beads were washed five times before resuspending in reducing SDS-PAGE sample buffer. After electrophoresis and transfer to a PVDF membrane, biotin-labeled immunoprecipitated proteins were visualized by incubation with horseradish peroxidase–conjugated streptavidin and ECL detection, as described previously (52).

Cell adhesion assays. Platelet adhesion to immobilized fibrinogen or immobilized mAb was performed in 100 mm polystyrene plates as described previously (54). Briefly, 100 mm plates were coated for 16 h at 4°C with 100 μg/ml fibrinogen, 10 μg/ml AP2, or 5 mg/ml BSA in PBS. After washing, the plates were blocked with 5 ml of 5 mg/ml BSA for 2 h at room temperature, and washed again with PBS. Human platelets were isolated by gel-filtration from PRP containing 1 μM prostaglandin E_1 and 1 U/ml apyrase (59), and adjusted to a final concentration of 3×10^8/ml in buffer containing 137 mM NaCl, 2.7 mM KCl, 1 mM $MgCl_2$, 5.6 mM glucose, 1 mg/ml BSA, 3.3 mM NaH_2PO_4, and 20 mM Hepes, pH 7.4. 1 ml of platelets was added to the protein-coated plates and incubated for 90 min at room temperature. After aspiration of unbound platelets, adherent cells were washed three times with 5 ml of ice-cold PBS containing 1 mM Na_3VO_4. Adherent platelets were lysed in RIPA buffer at 4°C for 30 min, and quantitated by measuring total protein using the BCA protein assay (Pierce Chemical Co.). Nonadherent platelets that had been incubated with BSA-coated plates were treated with 5 U/ml apyrase for 5 min at room temperature to minimize platelet activation, washed briefly in PBS at 12,000 *g* for 3 s to remove BSA present in the resuspension buffer, lysed in RIPA buffer, and quantified using the BCA assay as described above.

CHO cell adhesion assays were performed using vital-dye–labeled cells as described previously with slight modifications (60). 96-well tissue culture plates were coated overnight at 4°C with 0.1 ml of PBS containing varying concentrations of fibrinogen, 10 μg/ml AP2, or 1% BSA. The wells were then washed twice with PBS and blocked with 1% BSA in PBS at room temperature for 1 h. Transfected CHO cells were washed twice with serum-free media and labeled with 2 μM calcein-AM (Molecular Probes, Inc., Eugene, OR) at 37°C for 30 min. After washing with PBS, cells were counted and suspended in serum-free media at a concentration of $1–2 \times 10^6$/ml. 0.1 ml of cells was added to each well and incubated for 60 min at 37°C. Unbound cells were removed by washing three times with media, and adherent cells in 0.2 ml media were measured using a microplate fluorescence reader (CytoFluor II; PerSeptive Biosystems, Cambridge, MA) at an excitation wavelength 485 nm and an emission wavelength 530 nm. All experiments were performed in triplicate and repeated at least three times. Specificity of adhesion was verified by pretreating cells with AP2 or anti–PECAM-1 antibody at RT for 30 min, washing, and adding to ligand-coated wells.

Results

Patient RM has a variant form of Glanzmann thrombasthenia. To estimate the total cellular content of $\alpha_{IIb}\beta_3$ in the platelets of Glanzmann thrombasthenic patient RM, we performed immunoblot analysis using a mixture of well-characterized polyclonal antibodies specific for the α_{IIb} and β_3 integrin subunits. As shown in Fig. 1 *A*, RM platelets contain ~ 35–50% of $\alpha_{IIb}\beta_3$ found in normal control platelets. Expression of an unrelated surface glycoprotein, PECAM-1, was similar in RM and control lysates (not shown). Flow cytometric analysis revealed similarly reduced levels of $\alpha_{IIb}\beta_3$ on the surface of RM platelets (Fig. 1 *B*). α_{IIb} and β_3 appeared to associate to form a normal integrin complex, as the binding of the complex-specific mAb, AP2, was proportional to the level of surface expression, and addition of EDTA did not induce premature dissociation of the subunits at 22°C (not shown). Together, these data suggest that RM has a variant form of thrombasthenia in which significant levels of a stable, but dysfunctional $\alpha_{IIb}\beta_3$ complex is present on the platelet surface.

RM $\alpha_{IIb}\beta_3$ fails to adopt a ligand-binding competent conformation in response to platelet activation. To further evaluate the adhesive properties of $\alpha_{IIb}\beta_3$ on RM platelets, we measured the binding of the fibrinogen mimetic antibody, PAC-1, which binds to, and competes with fibrinogen for, the exposed ligand-binding domain on the activated $\alpha_{IIb}\beta_3$ complex (48). Though the strong platelet agonist, TRAP, induced normal platelet activation and α-granule secretion, as monitored by the expression of P-selectin on the cell surface (Fig. 2 *A*, *bottom panel*), little or no binding of PAC-1 to RM platelets was observed. To distinguish between a molecular defect in the extracellular domain of $\alpha_{IIb}\beta_3$ versus a failure of the complex to become activated from within the cell, we examined the ability of RGD peptides to engage the ligand binding pocket of $\alpha_{IIb}\beta_3$ and induce LIBS epitopes (47). As shown in Fig. 2 *B*, RGDW significantly enhanced the binding of two different LIBS-specific antibodies, D3 and anti-LIBS6, to RM $\alpha_{IIb}\beta_3$. In addition, PAC-1 binding could be induced by preincubating RM platelets with D3 (Fig. 2 *C*). The observation that RM $\alpha_{IIb}\beta_3$ (1) undergoes conformational change in response to RGD peptides and (2) binds large macromolecular ligand-mimetic antibodies when activated from outside the cell is consistent with the presence of either a mutation within the cytoplasmic domain of α_{IIb} or β_3 that abrogates affinity modulation, or a defect in one of the components of the signaling pathway that regulates the adhesive state of the $\alpha_{IIb}\beta_3$ complex.

The cytoplasmic domain of the RM integrin β_3 subunit is truncated. Direct DNA sequence analysis of PCR-amplified α_{IIb} and β_3 genes from patient RM genomic DNA revealed a heterozygous C2268T mutation within exon 13 of the β_3 gene that results in an Arg724Ter codon (Fig. 3 *A*). DNA sequence analysis of β_3 exon 10 revealed a second heterozygous mutation, deletion of T_{1811}, leading to a frameshift and termination at amino acid 642 (not shown). Each of these mutations were confirmed by sequencing individually subcloned PCR prod-

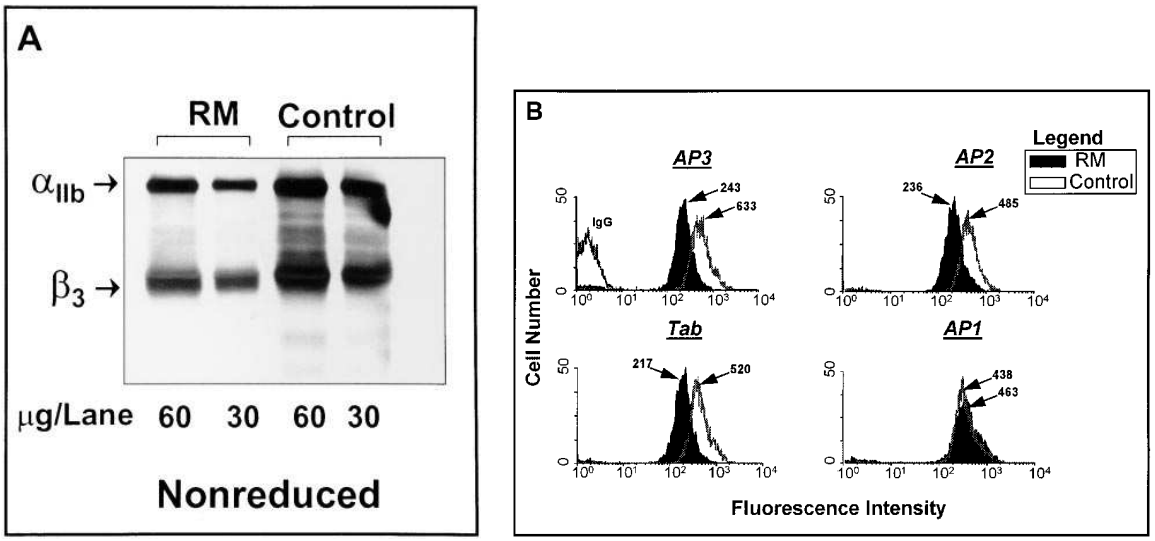

Figure 1. Expression of $\alpha_{IIb}\beta_3$ in Glanzmann thrombasthenic patient RM platelets. (*A*) Total cellular $\alpha_{IIb}\beta_3$ was estimated by preparing detergent lysates from normal control and patient RM platelets, and by analyzing the indicated amount of protein by SDS-PAGE/immunoblot using a mixture of rabbit polyclonal antibodies specific for α_{IIb} and β_3. By this method, RM platelets appear to contain ~ 30–40% of normal $\alpha_{IIb}\beta_3$ content. (*B*) Surface-expressed $\alpha_{IIb}\beta_3$ was estimated by flow cytometry using monoclonal antibodies specific for β_3 (*AP3*), α_{IIb} (*Tab*), or the intact $\alpha_{IIb}\beta_3$ complex (*AP2*). Surface-bound antibody was detected using FITC-conjugated goat anti–mouse IgG. RM platelets expressed ~ 35–50% of α_{IIb} and β_3 on the cell surface compared to normal control platelets. Surface expression of GPIb on both RM and control platelets was similar, as shown by the binding of the antibody, AP1. A subclass-matched mouse IgG_1 monoclonal antibody (IgG) was used to establish background binding.

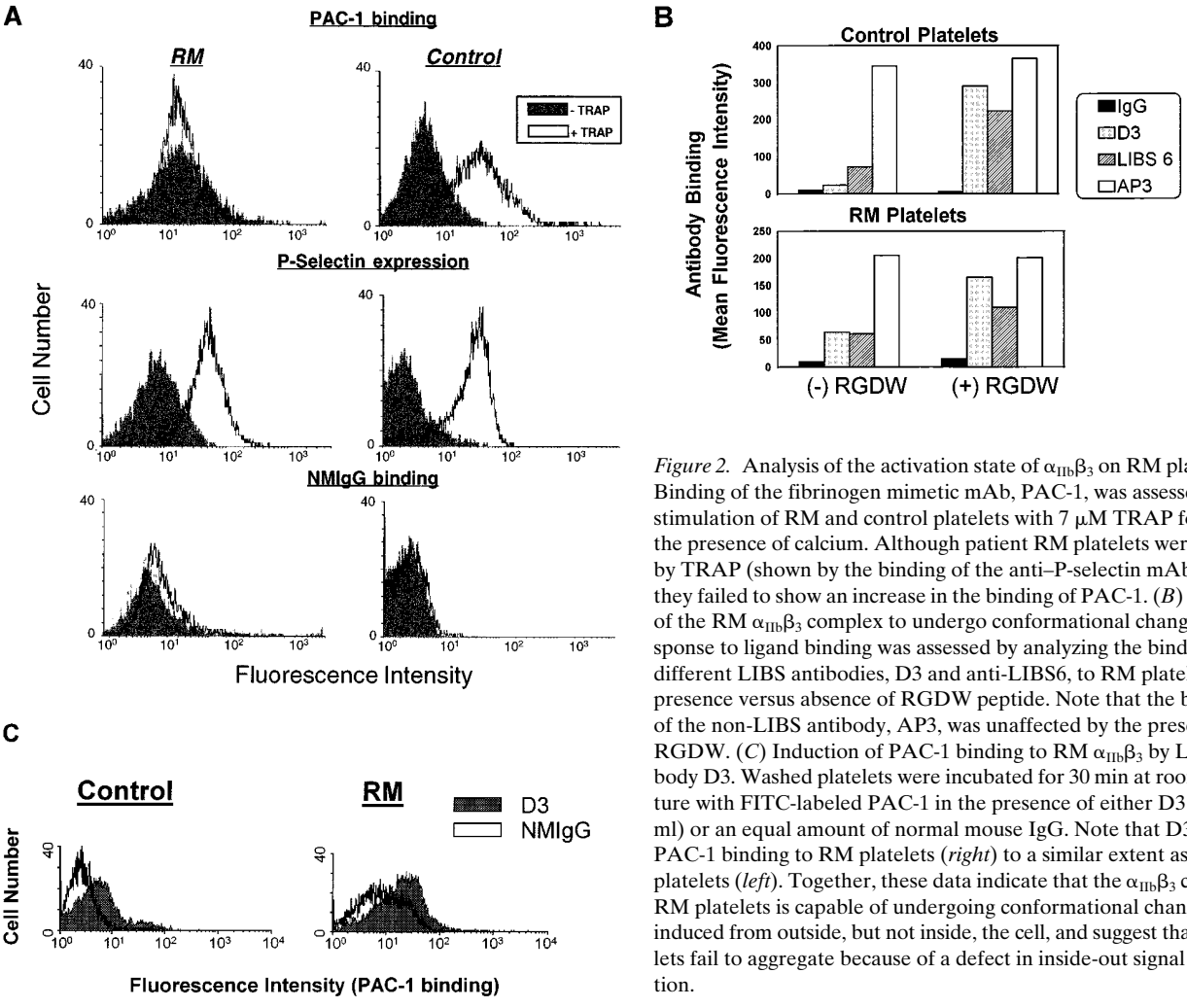

Figure 2. Analysis of the activation state of $\alpha_{IIb}\beta_3$ on RM platelets. (*A*) Binding of the fibrinogen mimetic mAb, PAC-1, was assessed after stimulation of RM and control platelets with 7 μM TRAP for 1 min in the presence of calcium. Although patient RM platelets were activated by TRAP (shown by the binding of the anti–P-selectin mAb, S-12), they failed to show an increase in the binding of PAC-1. (*B*) The ability of the RM $\alpha_{IIb}\beta_3$ complex to undergo conformational changes in response to ligand binding was assessed by analyzing the binding of two different LIBS antibodies, D3 and anti-LIBS6, to RM platelets in the presence versus absence of RGDW peptide. Note that the binding of the non-LIBS antibody, AP3, was unaffected by the presence of RGDW. (*C*) Induction of PAC-1 binding to RM $\alpha_{IIb}\beta_3$ by LIBS antibody D3. Washed platelets were incubated for 30 min at room temperature with FITC-labeled PAC-1 in the presence of either D3 (200 μg/ml) or an equal amount of normal mouse IgG. Note that D3 induced PAC-1 binding to RM platelets (*right*) to a similar extent as to control platelets (*left*). Together, these data indicate that the $\alpha_{IIb}\beta_3$ complex on RM platelets is capable of undergoing conformational changes when induced from outside, but not inside, the cell, and suggest that his platelets fail to aggregate because of a defect in inside-out signal transduction.

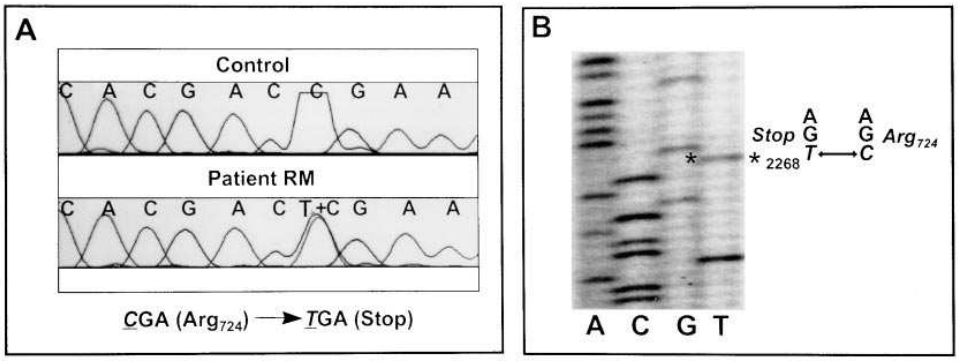

Figure 3. DNA sequence analysis of α_{IIb} and β_3 from RM genomic DNA. All exons, including the immediate intron–exon junctions, of both α_{IIb} and β_3 genes were amplified using PCR primer pairs originating in the intron sequence flanking each exon. The resulting PCR fragments were purified and subjected to direct cycle sequencing. The nucleotide sequence of exon 13 of the RM β_3 gene (*A*) reveals a C2268T heterozygous mutation (*arrow*) that results in Arg724Stop mutation in one allele. This nucleotide substitution was confirmed by manually sequencing a subclone of the exon 13 PCR product (*B*). PCR amplification of RM platelet mRNA, followed by sequence analysis (not shown), revealed that the C2268T mutation was homozygous at the RNA level, indicating that the other allele was silent. Nucleotide numbering is based on the cDNA sequence.

ucts. Amplification and analysis of β_3 platelet mRNA from nucleotides 1,666 to 2,420, a region that contains both mutations, indicated that only the allele encoding β_3Arg724Ter was present at the RNA level (Fig. 3 *B*). Whether the allele encoding ΔT_{1811} was not found at the RNA level because of poor transcription, or because of the production of an unstable transcript, was not determined. No mutations were found in the α_{IIb} gene. Genotypic analysis of RM's mother (not shown) revealed that she is a heterozygous carrier of the β_3Arg724 allele (her second other allele is wild type). Importantly, the β_3Arg724Ter allele encodes a truncated β_3 subunit, containing only eight membrane-proximal amino acid residues out of 47 that normally constitute the β_3 cytoplasmic domain (Fig. 4).

β_3Arg724Ter associates with α_{IIb}, traffics to the cell surface, and adopts a resting conformational state. To examine whether truncation of the β_3 cytoplasmic domain is responsible for the RM phenotype, an expression vector encoding the β_3Arg724Ter cDNA was constructed using site-directed mutagenesis and cotransfected with wild-type α_{IIb} cDNA into COS-7 cells. 72 h after transfection, surface-expressed $\alpha_{IIb}\beta_3$ was analyzed using a series of $\alpha_{IIb}\beta_3$-specific monoclonal antibodies. As shown in Fig. 5 *A*, α_{IIb} associated normally with β_3Arg724Ter, as shown by the ability of AP3 (β_3-specific) to coimmunoprecipitate α_{IIb}, and by the ability of the heterodimer to be recognized by the complex-dependent mAb, AP2. Flow cytometric analysis of stably-transfected CHO cell lines confirmed that α_{IIb} and β_3Arg724Ter form an integrin complex that is expressed normally on the cell surface (Fig. 5 *B*). These data are consistent with previous observations that the truncation of the β_3 cytoplasmic domain does not affect surface expression of recombinant $\alpha_{IIb}\beta_3$ (8, 33, 37), and suggest that the reduced level $\alpha_{IIb}\beta_3$ on the surface of RM platelets likely results from normal to near normal expression of the β_3Arg724Ter allele, coupled with failure of second allele to produce detectable levels of β_3. Incubation of CHO cells expressing recombinant β_3Arg724Ter with the activating antibody, D3, resulted in significantly increased PAC-1 binding, demonstrating that this truncated integrin is capable of exposing its ligand-binding site when engaged from outside the cell (Fig. 5 *C*). These results are similar to those obtained using RM platelets (see Fig. 2 *C*), and confirm that the ligand binding site in RM $\alpha_{IIb}\beta_3$ is intact.

Adhesive properties of recombinant $\alpha_{IIb}\beta_3$Arg724Ter. Previous studies have shown that CHO cells or platelets expressing wild-type $\alpha_{IIb}\beta_3$ can adhere to immobilized fibrinogen, even in the absence of previous cellular activation (61). As shown in Fig. 6 *A*, $\alpha_{IIb}\beta_3$Arg724Ter transfectants bound fibrinogen in a dose-dependent manner, and with the same efficiency as wild-type $\alpha_{IIb}\beta_3$ transfectants. Cell adhesion to fibrinogen was $\alpha_{IIb}\beta_3$-mediated, since it could be blocked by preincubating the cells with the anti-$\alpha_{IIb}\beta_3$ antibody, AP2 (Fig. 6 *B*). Unlike the wild-type transfectants, however, CHO cells expressing $\alpha_{IIb}\beta_3$Arg724Ter did not spread on either fibrinogen- or antibody-coated surfaces (Fig. 6 *C*), suggesting that integrin/cytoskeletal associations are necessary for cell spreading, and do not occur in the absence of the β_3 cytoplasmic domain.

Truncation of the β_3 cytoplasmic domain affects both cytoskeletal associations and transmembrane signaling in human platelets. To date, studies on the role of integrin cytoplasmic domains in outside-in signal transduction and cellular spreading have necessarily been performed using transfected cell lines expressing recombinant integrin constructs lacking one or

β_1	KLLMIIHDRREFAKFEKEKMNAKWDTGE *NPIY* KSAVTTVV *NPKY* EGK
β_2	KALIHLSDLREYRRFEKEKLKSQWNND- *NPLF* KSATTTVM *NPKF* AES
β_3	KLLITIHDRKEFAKFEEERARAKWDTAN *NPLY* KEATSTFT *NITY* RGT
RM β_3Arg724Ter	KLLITIHD

Figure 4. Comparison of cytoplasmic domain of the RM β_3 subunit with integrin β_1, β_2, and β_3 subunits. Note that the RM β_3 subunit cytoplasmic domain has only 8 of 47 amino acids normally present in the cytoplasmic domain, and is missing, among other things, the highly-conserved NPXY and NXXY motifs (italics).

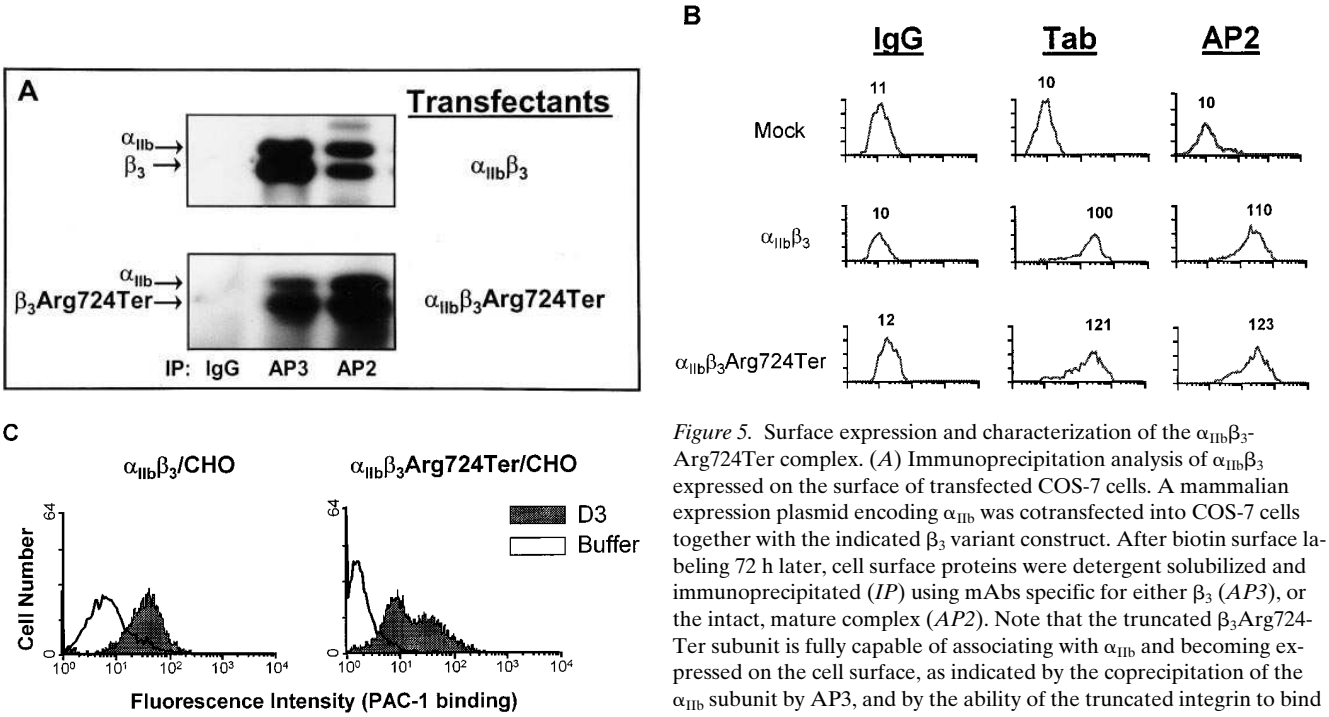

Figure 5. Surface expression and characterization of the $\alpha_{IIb}\beta_3$-Arg724Ter complex. (*A*) Immunoprecipitation analysis of $\alpha_{IIb}\beta_3$ expressed on the surface of transfected COS-7 cells. A mammalian expression plasmid encoding α_{IIb} was cotransfected into COS-7 cells together with the indicated β_3 variant construct. After biotin surface labeling 72 h later, cell surface proteins were detergent solubilized and immunoprecipitated (*IP*) using mAbs specific for either β_3 (*AP3*), or the intact, mature complex (*AP2*). Note that the truncated β_3Arg724-Ter subunit is fully capable of associating with α_{IIb} and becoming expressed on the cell surface, as indicated by the coprecipitation of the α_{IIb} subunit by AP3, and by the ability of the truncated integrin to bind the complex-specific mAb AP2. (*B*) $\alpha_{IIb}\beta_3$ expressed on the surface of stably-transfected CHO cell lines was measured flow cytometrically using the mAbs Tab (specific for α_{IIb}) or AP2 (specific for the intact, mature integrin complex). Preimmune normal mouse IgG was used as a control. Cloned CHO cell lines expressing equivalent amounts of $\alpha_{IIb}\beta_3$ or $\alpha_{IIb}\beta_3$Arg724Ter were chosen for this and subsequent analyses. (*C*) Binding of the activation-dependent fibrinogen-mimetic antibody, PAC-1, to the wild-type and RM $\alpha_{IIb}\beta_3$ complex. Transfected CHO cells were incubated with FITC-labeled PAC-1 in the presence or absence of 200 μg/ml of the LIBS mAb, D3, for 30 min at 37°C, and then analyzed by FACS. D3 increased the binding of FITC-labeled PAC-1 to CHO cells expressing wild-type $\alpha_{IIb}\beta_3$, as well as the mutant $\alpha_{IIb}\beta_3$Arg724Ter complex. These data indicate that the $\alpha_{IIb}\beta_3$Arg724Ter truncated integrin is fully capable of undergoing conformational change from a resting to an active, ligand-binding state.

more cytoplasmic domain components (see above, and references 8, 30, 31, 35). To study the effects of β_3 integrin truncation on cytoskeletally-directed cellular spreading and outside-in signaling in intact human platelets, we compared the ability of normal versus RM platelets to (1) bind to and spread on immobilized fibrinogen, and (2) tyrosine phosphorylate the focal adhesion kinase, pp125^{FAK}. As shown in Fig. 7, tissue culture plates containing immobilized fibrinogen or AP2 bound approximately the same number of platelets derived from Glanzmann patient RM or his mother (an obligate carrier), but whereas platelets from RM's mother went on to spread normally, RM platelets remained round and failed to spread, even after 90 min.

Previous studies have shown that adhesion of platelets to fibrinogen induces cell spreading and tyrosine phosphorylation of pp125^{FAK} (26). To determine whether platelets lacking amino acids 724–762 of the cytoplasmic domain of β_3 could, in the absence of platelet spreading, tyrosine phosphorylate pp125^{FAK}, platelets bound to AP2- or fibrinogen-coated wells were detergent solubilized, and pp125^{FAK} was recovered by immunoprecipitation. As shown in the lower panel of Fig. 8, although pp125^{FAK} was present at approximately equal levels in RM and control (mother's) platelets, it failed to become tyrosine phosphorylated in adherent RM platelets (Fig. 8, *top panel*). These data demonstrate that the cytoplasmic domain of the β_3 integrin subunit is required for at least two downstream events that take place after platelet adhesion to immobilized $\alpha_{IIb}\beta_3$ ligands: cell spreading and phosphorylation of pp125^{FAK}.

Discussion

The integrin $\alpha_{IIb}\beta_3$ plays a central role in platelet aggregation by serving as an adhesion receptor for fibrinogen and vWf. In addition to its role in mediating cell–cell and cell–matrix interactions, $\alpha_{IIb}\beta_3$ has been implicated recently in bidirectional transmembrane signaling. To further define the multiple functional roles that this integrin might play in platelet pathophysiology, we have taken the approach of identifying and characterizing naturally occurring mutations associated with Glanzmann thrombasthenia, an inherited bleeding disorder characterized by the failure of platelets to aggregate in response to physiological agonists (62). Examination of the biochemical and cell biological consequences of mutations responsible for this inherited integrin defect has historically provided important insights into the specific cellular functions mediated by integrins in vivo (40). Though mutations in other proteins that participate in metabolic or signaling pathways that enable platelet aggregation could theoretically result in the thrombasthenic phenotype (63), the molecular defects in all Glanzmann thrombasthenic patients examined to date have been found in the genes encoding either α_{IIb} or β_3 (39). Thus far, α_{IIb} and β_3 mutations have been identified that lead to defects in mRNA splicing (64–67), mRNA stability (68), divalent

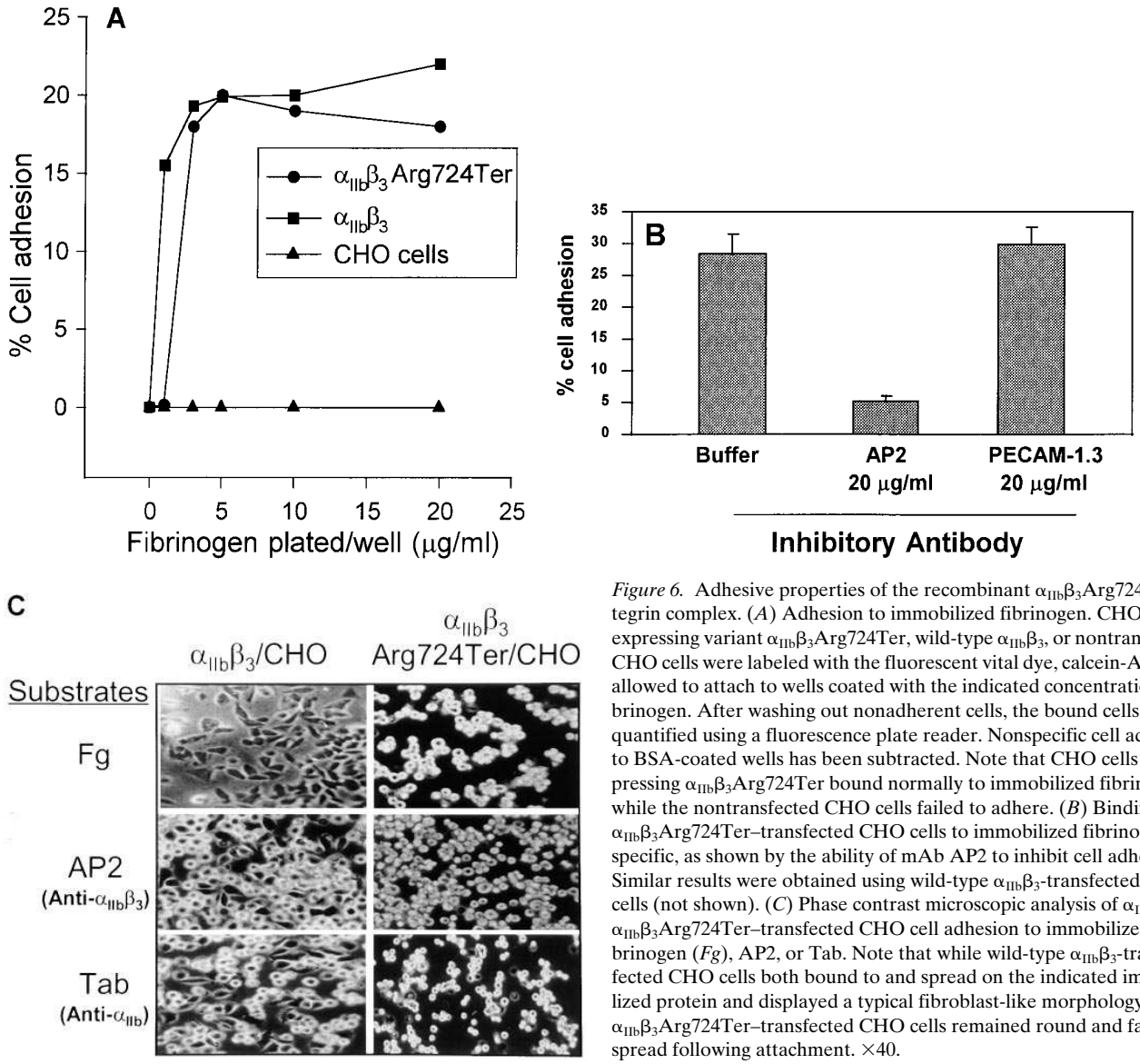

Figure 6. Adhesive properties of the recombinant $\alpha_{IIb}\beta_3$Arg724Ter integrin complex. (*A*) Adhesion to immobilized fibrinogen. CHO cells expressing variant $\alpha_{IIb}\beta_3$Arg724Ter, wild-type $\alpha_{IIb}\beta_3$, or nontransfected CHO cells were labeled with the fluorescent vital dye, calcein-AM, and allowed to attach to wells coated with the indicated concentration of fibrinogen. After washing out nonadherent cells, the bound cells were quantified using a fluorescence plate reader. Nonspecific cell adhesion to BSA-coated wells has been subtracted. Note that CHO cells expressing $\alpha_{IIb}\beta_3$Arg724Ter bound normally to immobilized fibrinogen, while the nontransfected CHO cells failed to adhere. (*B*) Binding of $\alpha_{IIb}\beta_3$Arg724Ter–transfected CHO cells to immobilized fibrinogen is specific, as shown by the ability of mAb AP2 to inhibit cell adhesion. Similar results were obtained using wild-type $\alpha_{IIb}\beta_3$-transfected CHO cells (not shown). (*C*) Phase contrast microscopic analysis of $\alpha_{IIb}\beta_3$- or $\alpha_{IIb}\beta_3$Arg724Ter–transfected CHO cell adhesion to immobilized fibrinogen (*Fg*), AP2, or Tab. Note that while wild-type $\alpha_{IIb}\beta_3$-transfected CHO cells both bound to and spread on the indicated immobilized protein and displayed a typical fibroblast-like morphology, $\alpha_{IIb}\beta_3$Arg724Ter–transfected CHO cells remained round and failed to spread following attachment. ×40.

cation binding (69–71), subunit association (72–74), intracellular trafficking (75), ligand binding (76), and integrin-mediated signal transduction (77, 78).

In the past several years, numerous studies using cultured hematopoietic, fibroblastic, and epithelial cells have clearly documented that inside-out integrin signaling is a major mechanism by which cell–cell and cell–extracellular matrix adhesion can be regulated. These same experimental systems have also been instrumental in highlighting the potential role of outside-in signaling through various integrins in the control of cell motility, growth, survival, and programmed cell death (for review see reference 79). The current study of the cell biological effects of a rare disease reinforces the notion that bidirectional integrin signaling is of physiological significance by documenting the hemostatic consequences of an integrin structural defect that presumably leads to deficient interactions of $\alpha_{IIb}\beta_3$ with the cytoskeletal and signaling machinery of the platelet.

Flow cytometric and Western blot analysis of platelets from RM, a young boy with a life-long history of easy bruising and recurrent mucosal bleeding, revealed that he has a variant form of Glanzmann thrombasthenia, expressing 40–50% of the normal level of α_{IIb} β_3 on the platelet surface (Fig. 1). Despite the relatively large number of fibrinogen receptors present, RM platelets failed to aggregate in response to all physiological agonists used, and did not bind to the activation-dependent fibrinogen mimetic antibody, PAC-1, upon platelet activation (Fig. 2 *A*). However, RM $\alpha_{IIb}\beta_3$, like wild-type $\alpha_{IIb}\beta_3$, underwent normal conformational changes in its extracellular region in response to either small RGD-containing peptides or LIBS antibodies, and could be induced from outside the cell by a LIBS antibody to bind PAC-1 (Fig. 2, *B* and *C*). Together, these data suggest that the ligand binding site of the $\alpha_{IIb}\beta_3$ complex on RM platelets is functionally intact, but that the integrin fails to respond to intracellular signals and adopt an active, ligand binding conformation.

To identify the molecular abnormality responsible for the inside-out integrin signaling defect in RM platelets, we examined the entire coding sequence of RM α_{IIb} and β_3 genes and found a heterozygous mutation in exon 13 of β_3: one of RM's alleles containing a C→T point mutation at base 2,268 of β_3

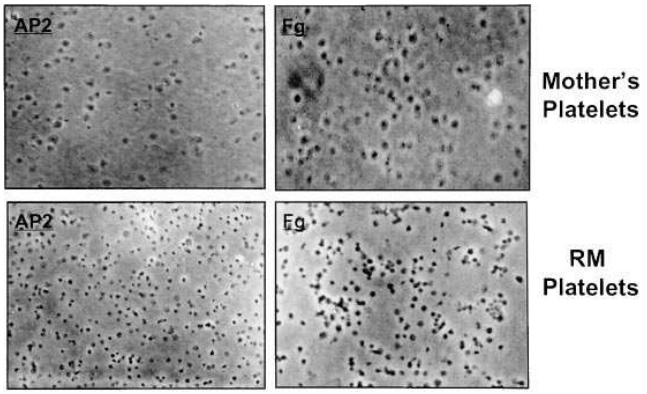

Figure 7. Patient RM platelets adhere to, but fail to spread on, immobilized fibrinogen. Washed platelets were added to fibrinogen- or AP2-coated plates for 90 min at room temperature. After washing away unbound platelets, the adherent platelets were examined by phase microscopy. Note that RM platelets bound, but failed to spread and adopt the typical fried egg appearance seen in the platelets from his mother (an obligate thrombasthenic carrier). ×60.

cDNA (Fig. 3). This point mutation results in a stop codon at amino acid residue 724, leading to a truncated form of β_3 with a cytoplasmic domain of only eight amino acids, instead of the normal 47 residues (Fig. 4). The other allele of RM was found to have a single base deletion within exon 10 at the genomic level, which results in a frame shift and termination within the same exon. This allele was incapable of producing a β_3 protein, as evidenced by the fact that only the allele encoding β_3Arg724Ter was present at the mRNA level. The decreased level of $\alpha_{IIb}\beta_3$ present on RM platelets (50–60% of normal), therefore, is most likely due to normal expression of a single allele encoding the β_3Arg724Ter form of GPIIIa—a supposition that was confirmed by our finding that recombinant β_3Arg724Ter associates with α_{IIb} in CHO cells stably transfected with both subunits, and is expressed at normal levels on the cell surface (Fig. 5).

Approximately 20% of $\alpha_{IIb}\beta_3$ is known to become associated with the cytoskeleton in thrombin-aggregated platelets (80–82), and several cytoskeletal and signaling proteins have been shown to interact with integrin β-subunit cytoplasmic domains, including paxillin and pp125^{FAK} (83), α-actinin (84), talin (85, 86), and the recently identified cytosolic integrin-binding proteins, cytohesin-1 (87) and β_3-endonexin (88). Fox et al. (89) have shown that the interaction of the $\alpha_{IIb}\beta_3$ complex with the underlying cytoskeleton, which is likely defective in RM platelets, stabilizes receptor-ligand interactions, and has proposed that activation-induced association of $\alpha_{IIb}\beta_3$ with the membrane skeleton and cytosolic signaling molecules represents an important step in regulating integrin-mediated cytoskeletal reorganization and subsequent cell spreading (90). In addition, the intrinsic activation state of the $\alpha_{IIb}\beta_3$ integrin complex may be controlled, in part, by interaction of α_{IIb} and β_3 cytoplasmic tails with each other. Haas and Plow (91) have shown, using terbium luminescence spectroscopy, that residues at the acidic carboxy terminus of α_{IIb} form a ternary complex involving divalent cation and amino acids 721–740 of the β_3 cytoplasmic domain—residues that are missing in RM β_3. Support for the existence of a salt bridge between the α_{IIb} and β_3 cytoplasmic domains has also been provided by the work of Hughes et al. (36), who found that disrupting charged residues within the membrane-proximal regions of the α_{IIb} and β_3 cytoplasmic domains alters the affinity state of the complex and affects downstream integrin-mediated functions, including tyrosine phosphorylation of pp125^{FAK}, cell spreading, and extracellular matrix assembly. Based on these observations, it is likely that the failure of RM platelets to carry out these functions, and ultimately his hemostatic defect, is due to the loss of the cytoplasmic domain of β_3.

Though the contribution of integrin cytoplasmic domains to cell adhesion, cytoskeletal reorganization, and signaling has been examined extensively in cultured cell lines expressing recombinant integrin subunits, there has been little opportunity to examine their function within the context of a primary human cell. Platelets from a thrombasthenic patient having a Ser752Pro point mutation within the cytoplasmic domain of β_3 failed to bind soluble fibrinogen after stimulation with either ADP or thrombin (77), but subsequent studies on the effects of this integrin β_3 cytoplasmic domain mutation on cytoskeletal interactions and signal transduction have been performed

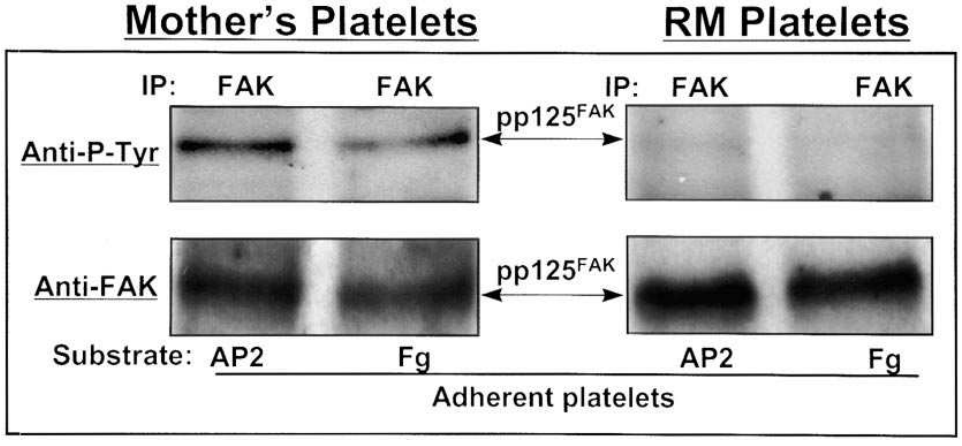

Figure 8. RM platelets fail to phosphorylate pp125^{FAK}. Platelets bound to AP2- or fibrinogen-coated wells (prepared as described in Fig. 7) were lysed in RIPA buffer and immunoprecipitated using 2 μl of a polyclonal antibody against FAK. FAK immunoprecipitates were resolved by SDS-PAGE, transferred to a PVDF membrane, and probed with either PY20 to assess the tyrosine phosphorylation state of platelet FAK (*top*) or anti-FAK antibody to assess antigen load (*bottom*). Note that RM platelets do not phosphorylate FAK after adhesion to either immobilized AP2 or immobilized fibrinogen. TRAP-induced phosphorylation of FAK in RM platelets in solution was also dramatically reduced (not shown).

using transfected cells. O'Toole et al. (8) showed that the Ser752Pro mutation or deletion of β_3 residues 724–762 resulted in loss of PAC-1 binding in transfected CHO cells, and found that the cytoplasmic domains of both α_{IIb} and β_3 were required for energy-dependent, cell type–specific affinity modulation, while Chen et al. (35) showed that introduction of the Ser752Pro form of β_3 into cells together with a constitutively active chimeric integrin α-subunit abolished ligand binding and reduced $\alpha_{IIb}\beta_3$-mediated cell spreading on immobilized fibrinogen, clot retraction, and focal adhesion formation. Loh et al. (37) similarly found that adherence to immobilized fibrinogen of a PMA-treated, EBV-transformed lymphocyte cell line expressing $\alpha_{IIb}\beta_3$Ser752Pro or $\alpha_{IIb}\beta_3$Arg724Ter was diminished significantly compared to wild-type control $\alpha_{IIb}\beta_3$, supporting the notion that the β_3 cytoplasmic domain is involved in integrin activation. More recently, Wu et al. (92) added fibrinogen matrix assembly to the list of postoccupancy events that require an intact β_3 cytoplasmic domain. In light of the fact that β_3Arg724Ter is likely to be expressed not only complexed with α_{IIb} on the platelet surface, but also in association with the α-subunit of the vitronectin receptor, it would be of future interest to examine the effect of this β_3 cytoplasmic domain truncation on bidirectional transmembrane signal transduction in a second primary cell, the endothelial cell.

There are data to suggest that the function of truncated integrins in transfected cells may differ significantly from that which occurs in their natural host cell. For example, Hibbs et al. (32) showed that transfected COS cells expressing a truncated form of the leukocyte integrin, $\alpha_L\beta_2$, bound appreciably to immobilized ICAM-1, whereas EBV-transformed human B cells expressing the same truncated integrin demonstrated no ICAM-1 binding ability. Similar cell type–specific influences have been noted previously for β_1 and β_3 integrins (8, 32, 38). Of direct relevance to this study, both we (Fig. 6) and Leong et al. (31) have shown that CHO cells expressing recombinant $\alpha_{IIb}\beta_3$Arg724Ter bind to, but fail to spread on, immobilized fibrinogen. Human platelets expressing $\alpha_{IIb}\beta_3$Arg724Ter exhibit the same behavior (Fig. 7), and establish that the cytoplasmic domain of β_3 is required for platelet spreading on immobilized fibrinogen. However, whereas adherent CHO cells expressing $\alpha_{IIb}\beta_3$Arg724Ter were able to phosphorylate $pp125^{FAK}$ to ~ 50–60% of its normal level (31), human platelets expressing this same truncated integrin complex retain virtually no ability to carry out $pp125^{FAK}$ tyrosine phosphorylation (Fig. 8), despite retention of the membrane-proximal region of the β_3 cytoplasmic domain that has been shown, using synthetic peptides that mimic β integrin cytoplasmic domains, to interact with $pp125^{FAK}$ (83). Taken together, we speculate that CHO cells possess one or more compensatory alternate pathways between integrin engagement and $pp125^{FAK}$ that permit its tyrosine phosphorylation—even when expressing only a truncated form of the $\alpha_{IIb}\beta_3$ complex—perhaps because of the fact that CHO cells are a fibroblast-derived cell line that expresses abundant levels of other (β_1) integrins. The differing signaling responses found in RM platelets, versus those present in $\alpha_{IIb}\beta_3$Arg724Ter CHO cells or inferred from the use of in vitro systems, reinforce the notion that the biochemical and cellular consequences of integrin mutations in transfected cells in culture are not automatically representative of events that occur in primary cells. Opportunities afforded by the ability to examine integrin function within the context of its normal cytosolic environment are likely to continue to provide complementary insights into the multiple functional roles that these adhesion and signaling receptors play in vascular cell biology.

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