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Ser-752 → Pro mutation in the cytoplasmic domain of integrin β_3 subunit and defective activation of platelet integrin $\alpha_{IIb}\beta_3$ (glycoprotein IIb-IIIa) in a variant of Glanzmann thrombasthenia

(molecular genetics/fibrinogen receptor/Arg-Gly-Asp/polymerase chain reaction)

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ABSTRACT **Integrins are membrane receptors which mediate cell–cell or cell–matrix adhesion. Integrin $\alpha_{IIb}\beta_3$ (glycoprotein IIb-IIIa) acts as a fibrinogen receptor of platelets and mediates platelet aggregation. Platelet activation is required for $\alpha_{IIb}\beta_3$ to shift from noncompetent to competent for binding soluble fibrinogen. The steps involved in this transition are poorly understood. We have studied a variant of Glanzmann thrombasthenia, a congenital bleeding disorder characterized by absence of platelet aggregation and fibrinogen binding. The patient's platelets did not bind fibrinogen after platelet activation by ADP or thrombin, though his platelets contained $\alpha_{IIb}\beta_3$. However, isolated $\alpha_{IIb}\beta_3$ was able to bind to an Arg-Gly-Asp-Ser affinity column, and binding of soluble fibrinogen to the patient's platelets could be triggered by modulators of $\alpha_{IIb}\beta_3$ conformation such as the Arg-Gly-Asp-Ser peptide and α-chymotrypsin. These data suggested that a functional Arg-Gly-Asp binding site was present within $\alpha_{IIb}\beta_3$ and that the patient's defect was not secondary to a blockade of $\alpha_{IIb}\beta_3$ in a noncompetent conformational state. This was evocative of a defect in the coupling between platelet activation and $\alpha_{IIb}\beta_3$ up-regulation. We therefore sequenced the cytoplasmic domain of β_3, following polymerase chain reaction (PCR) on platelet RNA, and found a T → C mutation at nucleotide 2259, corresponding to a Ser-752 → Pro substitution. This mutation is likely to be responsible for the uncoupling of $\alpha_{IIb}\beta_3$ from cellular activation because (*i*) it is not a polymorphism, (*ii*) it is the only mutation in the entire $\alpha_{IIb}\beta_3$ sequence, and (*iii*) genetic analysis of the family showed that absence of the Pro-752 β_3 allele was associated with the normal phenotype. Our data thus identify the C-terminal portion of the cytoplasmic domain of β_3 as an intrinsic element in the coupling between $\alpha_{IIb}\beta_3$ and platelet activation.**

Integrins belong to a supergene family of cell surface receptors which mediate cell–matrix or cell–cell adhesion. Integrins are involved in fundamental biological processes such as development, metastasis, immune response, inflammation, or hemostasis and thrombosis (1, 2). An important feature of some integrins is that their affinity for their ligands can be metabolically regulated, presumably by signal transduction. For example, $\alpha_L\beta_2$ (LFA-1), a leukocyte integrin, binds ICAM-1, a cell adhesion molecule, after T-cell antigen receptor cross-linking (3), and $\alpha_{IIb}\beta_3$, a platelet-specific integrin (4), binds soluble fibrinogen after platelet stimulation (5–7). The mechanisms of integrin activation, though still unclear, seem to require intracellular signaling as shown for $\alpha_{IIb}\beta_3$ (8, 9), as well as for $\alpha_L\beta_2$ (10). At least in the case of $\alpha_{IIb}\beta_3$ activation, modulation of affinity for its ligands is coordinated with conformational changes of the integrin (7, 11). What remains obscure is the exact link between intracellular signals and $\alpha_{IIb}\beta_3$ conformation modulation.

Glanzmann thrombasthenia is a hereditary bleeding disorder characterized by the absence of platelet aggregation due to either an absence or a functional alteration of the integrin $\alpha_{IIb}\beta_3$ (12). In several cases, the genetic basis for the quantitative defect was found to be associated with alterations within the genes of $\alpha_{IIb}\beta_3$ subunits—i.e., either α_{IIb} (glycoprotein IIb) (13, 14) or β_3 (glycoprotein IIIa) (14, 15). In addition, the qualitative defects in $\alpha_{IIb}\beta_3$ found in variants of Glanzmann thrombasthenia have also become the focus of investigations, and two mutations in Glanzmann variants that disrupted ligand binding sites of β_3 have been characterized (16–18). Here we report a variant of Glanzmann thrombasthenia whose biochemical and genetic analyses are consistent with the idea that the functional defect is due to replacement of serine by proline in position 752 in the cytoplasmic domain of β_3 and that this mutation impairs the coupling between cellular activation and up-regulation of $\alpha_{IIb}\beta_3$ affinity for fibrinogen. To our knowledge this is the first point mutation reported affecting integrin activation.

METHODS

Patient's Case. Patient P.'s case of variant Glanzmann thrombasthenia has been previously reported (19). Briefly, patient P. exhibited a life-long bleeding tendency with failure of platelets to aggregate and to bind fibrinogen in response to agonists such as ADP, collagen, or thrombin. The patient's platelets could support clot retraction. The presence of $\alpha_{IIb}\beta_3$ in the patient's platelets was established by immunocytochemistry.

Protein Analysis and Monoclonal Antibody Binding. Blood was collected in the presence of 5 mM EDTA, and platelets were prepared by conventional methods (20). Platelet proteins were solubilized in 2% $NaDodSO_4$ and two-dimensional nonreduced/reduced $NaDodSO_4$/PAGE was performed as described (21). Total platelet proteins (200 μg) were solubilized in 2% $NaDodSO_4$ and separated in a first dimension in a 6% polyacrylamide tube gel. Disulfide bonds were reduced by soaking the gel in 10% (vol/vol) 2-mercaptoethanol, and the proteins were then separated again in a perpendicular direction in a 7–12% polyacrylamide slab gel. Proteins were detected by staining with Coomassie blue-R.

The monoclonal antibody AP-2, which is specific for glycoprotein IIb-IIIa (22), the $\alpha_{IIb}\beta_3$ integrin (a generous gift

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Abbreviations: RGDS, Arg-Gly-Asp-Ser; nt, nucleotide(s).

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of T. J. Kunicki, The Blood Center of Southeastern Wisconsin, Milwaukee, WI), was radioiodinated and its binding to washed platelets was determined as previously reported (23). In some experiments AP-2 binding was carried out on platelets stimulated with thrombin at 0.125 unit/ml for 10 min and the reaction was stopped with hirudin (20).

Arg-Gly-Asp-Ser (RGDS) Affinity Chromatography. Purification of $\alpha_{IIb}\beta_3$ by affinity chromatography on RGDS-Sepharose was performed according to Steiner *et al.* (24). Proteins from 3×10^9 platelets solubilized in 1% Triton X-100 were enriched in $\alpha_{IIb}\beta_3$ by concanavalin-A lectin chromatography and then passed over an aminoethylglycine-RGDS-Sepharose column. After washing, the material retained on the column was specifically eluted with buffer containing 1 mM free RGDS (Sigma), then concentrated and analyzed by $NaDodSO_4$/PAGE under reducing conditions followed by silver staining.

Fibrinogen Binding and Platelet Aggregation. Binding of ^{125}I-labeled fibrinogen to platelets was measured as described (20, 25) with platelets treated as follows: Fresh washed platelets were activated by 10 μM ADP or thrombin at 0.125 unit/ml; activation of $\alpha_{IIb}\beta_3$ with RGDS (26) was carried out exactly as described by incubating the tetrapeptide with resting platelets, which were then fixed in the presence of paraformaldehyde before RGDS was removed by washing; chymotrypsin partial digestion was done at 22°C, under conditions chosen to trigger $\alpha_{IIb}\beta_3$ affinity for fibrinogen in the absence of platelet activation (20). Binding of fibrinogen was measured at saturating concentrations of the ligand (350–450 μg/ml) while platelet concentration was 3×10^8 per ml. Nonspecific binding was assessed by measuring fibrinogen binding in the presence of 5 mM EDTA or 1 mM RGDS. Specific binding was calculated as the difference between total and nonspecifically bound fibrinogen.

Platelet aggregations were performed in parallel to fibrinogen binding on washed platelets as described (20), in the presence of 20 μM ADP or thrombin at 0.125 unit/ml as agonist.

Amplification and Analysis of DNA and Platelet mRNA Sequences. The following primers were used for amplification of β_3 and α_{IIb} platelet mRNAs: The 3′ primers for β_3 were 3aA [5′-AGGAGGAATTCTGGGACAAAGGC-3′, complementary to nucleotides (nt) 2664–2642 of the β_3 mRNA] (27), 3aC (5′-GCAGGGCGGCAAGGCCAATGAGCAGAATGG-3′, nt 2235–2206), 3aE (5′-GCAGGTGTCAGTACGCGTGGTACAGTTGC-3′, nt 1799–1771 of the mRNA), and 3aG (5′-CGAATCATCTGGCCGGAGCCGG-3′, nt 389–368). The 3′ primers for α_{IIb} were 2bM (5′-CCAAAGCTTGGAGGCAACTT-3′, complementary to nt 3206–3187 of the α_{IIb} mRNA) (28), 2bA (5′-TCGAAGCTTACGAGAACTGGAT-3′, nt 2736–2715), 2bC (5′-TCGGAATTCCTATCTGGGCGTT-3′, nt 2201–2180), 2bE (5′-AGGAAGCTTGTGCCCAGTGGCT-3′, nt 1591–1570), 2bG (5′-GAGCTGTGTGCCAGTCAGCA-3′, nt 1150–1131), and 2bI (5′-AGAATTCCCAAGCACCAGCTCTC-3′, nt 652–630). The 5′ primers for β_3 were 3aB (5′-GAAAAAGCTTAAGGACACTGGC-3′, corresponding to nt 2024–2045), 3aD (5′-GTGACGACTTCTCCTGTGTCCGCTACAAG-3′, nt 1666–1694), 3aF (5′-CCAGGTCACTCAAGTCAG-3′, nt 332–349), and 3aH (5′-CGCGGGAGGCGGACGAGATGCG-3′, nt 4–25). The 5′ primers for α_{IIb} were 2bN (5′-CCTGCTCTACATCCTGGATA-3′, nt 2527–2546), 2bB (5′-GGAGAATTCGACCAGGGTGGTG-3′, nt 2128–2149), 2bD (5′-GAGAAGCTTCAACATCCAGATG-3′, nt 1540–1561), 2bF (5′-ACCGAATTCTGGCCGAAGTGGG-3′, nt 1050–1071), 2bH (5′-GCGGAATTCAGCTCCGTGGTCA-3′, nt 599–620), and 2bJ (5′-CCAAAGCTTTGTGTCCACTGCA-3′, nt 6–27). Sequences for oligonucleotides 3aC, 3aD, 3aE, 3aF, 3aG, and 3aH were kindly provided by P. J. Newman.

PCR on platelet RNA (RNA-PCR) was carried out as follows. A first single-stranded cDNA was synthesized from 1 μg of total platelet RNA [extracted as previously described (29)] with Moloney murine leukemia virus reverse transcriptase (BRL) and 1 mM 3′ oligonucleotide primer. PCR was performed according to Saiki *et al.* (30) with 200 nM 3′ and 5′ primers. The cycling program included a 5-min 95°C denaturation step followed by addition of 2.5 units of *Taq* DNA polymerase (Amersham) and 30 temperature cycles consisting each of a 94°C/1.5-min denaturation step, a 50°C/1.5-min annealing step, and a 72°C/3-min extension step (the last cycle including a 72°C/10-min termination step) in an IHB thermal reactor (Hybaid, Middlesex, U.K.).

DNA-PCR on β_3 exon XIV (31), which contains the cytoplasmic domain of β_3, was performed with the 3′ primer 3aA and the 5′ primer 3aB′ (5′-AGGAAGTCACTGTAAGATGCTATT-3′, corresponding to intronic nt −40 to −17 upstream from exon XIV). The experimental conditions and the cycling program were the same as for RNA-PCR without the reverse transcription step.

Acc I (Boehringer Mannheim) restriction digestion was performed on purified PCR products according to the supplier's recommendations.

Nucleotide sequences were determined by double-strand sequencing using the dideoxy-chain-termination method with Sequenase version 2.0 and T7 dGTP kits (United States Biochemical). Templates were either gel-purified PCR products or plasmids after subcloning of PCR fragments in pBluescript II SK(+) (Stratagene).

RESULTS AND DISCUSSION

Two-dimensional nonreduced/reduced $NaDodSO_4$/PAGE analysis of the patient's platelet proteins did not reveal any major structural alteration of either α_{IIb} or β_3 subunits and showed that both were expressed to appreciable levels in variant platelets, confirming our preliminary results (19, 32) (Fig. 1). Exposure of $\alpha_{IIb}\beta_3$ on the platelet surface was estimated by the binding to platelets of ^{125}I-labeled AP-2, a monoclonal antibody specific for the $\alpha_{IIb}\beta_3$ complex (22) (Table 1): in the patient, surface $\alpha_{IIb}\beta_3$ was 44% of the normal value when measured on resting platelets and increased up to

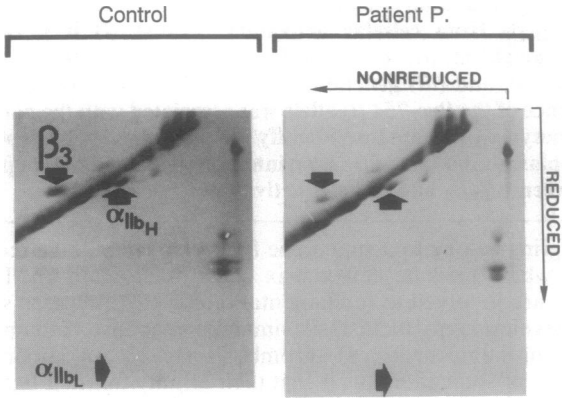

FIG. 1. Two-dimensional nonreduced/reduced $NaDodSO_4$/PAGE of normal (control) and variant (patient P.) platelet proteins stained by Coomassie blue. Normal α_{IIb} heavy chain (α_{IIb_H}) and α_{IIb} light chain (α_{IIb_L}) and variant α_{IIb_H} and α_{IIb_L} (arrows) exhibited identical M_r values (123,000 and 25,000, respectively). Both normal and variant β_3 subunits showed the typical 90,000-to-104,000 shift in M_r after reduction of disulfide bonds. A slight decrease in staining of both α_{IIb} and β_3 subunits in the patient's sample compared to the control is indicative of a moderate quantitative defect. Polypeptides cleaved in two or more chains after disulfide reduction migrate below the diagonal, while polypeptides retarded in the second dimension by reduction of intrachain disulfides migrate above the diagonal.

Table 1. Number of $\alpha_{IIb}\beta_3$ molecules exposed on the surface of resting and stimulated platelets estimated by binding of the monoclonal antibody AP-2

Subject	No. of molecules per platelet		
	Resting platelets	Stimulated platelets	Internal pool
Controls	36,400 ± 6,500	49,700 ± 14,100	13,300
Patient P.	16,000 ± 4,600 (44%)	27,800 ± 4,800 (56%)	11,800 (88%)
Daughter	20,200 ± 5,100 (55%)	30,000 (60%)	9,900 (70%)

Washed platelets were preincubated for 3 min at 37°C with α-thrombin at 0.1 unit/ml or buffer, then thrombin was inactivated with hirudin at 2 units/ml. Resting platelets and platelets stimulated by 0.125 unit of thrombin were then incubated with ^{125}I-labeled AP-2 for 30 min at 22°C. The number of platelet-bound AP-2 molecules is indicated as the mean ± SD. For resting and activated control platelets, values from 44 and 7 independent donors, respectively, were determined. Values for the patient were obtained in two to four separate experiments. Percentages were calculated relative to the corresponding control value. Two experiments were conducted on the patient's daughter, only one of which was carried out on thrombin-activated platelets. Internal pool of $\alpha_{IIb}\beta_3$ was calculated by subtracting the value obtained for resting platelets from that of stimulated platelets.

56% after platelet stimulation by α-thrombin. Interestingly, the calculated value for internal $\alpha_{IIb}\beta_3$ (11,800) was comparable to what was found with normal platelets (13,300). These results indicate that variant $\alpha_{IIb}\beta_3$ is exposed at the platelet surface, with a near normal internal pool that is efficiently translocated to the plasma membrane upon platelet stimulation. Thus the inability of variant platelets to bind fibrinogen and to aggregate in response to physiological stimuli such as ADP, collagen, and thrombin could not be attributed to a gross anomaly in the structure or cellular distribution of variant $\alpha_{IIb}\beta_3$. The functional defect was unlikely to be due to the moderate quantitative defect of the variant $\alpha_{IIb}\beta_3$, which was within the range observed for Glanzmann type I heterozygotes who exhibit normal platelet aggregation (12).

Because a possible cause for the functional defect of variant $\alpha_{IIb}\beta_3$ was an alteration of its ligand-binding sites, as reported for other Glanzmann variants (15, 16), the integrin was subjected to an RGDS-affinity chromatography. Variant $\alpha_{IIb}\beta_3$ bound to immobilized RGDS and was efficiently eluted by the free peptide (Fig. 2). This experiment suggested that soluble variant $\alpha_{IIb}\beta_3$ was capable of interaction with the adhesive RGDS peptide, and thus that the functional defect in variant $\alpha_{IIb}\beta_3$ was probably not due to the direct alteration of the ligand-binding capacity of the integrin.

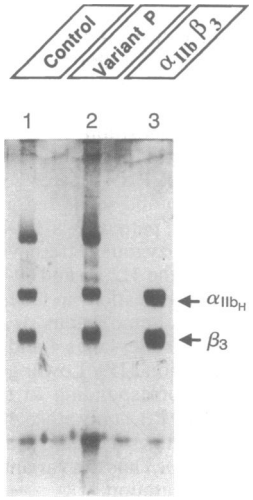

FIG. 2. NaDodSO$_4$/PAGE analysis of $\alpha_{IIb}\beta_3$ eluted from an RGDS-affinity column. Platelet proteins bound to the RGDS column and eluted by free RGDS were analyzed by NaDodSO$_4$/PAGE after disulfide reduction. Lane 1, control subject; lane 2, patient P.; lane 3, purified $\alpha_{IIb}\beta_3$ as a standard. High molecular weight bands observed in both the control and variant samples correspond to a contaminant often seen when analyzing small platelet samples.

Induction of $\alpha_{IIb}\beta_3$ affinity for fibrinogen binding during platelet stimulation is coordinated with induction of neoepitopes on the molecule (7, 10), suggesting that conformational changes accompany activation. Sims *et al.* (33) have shown that platelet activation induces a spatial reorganization of $\alpha_{IIb}\beta_3$ exoplasmic domains, a phenomenon which may be the basis for the conversion of this integrin into a functional adhesion receptor. Moreover, it was shown that the affinity of $\alpha_{IIb}\beta_3$ for fibrinogen is increased in the presence of "activators" acting directly on the integrin, such as some monoclonal antibodies (34, 35), α-chymotrypsin (20), soluble adhesive peptides such as RGDS (26), or phosphatidic acid and lysophosphatidic acid (36). Thus conformation mobility of $\alpha_{IIb}\beta_3$ is probably an essential element driving the increase in avidity of the integrin. We therefore wondered if the defect in patient P. could be due to a blockade in conformational mobility of $\alpha_{IIb}\beta_3$, and we designed experiments to test this possibility. While platelet agonists such as ADP or thrombin, which act on $\alpha_{IIb}\beta_3$ activation by intracellular signaling (8), were able to induce only very limited fibrinogen binding to variant platelets (≤5% of control platelets), direct $\alpha_{IIb}\beta_3$ activators such as the soluble adhesive RGDS peptide or α-chymotrypsin induced significant fibrinogen binding, up to 45% of the control values (Fig. 3). Altogether, these results suggested that the competence for fibrinogen binding of variant $\alpha_{IIb}\beta_3$ (*i*) was preserved, (*ii*) could be up-regulated by agents acting directly on the integrin by conformational changes, but (*iii*) could not be up-regulated by intracellular signaling. The inability of variant $\alpha_{IIb}\beta_3$ to undergo change in affinity for fibrinogen binding *in situ* was unlikely to be the consequence of a conformation blocked in a nonfunctional state. We thus postulated a defect in an as-yet-undefined activation pathway of $\alpha_{IIb}\beta_3$ or in the coupling between $\alpha_{IIb}\beta_3$ and platelet activation.

Data have accumulated suggesting that all major intracellular signal transduction events are normal in the platelets of patient P., including normal shape change and exocytosis of internal granules after cell stimulation (19, 24). We therefore considered as a reasonable possibility that the intracellular signaling pathways leading to $\alpha_{IIb}\beta_3$ activation were intact but that the receptor was unable to couple with platelet activa-

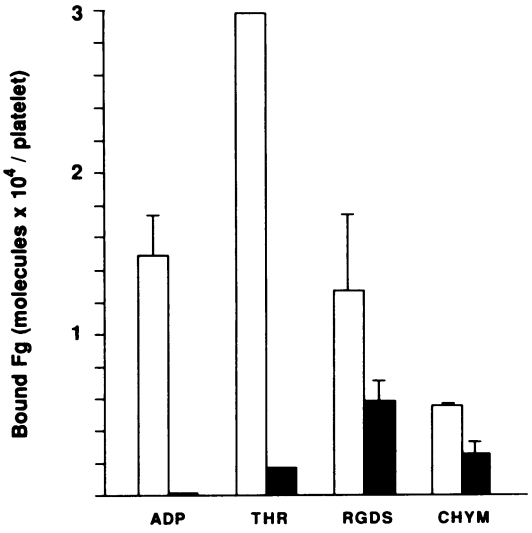

FIG. 3. Comparison of the fibrinogen-binding capacity of platelets activated with the cellular activators, ADP and thrombin, or with the direct activators of $\alpha_{IIb}\beta_3$, RGDS and chymotrypsin. ^{125}I-labeled fibrinogen (Fg) binding was measured on normal (open bars) or variant (black bars) platelets preincubated with 10 μM ADP (ADP), human α-thrombin at 0.125 unit/ml (THR), or 1 mM RGDS (RGDS), or after α-chymotryptic partial cleavage of $\alpha_{IIb}\beta_3$ (CHYM). Specifically bound fibrinogen is expressed in 10,000 molecules per platelet.

tion. We therefore examined the cytoplasmic domain of the β_3 subunit, because of its topographically favorable location and because integrin β subunits have been suspected of interacting with cytoplasmic elements (37). We first amplified a segment of β_3 mRNA corresponding to the cytoplasmic domain of the protein by reverse transcription followed by PCR. After sequencing, we found a T → C mutation at nt position 2259 (Fig. 4 *Upper*). The corresponding amino acid alteration was the replacement of Ser-752 by Pro in the mature protein. This substitution is likely to be significant for several reasons. First, the T-2259 → C substitution, which suppresses an *Acc* I restriction site, was not found in a total of 150 control chromosomes analyzed by PCR and *Acc* I restriction analysis, ruling out a polymorphism (not shown). Second, sequencing of the rest of β_3 and of full-length α_{IIb} showed no other mutation in the receptor, in particular in the ligand-binding domains of either subunit, or in the cytoplasmic tail of α_{IIb}. Third, molecular genetic analysis was consistent with the mutation being associated with the $\alpha_{IIb}\beta_3$ activation defect. *Acc* I restriction analysis conducted on the variant's genomic DNA after amplification by DNA-PCR of exon XIV of the β_3 gene showed a pattern consistent with one *Acc* I-resistant allele corresponding to the C-2259 mutation and one *Acc* I-sensitive allele, normal at position 2259 (Fig. 4 *Lower*). However, variant β_3 mRNA, when amplified on platelet RNA by RNA-PCR, was resistant to *Acc* I restriction, indicating that only the C-2259 allele was expressed. Thus patient P. is a compound heterozygote, one β_3 allele carrying the C-2259 mutation, the other allele (termed "null") not being expressed in platelets. This genotype is consistent with the presence of approximately 50% $\alpha_{IIb}\beta_3$ in the patient's platelets. *Acc* I restriction analysis in the family showed that the patient's daughter, whose platelets aggregated normally, did not inherit the C-2259 mutation (Fig. 5). In contrast, *Taq* I restriction fragment length polymorphism analysis (38) conducted on DNA-PCR of exon VIII of the β_3 gene showed that she inherited the null allele from her father (not shown), consistent with the moderate quantitative defect in $\alpha_{IIb}\beta_3$ observed in her platelets (60%), as measured by AP-2 (Table 1).

Our data strongly support the assumption that the Pro-752 mutation in the β_3 cytoplasmic domain is likely to be responsible for uncoupling $\alpha_{IIb}\beta_3$ from cellular activation. The functional relevance of the cytoplasmic domain of β_3 is consistent with deletion studies conducted on another integrin, LFA-1, which have also stressed the importance of the cytoplasmic domain of β_2 in regulating ligand binding (10). In addition we have recently found that the β_3 cytoplasmic domain is highly conserved among different species (unpublished results), consistent with a physiologic role. It is tempting to speculate on a direct role of Ser-752 in the

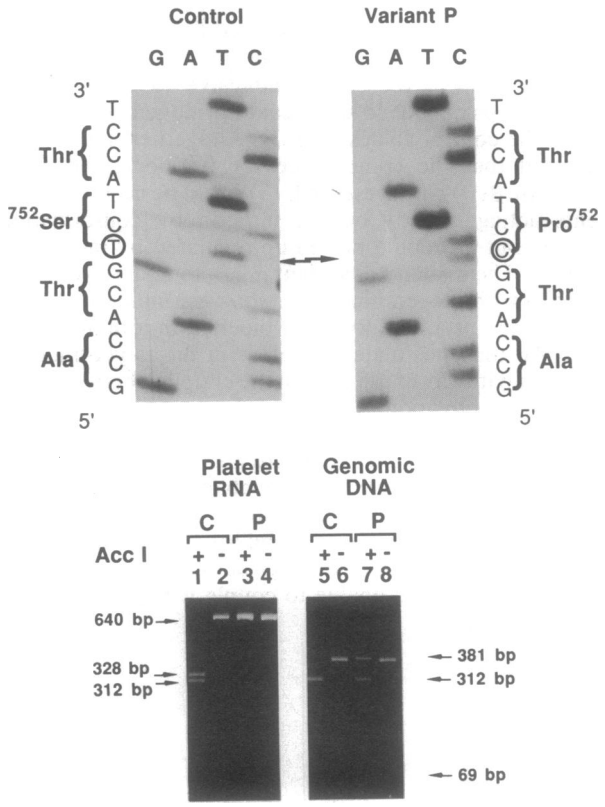

FIG. 4. (*Upper*) Nucleotide sequence of the portion of the variant's β_3 mRNA encoding the cytoplasmic domain. Arrows indicate bands corresponding to control and variant's nucleotides (circled) in position 2259. Codons in the open reading frame are indicated by brackets, and the corresponding amino acids are noted. Only the positions of serine in the control and the corresponding proline in the variant are indicated. (*Lower*) *Acc* I restriction analysis of PCR-amplified β_3 RNA and genomic DNA. PCR products from control (C) or variant (P) were incubated in the presence (+) or absence (−) of the restriction enzyme *Acc* I, separated by electrophoresis on a 2% agarose gel, and stained by ethidium bromide. Lanes 1–4, platelet RNA was used as template. The 640-base-pair (bp) RNA-PCR product of control, containing a T in position 2259, is cut into a 328-bp and a 312-bp band with *Acc* I (lane 1), while in the variant the 640-bp product remains mostly uncut (lane 3). Barely detectable are a 328-bp and a 312-bp band, indicating very little expression of a T-2259 allele in the variant. Lanes 5–8, genomic DNA was used as template. The 381-bp β_3 exon XIV DNA-PCR product from the control was cut by *Acc* I into a 312-bp and a 69-bp fragment (lane 5). The 69-bp band is not well seen on this gel. In lane 7, *Acc* I digestion generated a pattern of both an uncut 381-bp product and cut 312-bp and 69-bp bands, consistent with heterozygosity at position 2259.

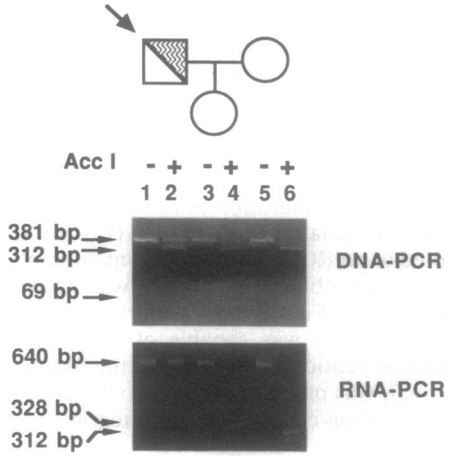

FIG. 5. *Acc* I digestion analysis for detection of the C-2259 mutation in the variant's family. The pedigree of the family is illustrated in the top part of the figure. Patient P. is identified by the arrow and the grey field illustrates the C-2259 (Pro-752) allele. As in Fig. 4, a portion of exon XIV encompassing position 2259 was PCR-amplified from genomic DNA (upper gel) and from the corresponding portion of the β_3 mRNA from platelet RNA (lower gel). PCR products were analyzed by *Acc* I digestion and separated in a 2% Seakem/2% Nusieve agarose gel. Upper gel, patient P.'s 381-bp exon XIV product, lane 1, gives rise to the heterozygous pattern with both uncut 381-bp band of the Pro-752 allele and the 312-bp and 69-bp fragments for the other (null) allele after *Acc* I digestion, in lane 2. Both the patient's daughter and his wife have a homozygous *Acc* I pattern with only the 312-bp and 69-bp bands visible, lanes 4 and 6, consistent with their β_3 alleles having a T in position 2259. Lower gel, while only the *Acc* I-resistant 640-bp band corresponding to the C-2259 transcript is seen in lane 2 (patient P.), only the *Acc* I-sensitive 328-bp and 312-bp bands corresponding to T-2259 transcript(s) are seen in lanes 4 (daughter) and 6 (wife). Thus the variant's daughter did not inherit the C-2259 (Pro-752) mutation.

regulation of activation of $\alpha_{IIb}\beta_3$: phosphorylation and/or dephosphorylation of the β_3 cytoplasmic tail has been recently proposed as a mechanism for regulation of $\alpha_{IIb}\beta_3$ activation (39, 40), though this conclusion has been challenged more recently (41). However, the residue substituting for Ser-752 is a proline, which is known to disrupt secondary structures; thus it remains to be investigated whether the absence of signal-receptor coupling is due to the loss of Ser-752, the hydroxyl group of which may be functionally relevant, or to the disruption by Pro-752 of a functional domain.

It was recently proposed, on the basis of serial deletions of $\alpha_{IIb}\beta_3$ in transfected cells, that the cytoplasmic tail of α_{IIb} has a negative regulatory role on integrin activation, possibly through interaction with an α_{IIb}-specific cellular inhibitor (42). The work presented here is in favor of a positive regulation of the receptor activity of $\alpha_{IIb}\beta_3$ by the cytoplasmic tail of β_3: we thus propose a model in which the affinity of $\alpha_{IIb}\beta_3$ for fibrinogen is regulated through a double (or multiple) mechanism involving positive and negative "switches" located in the two cytoplasmic domains of the integrin. This could provide platelets with a means of regulating $\alpha_{IIb}\beta_3$ activation more subtly than through a one-way mechanism. This model could be of relevance not only for $\alpha_{IIb}\beta_3$ but for other "activatable" integrins, such as LFA-1.

We have not been able to perform a direct test of the effect of the β_3 Pro-752 mutation on $\alpha_{IIb}\beta_3$ activation, first because there is no *in vitro* test for $\alpha_{IIb}\beta_3$ metabolic activation, and second because there is no cell line available in which transfected $\alpha_{IIb}\beta_3$ can be activated by an intracellular pathway (43). This stresses the importance of a genetic approach in elucidating such a complex mechanism and further validates the use of variants of Glanzmann thrombasthenia in helping to understand the structure–function relationships of integrins.

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