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The Interaction of Integrin $\alpha_{IIb}\beta_3$ with Fibrin Occurs through Multiple Binding Sites in the α_{IIb} β-Propeller Domain*[S]

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Background: During thrombus formation, platelet integrin $\alpha_{IIb}\beta_3$ binds fibrin; however, the mechanism of this interaction is unclear.
Results: Mutations of discontinuous negatively charged and aromatic residues in the α_{IIb} β-propeller domain impair fibrin clot retraction and cell adhesion.
Conclusion: Integrin $\alpha_{IIb}\beta_3$ has multiple binding sites for fibrin.
Significance: Uncovered recognition specificity of $\alpha_{IIb}\beta_3$ for fibrin may be used to select inhibitors of this interaction.

The currently available antithrombotic agents target the interaction of platelet integrin $\alpha_{IIb}\beta_3$ (GPIIb-IIIa) with fibrinogen during platelet aggregation. Platelets also bind fibrin formed early during thrombus growth. It was proposed that inhibition of platelet-fibrin interactions may be a necessary and important property of $\alpha_{IIb}\beta_3$ antagonists; however, the mechanisms by which $\alpha_{IIb}\beta_3$ binds fibrin are uncertain. We have previously identified the γ370–381 sequence (P3) in the γC domain of fibrinogen as the fibrin-specific binding site for $\alpha_{IIb}\beta_3$ involved in platelet adhesion and platelet-mediated fibrin clot retraction. In the present study, we have demonstrated that P3 can bind to several discontinuous segments within the α_{IIb} β-propeller domain of $\alpha_{IIb}\beta_3$ enriched with negatively charged and aromatic residues. By screening peptide libraries spanning the sequence of the α_{IIb} β-propeller, several sequences were identified as candidate contact sites for P3. Synthetic peptides duplicating these segments inhibited platelet adhesion and clot retraction but not platelet aggregation, supporting the role of these regions in fibrin recognition. Mutant $\alpha_{IIb}\beta_3$ receptors in which residues identified as critical for P3 binding were substituted for homologous residues in the I-less integrin $\alpha_M\beta_2$ exhibited reduced cell adhesion and clot retraction. These residues are different from those that are involved in the coordination of the fibrinogen γ404–411 sequence and from auxiliary sites implicated in binding of soluble fibrinogen. These results map the binding of fibrin to multiple sites in the α_{IIb} β-propeller and further indicate that recognition specificity of $\alpha_{IIb}\beta_3$ for fibrin differs from that for soluble fibrinogen.

Integrin $\alpha_{IIb}\beta_3$, a major membrane protein expressed on the surface of platelets, plays central roles in normal hemostasis and pathological thrombosis. On stimulated platelets, $\alpha_{IIb}\beta_3$ serves as a specific receptor for the plasma protein fibrinogen. Fibrinogen binding to activated $\alpha_{IIb}\beta_3$ induces platelet aggregation, the essential cellular event in the formation of the primary hemostatic plug. Furthermore, platelets bind fibrin, a product of the enzymatic transformation of soluble fibrinogen into insoluble fibrin, which is formed early and dominates the entire process of thrombus growth (1–3). Because the same molecular pathways mediate pathological thrombus formation, the interaction between $\alpha_{IIb}\beta_3$ and fibrinogen has been targeted for antithrombotic therapy (for a review, see Ref. 4). It has also been proposed that inhibition of platelet interactions with fibrin may be a necessary and important property of $\alpha_{IIb}\beta_3$ antagonists (5). The initial interaction of soluble fibrinogen with $\alpha_{IIb}\beta_3$ occurs via the COOH-terminal sequence in the globular γC domains of fibrinogen with γ^{404}GAKQAGDV^{411} (γC peptide) providing critical coordination residues that bind to the interface between the α_{IIb} β-propeller domain of the α integrin subunit and the β_3 I domain of the β subunit (6). The γC sequence is unique to fibrinogen, and binding of fibrinogen to $\alpha_{IIb}\beta_3$ through γC is highly specific (7, 8). Although four integrin recognition RGD sequences are present in fibrinogen and the RGD peptide inhibits $\alpha_{IIb}\beta_3$ adhesive reactions and can bind within the same pocket that is occupied by γGAKQAGDV, none of the RGDs in fibrinogen are required for platelet aggregation (9).

Fibrinogen binding to $\alpha_{IIb}\beta_3$ is a multistep process: initial reversible contact is followed by irreversible binding such that the bound ligand no longer readily dissociates (10, 11). The binding of fibrinogen to the receptor is accompanied by the alteration of fibrinogen conformation and leads to unmasking of cryptic sequences that potentially can serve as new $\alpha_{IIb}\beta_3$-binding sites (12, 13). Also, as the thrombus formation proceeds, the interaction of $\alpha_{IIb}\beta_3$ with fibrin engages new contacts that lead to clot retraction. Thus, the overall process of throm-

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bus formation *in vivo* involves the interactions of $\alpha_{IIb}\beta_3$ with different forms of fibrinogen: soluble fibrinogen and an insoluble fibrin(ogen) matrix. The evidence accumulated so far suggests that these interactions involve differential recognition specificity. In contrast to platelet aggregation, the γC sequence is not absolutely required for adhesion to immobilized fibrinogen and fibrin clot retraction (14, 15). Furthermore, RGDs do not contribute to $\alpha_{IIb}\beta_3$-mediated clot retraction. Recombinant human fibrinogen in which all RGDs in the Aα chains were mutated and γ^{408}AGDV^{411} in the γC domains were truncated exhibits delayed but otherwise normal clot retraction (16). Also, neither RGD nor γC peptides inhibit clot tension development during retraction (17), and some anti-$\alpha_{IIb}\beta_3$ mAbs inhibit clot retraction but not fibrinogen binding and vice versa (17–19). In addition, fibrinogen from mice in which the γC domain was targeted to delete γ^{407}QAGDV^{411} does not support platelet aggregation but still mediates normal clot retraction (20). Finally, some $\alpha_{IIb}\beta_3$ antagonists have different efficacies in inhibiting clot retraction despite the equivalent antiaggregatory potency (21). Taken together, these data indicate that the site(s) involved in the initial binding of fibrinogen to $\alpha_{IIb}\beta_3$ during platelet aggregation is different from those that participate in the interaction of platelets with the insoluble fibrin(ogen) matrix during thrombus growth and clot retraction.

The existence of alternative binding sites in addition to γC and RGD that are involved in binding of fibrinogen to $\alpha_{IIb}\beta_3$ was first suggested by Parise *et al.* (22). They found that $\alpha_{IIb}\beta_3$ binding to fibrinogen immobilized on agarose was not inhibited by either RGD or γ^{400}HHLGGAKQAGDV^{411} (named the H12 peptide). The subsequent studies have localized two sites in the γC domain that may mediate the interaction of $\alpha_{IIb}\beta_3$ with insoluble forms of fibrin(ogen). The mutations within the γ316–322 sequence of recombinant fibrinogen diminished platelet aggregation and platelet adhesion under flow (23, 24). We have previously identified the sequence γ^{370}ATWKTRWYSMKK^{381} (termed P3) as the binding site for $\alpha_{IIb}\beta_3$ in adhesion and clot retraction (15, 25). We further found that the mechanism by which $\alpha_{IIb}\beta_3$ binds P3 is distinct from the γC recognition. First, P3-mediated adhesion of platelets to fibrinogen fragments lacking the γC residues ^{406}KQAGDV^{411} does not require their prior stimulation, whereas the engagement of γC by $\alpha_{IIb}\beta_3$ is activation-dependent (15). Second, P3 is fibrin-specific in that it is poorly exposed on the surface of intact soluble fibrinogen but becomes fully available after the transformation of fibrinogen to fibrin or after deposition of fibrinogen on various surfaces, including aggregated platelets (12, 26). Third, P3 binding to $\alpha_{IIb}\beta_3$ depends on its positively charged residues (25). Because P3 contains no sequences resembling the γ^{404}GAKQAGDV^{411} or RGD motif, it is reasonable to assume that the binding site(s) for P3 in $\alpha_{IIb}\beta_3$ is unlike that utilized by RGD or γC. Here, we performed the binding analyses to demonstrate that $\alpha_{IIb}\beta_3$ contains multiple binding sites for P3. Furthermore, using synthetic peptide libraries and mutational analyses, we have localized these sites in the α_{IIb} β-propeller domain of the receptor.

EXPERIMENTAL PROCEDURES

Proteins, Peptides, and Monoclonal Antibodies—Human fibrinogen, thrombin, and plasmin were obtained from Enzyme Research Laboratories (South Bend, IN). The D98 fragment of fibrinogen (98 kDa) was prepared by digestion of fibrinogen with plasmin and purified as described (27). The DD dimer fragment was purified from the cross-linked fibrin as described (28). Recombinant fibrinogen with the binding site for $\alpha_{IIb}\beta_3$ in the γC domain of fibrinogen γ408–411 (Fg γ407)[2] deleted was produced as described previously (14). The platelet integrin $\alpha_{IIb}\beta_3$ was isolated from outdated human platelets (The Blood Center, Hammond, LA) using an affinity chromatography with concanavalin A-agarose (25). The peptides corresponding to the α_{IIb} sequences (α_{IIb} 64–78, α_{IIb} 94–108, α_{IIb} 153–162, α_{IIb} 229–237, α_{IIb} 241–255, α_{IIb} 361–375, α_{IIb} 421–435, and α_{IIb} 10–20) were synthesized by Peptide 2.0 (Chantilly, VA). The peptide duplicating the fibrinogen sequence γ370–381 (P3) was synthesized and labeled with ^{125}I using IODO-GEN (Thermo Scientific Pierce Protein Research Products, Rockford, IL).

The 9-fluorenylmethoxycarbonyl (Fmoc)-protected and pentafluorophenyl-activated amino acids for synthesis of the peptide libraries were purchased from Bachem (King of Prussia, PA). Pentafluorophenyl-activated Trp was obtained from Novabiochem. mAb AP3 against the β3 integrin subunit was isolated from hybridoma cells obtained from ATCC (Manassas, VA). The anti-α_{IIb} mAb sc-51654 was from Santa Cruz Biotechnology (Dallas, TX), mAb LM609 was from Millipore (Billerica, MA), and mAb 7E3 was a gift from Dr. B. Coller. The plasmids pcDNA3.1/Neo and pcDNA3.1/Hygro containing the full-length cDNA encoding the human α_{IIb} and β_3 integrin subunits, respectively, were provided by Dr. T. O'Toole. The primers for mutagenesis were obtained from Integrated DNA Technologies, Inc. (Coralville, IA). PfuTurbo DNA polymerase was from Agilent (Santa Clara, CA), and Lipofectamine 2000 was from Invitrogen.

Surface Plasmon Resonance (SPR)—The interaction of $\alpha_{IIb}\beta_3$ with P3 was examined using a BIAcore 2000 SPR-based biosensor (Biacore AB, Uppsala, Sweden). Purified $\alpha_{IIb}\beta_3$ was coupled to a CM5 sensor chip (Biacore) using the amine coupling kit according to the manufacturer's protocol. The sensor chip was coated to achieve ~1500 response units, which correspond to ~62 μM $\alpha_{IIb}\beta_3$. Different concentrations of P3 in HBSP buffer (10 mM HEPES buffer with pH 7.4, 150 mM NaCl, and 0.005% v/v Surfactant P20) (Biacore) containing 1 mM $CaCl_2$ and 1 mM $MgCl_2$ were passed over the flow cell at 10 μl/min, and the association between immobilized protein and peptide was detected as the change in the SPR response. All data were corrected for the response obtained using a blank reference flow cell that was activated with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide/*N*-hydroxysuccinimide and then blocked with ethanolamine. The chip surface was regenerated with 2 M NaCl and 50 mM NaOH followed by re-equilibration with the binding buffer. Experimental data were analyzed using the BIAevaluation 4.1 program supplied with the instrument. The data for the

[2] The abbreviations used are: Fg, fibrinogen; SPR, surface plasmon resonance.

construction of Scatchard plots were obtained from the equilibrium portions of SPR sensorgrams, and the dissociation equilibrium constant (K_d) was estimated by analysis of the binding curve using the steady-state affinity model provided by the same software.

Synthesis of Cellulose-bound Peptide Libraries and Screening for P3 Binding—The α_{IIb} β-propeller (residues 1–451)-derived peptide libraries were prepared by parallel spot synthesis as described (25, 29). Peptides were COOH-terminally attached to cellulose via a $(\beta\text{-Ala})_2$ spacer and were acetylated NH_2-terminally. The cellulose membranes with covalently coupled peptides were incubated for 1 min in methanol and then washed with TBS buffer. After blocking with 1% BSA for 2 h at 22 °C, the membranes were incubated with 10 μg/ml ^{125}I-labeled P3 (10^5 cpm/ml) in phosphate-buffered saline (PBS) for 3 h at 22 °C. After washing with TBS containing 0.05% Tween 20, the membranes were dried, and P3 binding was detected by autoradiography and analyzed by densitometry as described (25).

Cells and Stable Transfection of Integrin Subunit Constructs—Platelets were collected from fresh aspirin-free human blood in the presence of 2.8 μM prostaglandin E_1 and isolated by differential centrifugation followed by gel filtration on Sepharose 2B in divalent cation-free Tyrode's buffer, pH 7.2 containing 0.1% BSA. Human embryonic kidney 293 (HEK293) cells were stably transfected with pcDNA3.1 plasmids with inserted wild-type (WT) α_{IIb} and β_3 or mutant α_{IIb} and WT β_3 using Lipofectamine 2000 reagent (Invitrogen). After 48 h at 37 °C in 5% CO_2, cells were harvested and cultured in medium with 0.5 mg/ml G418 (Invitrogen) and 0.25 mg/ml hygromycin (Invitrogen). After 14 days, surviving cells were collected and sorted. The expression of $\alpha_{IIb}\beta_3$ on the surface of the cells was evaluated by FACS analyses using anti-β_3 mAb AP3 (10 μg/ml) and a FACSCalibur flow cytometer (BD Biosciences). The $\alpha_{IIb}\beta_3$-expressing HEK293 cells were maintained in DMEM/F-12 (Invitrogen) supplemented with 10% FBS, 2 mM glutamine, 15 mM HEPES, 0.25 mg/ml G418, and 0.1 mg/ml hygromycin.

Immunoprecipitation—Cells (5×10^6) were labeled with 100 μg Immunopure Sulfo-NHS-LC-Biotin (Thermo Scientific, Rochester, NY) in 200 μl of PBS for 30 min at 22 °C. The cells were solubilized with a lysis buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100, 1 mM $CaCl_2$, 1 mM PMSF, 100 μg/ml leupeptin, 10 mM benzamidine) for 30 min at 22 °C. The lysates were incubated with 10 μg of normal mouse IgG (Sigma) and 50 μl of Zysorbin-G (Zymed Laboratories Inc., San Francisco, CA) for 2 h at 4 °C. After centrifugation, the supernatant was incubated with anti-α_{IIb} mAb sc-51654 (10 μg) for 2 h at 4 °C. The integrin-mAb complex was captured by incubating with 50 μl of protein A-Sepharose (Amersham Biosciences) for 2 h at 4 °C. The immunoprecipitated proteins were eluted with SDS-polyacrylamide gel electrophoresis loading buffer and analyzed by Western blotting. The Immobilon-P membranes (Millipore, New Bedford, MA) were incubated with streptavidin conjugated to horseradish peroxidase and developed using enhanced SuperSignal chemiluminescent substrate (Pierce).

Fibrin Clot Retraction—Clot retraction assays using isolated platelets were performed as described previously (15). Briefly, the reaction mixture (total volume, 1.0 ml) consisted of 3×10^8 platelets in isotonic HEPES buffer (20 mM HEPES, pH 7.3, 137 mM NaCl, 2.7 mM KCl, 1 mM $MgCl_2$, 3.3 mM NaH_2PO_4 containing 35 mg/ml BSA and 1 mg/ml glucose), 0.25 mg/ml fibrinogen, and 1 mM $CaCl_2$ in glass tubes coated with Sigmacote (Sigma). For clot retraction assays with recombinant Fg γ407, the volume of the reaction mixture was reduced to 0.25 ml. Clot retraction was initiated by adding of 1 unit/ml thrombin at 22 °C. Clot retraction mediated by wild-type HEK293 cells and generated mutant cell lines was performed as described (30). The reaction mixture consisted of 2×10^6 cells in 1 ml containing 10 mM tranexamic acid, 0.25 mg/ml fibrinogen, and 2 mM $CaCl_2$. Clot retraction was initiated by adding of 1 unit/ml thrombin at 37 °C. To block the effect of $\alpha_v\beta_3$, cells were first preincubated with mAb LM609 (10 μg/ml) for 10 min at 22 °C.

Clot retraction triggered by platelets and HEK293 cells was monitored by taking photographs of clots at several time intervals using a digital camera. The images were scanned, and the areas occupied by clots were calculated using NIH ImageJ software. The effect of the α_{IIb}-derived peptides on platelet-mediated clot retraction was evaluated by determining several parameters (lag phase and IC_{50}) obtained from the kinetic curves of retraction as described previously (15). The value of IC_{50} is defined as a concentration of the inhibitor that produces 50% of maximal inhibition. The lag phase is defined as the time spanned from the onset of the process until the first visible changes in clot morphology are observed.

Adhesion Assays—The wells of 96-well tissue culture plates (Costar, Cambridge, MA) were coated with the fibrinogen fragment D98 for 3 h at 37 °C or overnight at 4 °C. The wells were postcoated with 1% BSA inactivated at 75 °C for platelet adhesion assays or 1% polyvinyl alcohol for HEK293 cells. Cells were labeled with 10 μM calcein AM (Molecular Probes, Inc., Eugene, OR) for 30 min at 37 °C. Platelets were washed in isotonic HEPES buffer and resuspended at 1×10^8/ml in the same buffer supplemented with 1% BSA, 1 mM $MgCl_2$, and 1 mM $CaCl_2$. Calcein-labeled wild-type and $\alpha_{IIb}\beta_3$-expressing HEK293 cells were washed and resuspended in DMEM/F-12 medium at 1×10^5 cells/ml. Aliquots (100 μl) of cells were added to the wells and incubated at 37 °C for 30 min. The nonadherent cells were removed by two washes with PBS, and fluorescence was measured in a fluorescence plate reader (Applied Biosystems, Framingham, MA). The number of adherent cells was determined using the fluorescence of aliquots with a known number of labeled cells.

Platelet Aggregation—Platelet aggregation studies were performed using isolated platelets as described previously (13). 1×10^8/ml platelets were incubated with different concentrations of the α_{IIb}-derived peptides or RGDV peptide for 10 min before the initiation of aggregation. Platelet aggregation with 0.25 mg/ml fibrinogen in the presence of 10 μM ADP and 10 μM epinephrine was measured in a platelet aggregometer (Chronolog Corp., Haverton, PA) at 37 °C with continuous stirring at 1200 rpm. The maximal aggregation, achieved within 5 min after addition of agonists, was determined and expressed as a percentage of aggregation in the absence of peptides. All aggregation assays were conducted within 3 h after venipuncture.

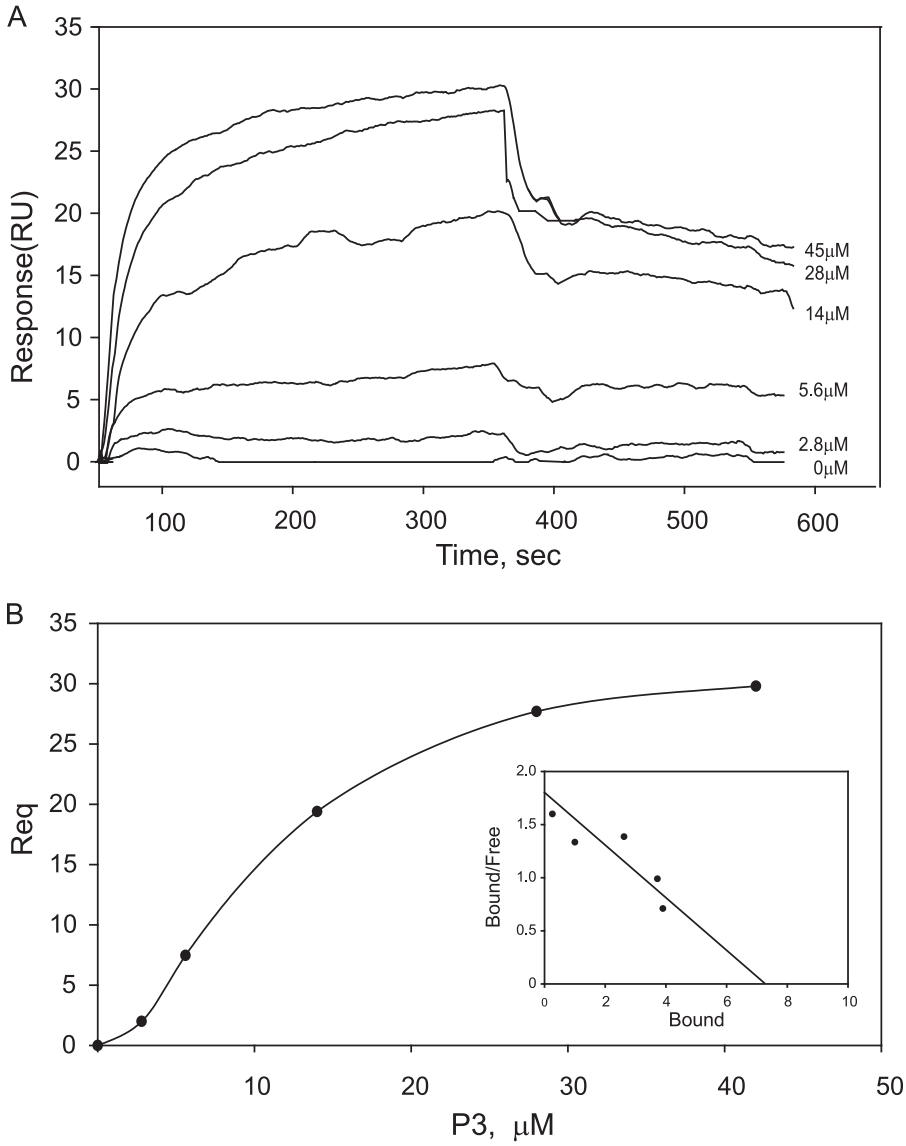

FIGURE 1. **The interaction of $\alpha_{IIb}\beta_3$ with the P3 peptide analyzed by SPR.** *A*, representative profiles of the SPR responses for P3 peptide concentrations ranging from 0 to 45 μM binding to purified $\alpha_{IIb}\beta_3$ coupled onto a CM5 sensor chip. *RU*, response units. *B*, saturable binding curve and Scatchard plot (*inset*) of P3 binding to $\alpha_{IIb}\beta_3$. *Req* is the response at equilibrium. The *abscissa* in the Scatchard plot is the ratio of the number of P3 molecules bound per molecule of $\alpha_{IIb}\beta_3$. The ratio of bound to free peptide is given on the *ordinate*.

RESULTS

Multiple Binding Sites for the P3 Peptide in $\alpha_{IIb}\beta_3$—To characterize the interaction between $\alpha_{IIb}\beta_3$ and the P3 peptide, binding studies using SPR were performed. For these analyses, the isolated $\alpha_{IIb}\beta_3$ was coupled to the CM5 sensor surface, and the SPR profiles across a range of P3 concentrations flowed over protein surfaces were examined. Fig. 1*A* demonstrates the sensorgrams for the binding of P3 in the range of 0–45 μM. The maximal responses achieved in the equilibrium portions of the sensorgrams for each P3 concentration were determined, and binding data were used to construct Scatchard plots and calculate the number of binding sites and the equilibrium dissociation constants (K_d). The binding of P3 to $\alpha_{IIb}\beta_3$ was saturable at 45 μM (maximal testable concentration) and occurred with K_d of 19.4 ± 2 μM (Fig. 1*B*). The stoichiometry of P3 binding to $\alpha_{IIb}\beta_3$ obtained from extrapolation of the linear parts of Scatchard plots was found to be 7 ± 0.6:1 (Fig. 1*B*, *inset*), indicating that the binding of P3 to $\alpha_{IIb}\beta_3$ may occur at several sites.

Screening of the α_{IIb} Propeller-derived Peptide Libraries for P3 Binding—Previous studies demonstrated that, in addition to a well defined set of amino acid residues in the α_{IIb} β-propeller and the β_3 I-like domain involved in ligation of RGD and γ^{404}GAKQAGDV^{411}, numerous residues scattered throughout the α_{IIb} β-propeller domain provide contact sites for soluble fibrinogen (31–33). Therefore, we focused on this region of the receptor. To localize the binding sites for P3 in our initial analyses, we screened the cellulose-bound peptide library representing the complete sequence of the α_{IIb} β-propeller. The library consisting of 9-mer peptides with a 3-residue offset spanning the entire α_{IIb} β-propeller (residues 1–451) (Fig. 2*A*) was synthesized, and the membrane with covalently attached peptides was probed with ^{125}I-labeled P3 peptide. The results of

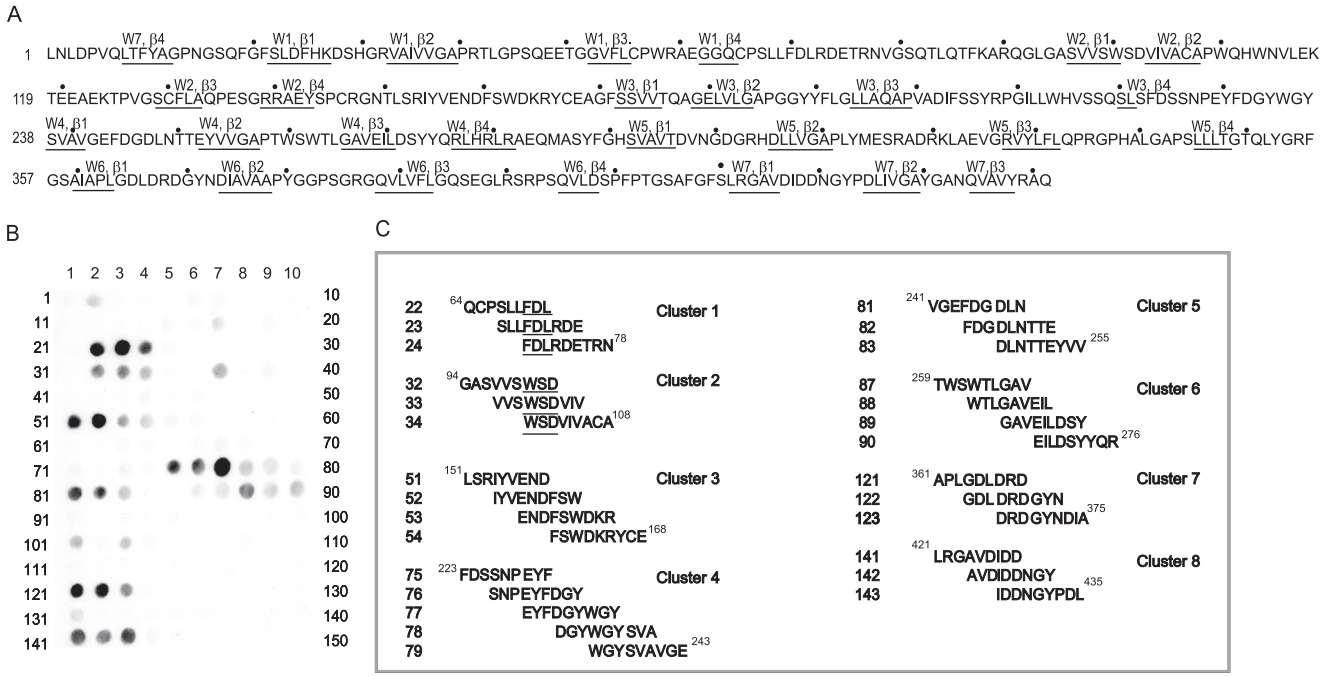

FIGURE 2. **The binding of P3 to the peptide library spanning the sequence of the α_{IIb} β-propeller.** *A*, the amino acid sequence of the of α_{IIb} β-propeller (residues 1–451). The β-strands from the seven blades are marked and *underlined*. The numbering of residues is shown with a *dot*, which marks every 10th residue. *B*, a peptide library consisting of 9-mer peptides derived from the α_{IIb} β-propeller (residues 1–451) was screened for P3 binding. The membrane with covalently attached peptides was incubated with ^{125}I-labeled P3 and subjected to autoradiography. *C*, clusters of the overlapping sequences selected based on the highest P3 binding activity are shown. The *numbers* of peptides correspond to the *numbering* of spots in *B*.

autoradiography revealed that several peptides bound P3 (Fig. 2, *B* and *C*). The majority of the P3-binding spots formed clusters; *i.e.* they were formed by the stretches of overlapping peptides. The eight P3-binding clusters correspond to the following sequences: α_{IIb} 64–78 (cluster 1), α_{IIb} 94–108 (cluster 2), α_{IIb} 151–174 (cluster 3), α_{IIb} 223–243 (cluster 4), α_{IIb} 241–254 (cluster 5), α_{IIb} 259–276 (cluster 6), α_{IIb} 361–375 (cluster 7), and α_{IIb} 421–435 (cluster 8). The observation that the central spots within clusters display the highest intensity suggests that common sequences in these peptides may be responsible for their activities. For example, within cluster 1 formed by three overlapping peptides (spots 22–24), the central spot has the highest P3 binding activity (Fig. 2*C*). The overlapping parts of these peptides contain the sequence FDL, which potentially may be responsible for P3 binding. In cluster 2, the overlapping peptides contain the common WSD sequence. Other clusters with more than three spots (clusters 4 and 6) may contain more extended recognition sites.

To ensure that these types of analyses detect integrin-ligand recognition events reported previously (33), we have independently re-examined the binding of the DD fragment to the α_{IIb} β-propeller. This fibrin-derived fragment contains both the H12 and P3 sequences, and thus, its binding to $\alpha_{IIb}\beta_3$ may potentially involve both fibrinogen- and fibrin-specific sites. Screening membranes with ^{125}I-DD revealed that, among 16 binders, 10 corresponded to the segments identified by Kamata *et al.* (33) as critical for binding of soluble fibrinogen to $\alpha_{IIb}\beta_3$ (supplemental Fig. 1S). Furthermore, among 40 individual amino acid residues identified as critical in that study (33), 36 were present in the DD binders (supplemental Fig. 1SB). Several residues, including Tyr^{143}, Pro^{145}, Asp^{163}, Leu^{183}, and Thr^{207}, mutations of which were identified in Glanzmann thrombasthenia (34),[3] were also found in the DD binders. In addition, in agreement with previous data (33) indicating that mutations in the α_{IIb} 298–304 region (a predicted binding site for fibrinogen (36)) do not affect fibrinogen binding, no interaction of the DD fragment with this segment was detected. Among other DD-binding regions, five segments (α_{IIb} 25–33, α_{IIb} 52–60, α_{IIb} 349–363, α_{IIb} 379–393, and α_{IIb} 442–451) were not previously found to be important for fibrinogen binding, and two (α_{IIb} 400–408 and α_{IIb} 415–423) were not analyzed (33). Thus, the screening experiments with the DD fragment largely supported conclusions drawn from previous mutational analyses using soluble fibrinogen (33) and, thus, substantiated the validity of this mapping strategy. However, they also revealed the differences between the binding of the DD fragment and P3 to the α_{IIb}-derived peptide libraries; *i.e.* only three regions, α_{IIb} 109–117, α_{IIb} 228–239, and α_{IIb} 256–267, bound both ligands.

The α_{IIb} β-Propeller-derived Peptides Inhibit Platelet-mediated Clot Retraction and Adhesion but Not Platelet Aggregation—To assess whether the P3-binding peptides identified in the screening experiments above can mirror the effect of P3 in functional analyses, the peptides duplicating the sequences of the P3 binders, including α_{IIb} 64–78, α_{IIb} 94–108, α_{IIb} 153–162, α_{IIb} 229–237, α_{IIb} 241–255, α_{IIb} 361–375, and α_{IIb} 421–435, were synthesized and examined for their ability to inhibit clot retraction and platelet adhesion. The peptide duplicating cluster 6 was not synthesized because only 1 residue

[3] Glanzmann Thrombasthenia Database (2013) sinaicentral.mssm.edu/intranet/research/glanzmann/play?page=nomenclature.

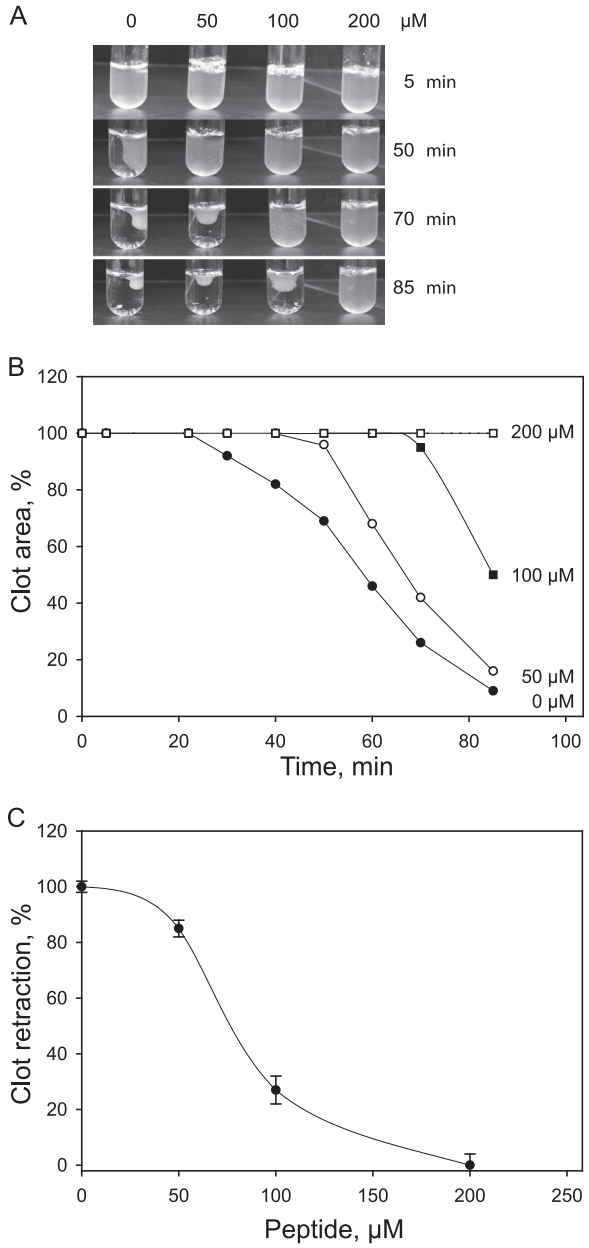

FIGURE 3. **Effect of the α_{IIb} 241–255 peptide on platelet-mediated fibrin clot retraction.** *A*, platelets were mixed with 0.25 mg/ml fibrinogen in isotonic HEPES buffer containing 1 mM $CaCl_2$ and different concentrations of the α_{IIb} 241–255 peptide (0–200 μM), and fibrin clots were formed by adding 1 unit/ml thrombin at 22 °C. Clot retraction was observed by taking photographs at different times (0–85 min). The *left lane* of tubes (*0*) shows clot retraction in the absence of peptide. A representative experiment is shown. *B*, clot areas in each tube were measured from images in *A*, and a percentage of clot retraction was calculated. Kinetic curves of retraction in the absence (●) or presence of 50 (○), 100 (■), and 200 (□) μM α_{IIb} 241–255 were generated by plotting clot areas *versus* time. *C*, dose-dependent inhibition of clot retraction by α_{IIb} 241–255 is shown. Clot retraction in the presence of selected concentrations of the peptide was determined at 85 min. At this time, clot retraction in the absence of peptide was complete (100% retraction), and clot retraction in the presence of each concentration of peptide was at different stages of completion. At 200 μM, no clot retraction was observed. The data shown are means and S.D. (*error bars*) from three experiments.

(Trp^{262}) in its most active part (α_{IIb} 259–271, spots 87–89) is exposed on the surface. Fig. 3 shows the effect of α_{IIb} ^{241}VGEFDGDLNTTEYVV^{255} as an example. The increasing concentrations of peptide progressively blocked clot retraction (Fig. 3, *A* and *B*), and at 200 μM, fibrin clots did not retract after 4 h. The IC_{50} value calculated from the progress curves of retraction was 72 ± 6 μM (Fig. 3*C* and Table 1). All other peptides, except α_{IIb} 361–375, were capable of inhibiting clot retraction albeit with different efficiencies. The IC_{50} value and lag phase determined for the concentration of each peptide allowed the comparison of their potency (Table 1). The α_{IIb} 241–255 was most active followed by α_{IIb} 94–108, α_{IIb} 64–78, α_{IIb} 421–435, α_{IIb} 153–162, and α_{IIb} 229–237. In additional experiments, we examined the effect of selected α_{IIb}-derived peptides used in combinations. As shown in supplemental Fig. 3S, the mixtures of two or four peptides added to platelets at the concentrations to achieve a final concentration equal to that used with each individual peptide did not produce an additive effect in inhibition of clot retraction. This observation is consistent with a model in which the identified α_{IIb}-derived peptides bind the same P3 site in fibrinogen.

Previous studies demonstrated that, although mutation of each of the RGD sequences in the Aα chains of recombinant fibrinogens did not affect clot retraction, deletion of the γC sequence 408–411 resulted in the slight delay of retraction when compared with normal fibrinogen (16). However, after the delay, retraction rates and the final size of clots for both mutant Fg γ407 and normal fibrinogen were similar, consistent with a mechanism in which γC may contribute to an initial phase of clot retraction but not to a subsequent step. We compared the potency of two representative α_{IIb} β-propeller-derived peptides, 94–108 and 241–255, to inhibit retraction of clots formed by mutant γ407 and normal fibrinogens. In agreement with published data (16), the retraction rates for both fibrinogens after 20 min were similar (Fig. 4, *A* and *B*). The inhibitory effect of peptides on Fg γ407-mediated clot retraction was stronger than that on retraction of clots generated from normal fibrinogen (Fig. 4 and supplemental Fig. 2S; shown for α_{IIb} 241–255), suggesting that peptides mainly block the γC-independent step of clot retraction.

The effect of α_{IIb}-derived peptides on platelet adhesion was tested using the immobilized D98 fragment. This fibrinogen fragment lacks the COOH-terminal γ404–411 sequence, and therefore, $\alpha_{IIb}\beta_3$-dependent platelet adhesion is mediated solely by P3 (15). The effect of peptide α_{IIb} 94–108 is shown as an example (Fig. 5*A*). The peptides inhibited adhesion of resting platelets in a dose-dependent manner and at 250 μM produced ~40–60% inhibition (Fig. 5*B* and Table 1). The inhibition was specific as α_{IIb}-derived peptide α_{IIb} 10–20 had no activity. As anticipated, the inhibitory effect of peptides on platelet adhesion to intact fibrinogen was less potent apparently due to the presence of strong γ404–411-binding sites (Table 1). In agreement with the clot retraction data, the equimolar mixtures of peptides did not produce additional inhibition of platelet adhesion to D98 (supplemental Fig. 4S).

To examine whether the α_{IIb}-derived peptides were able to inhibit binding of soluble fibrinogen, we tested their effect on platelet aggregation. Isolated platelets were preincubated with different concentrations of synthetic peptides or RGDV (positive control) for 10 min before the initiation of aggregation. Among selected peptides, α_{IIb} 64–78 inhibited platelet aggregation by ~30% at 200 μM (supplemental Fig. 5S). No inhibition

TABLE 1

Effect of the α_{IIb} β-propeller-derived peptides on platelet-mediated fibrin clot retraction and platelet adhesion to fibrinogen or its D98 fragment

The potency of each peptide in adhesion and clot retraction assays was determined as described under "Experimental Procedures."

Peptide	Sequence	Adhesion to D98[a]	Adhesion to Fg[a]	Clot retraction	
				IC_{50}	Lag phase[b]
		%	%	μM	min
Control, no peptide		100	100		23 ± 0.5
α_{IIb} 64–78	QCPSLLFDLRDETRN	57 ± 19	74 ± 5.1	144 ± 6	35 ± 2.0
α_{IIb} 94–108	GASVVSWSDVIVACA	43 ± 5	81 ± 5.2	116 ± 9	38 ± 2.0
α_{IIb} 153–162	RIYVENDFSW	67 ± 15	90 ± 2.2	200 ± 6	25 ± 1.0
α_{IIb} 229–237	EYFDGYWGY	60 ± 20	78 ± 2.4	262 ± 10	26 ± 0.5
α_{IIb} 241–255	VGEFDGDLNTTEYVV	71 ± 2	68 ± 9.0	72 ± 6	40 ± 4.0
α_{IIb} 361–375	APLGDLDRDGYNDIA	55 ± 6	105 ± 9.7	No inhibition	23 ± 0.5
α_{IIb} 421–435	LRGAVDIDDNGYPDL	55 ± 14	73 ± 3.8	165 ± 7	35 ± 2.0
α_{IIb} 10–20	FYAGPNGSQFG	100	100	No inhibition	23 ± 0.5

[a] The potency of the α_{IIb}-derived peptides expressed as the maximal platelet adhesion to D98 or Fg attained in the presence of 250 μM each peptide.
[b] Lag phase of clot retraction in the presence of 50 μM each peptide was determined as the time spanned from the onset of the process until the first visible changes in clot morphology are observed.

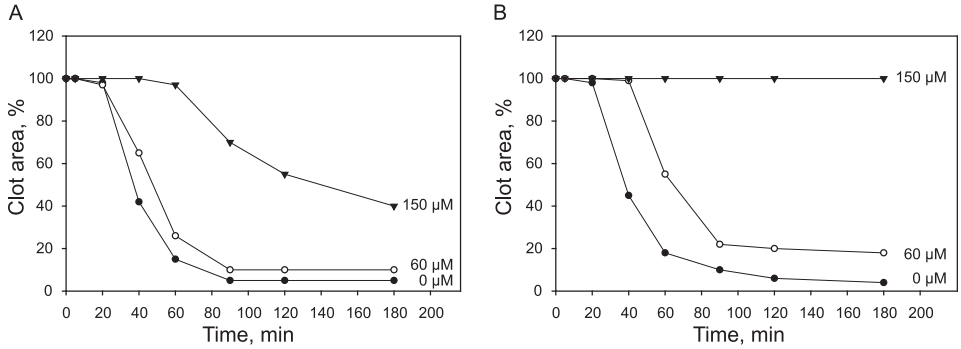

FIGURE 4. **Effect of the α_{IIb} 241–255 peptide on platelet-mediated retraction of clots formed from mutant γ407 and normal recombinant fibrinogens.** Platelets (2×10^8/ml) were mixed with 0.25 mg/ml normal fibrinogen (*A*) and Fg γ407 (*B*) in isotonic HEPES buffer containing 1 mM $CaCl_2$ and 60 μM (○) or 150 μM (▼) α_{IIb} 241–255 (final volume, 0.25 ml). Fibrin clots were formed by adding 1 unit/ml thrombin at 22 °C, and clot retraction was observed by taking photographs at different times. Kinetic curves of retraction of clots formed from normal fibrinogen in the absence (●) or presence of α_{IIb} 241–255 were generated by plotting clot areas *versus* time.

of aggregation by other peptides was detected. As expected, RGDV blocked aggregation in a dose-dependent manner and completely inhibited it at 100 μM. These observations lend further support to the idea that the sites responsible for binding of soluble fibrinogen are different from those involved in the interaction of $\alpha_{IIb}\beta_3$ with insoluble forms of fibrin(ogen).

Localization of Critical Amino Acid Residues in the P3-binding Clusters—With the above data indicating that the α_{IIb}-derived peptides are able to inhibit platelet-mediated clot retraction, we sought to identify critical residues for P3 binding. Additional peptide libraries in which each residue in the identified clusters was mutated to Ala were synthesized and examined for P3 binding (supplemental Fig. 6S). On the basis of densitometry analyses, mutation of Leu^{69}, Phe^{70}, Asp^{71}, Asp^{74}, Glu^{75} (cluster 1), Trp^{100}, Asp^{102} (cluster 2), Glu^{157}, Asp^{159}, Glu^{168}, Trp^{162} (cluster 3), Trp^{235}, Glu^{243} (cluster 4), Asp^{247}, Glu^{252}, Tyr^{253} (cluster 5), Asp^{369} (cluster 7), Asp^{426}, Asp^{428}, and Asp^{429} (cluster 8) to Ala resulted in a >70% loss of P3 binding (Table 2). Furthermore, mutations of Leu^{68}, Trp^{162}, Tyr^{166}, Asp^{224}, Glu^{229}, Tyr^{230}, Phe^{231}, Tyr^{237}, Asp^{365}, Asp^{367}, Tyr^{371}, Asp^{373}, Gly^{423}, and Asp^{434} reduced P3 binding by ~40–70% (Table 2). The finding that the majority of critical residues in the α_{IIb} β-propeller are negatively charged is consistent with the high positive charge of P3. In addition, hydrophobic residues (Leu and Phe) and aromatic residues (Trp and Tyr) were found to contribute to binding. The role of these residues is also highlighted by the finding that not all negatively charged peptides in the α_{IIb} β-propeller scan bound P3 (*e.g.* ^{43}LGPSQEETG^{51}, ^{115}VLEKTEEAE^{123}, and ^{295}VTDVNGDGR^{303}).

The analyses of the three-dimensional structure of the α_{IIb} β-propeller (Protein Data Bank code 2VDO (6)) indicated that not all residues in the P3 binders are exposed on the surface (supplemental Fig. 6S). The following residues that are exposed on the surface of the α_{IIb} β-propeller and when mutated to Ala exhibited significant loss of P3 binding were selected as initial candidates for subsequent analyses: Leu^{68}, Leu^{69}, Phe^{70}, Asp^{71}, Asp^{74}, Glu^{75} (cluster 1), Trp^{100}, Asp^{102} (cluster 2), Glu^{157}, Asp^{159}, Trp^{162} (cluster 3), Asp^{224}, Glu^{229}, Phe^{231}, Trp^{235} (cluster 4), Glu^{243}, Asp^{247}, Glu^{252} (cluster 5), Asp^{365}, Asp^{367}, Asp^{369}, Tyr^{371} (cluster 7), Asp^{426}, Asp^{428}, and Asp^{429} (cluster 8).

HEK293 Cells Expressing Mutant $\alpha_{IIb}\beta_3$ Receptors Support Reduced Cell Adhesion and Clot Retraction—To determine whether the residues identified as critical in the experiments above are involved in the $\alpha_{IIb}\beta_3$ function in adhesion and clot retraction, we replaced them with corresponding residues of the I-less α_M β-propeller of integrin $\alpha_M\beta_2$. The rationale for this strategy is based upon the observations that, although both the α_{IIb} and I-less α_M β-propeller domains have relatively high homology (30% identical residues and 48% conservative substitutions) and both $\alpha_{IIb}\beta_3$ and $\alpha_M\beta_2$ bind fibrinogen, it is the α_MI domain inserted between the second and third repeats of the α_M propeller that is responsible for the binding of fibrinogen by

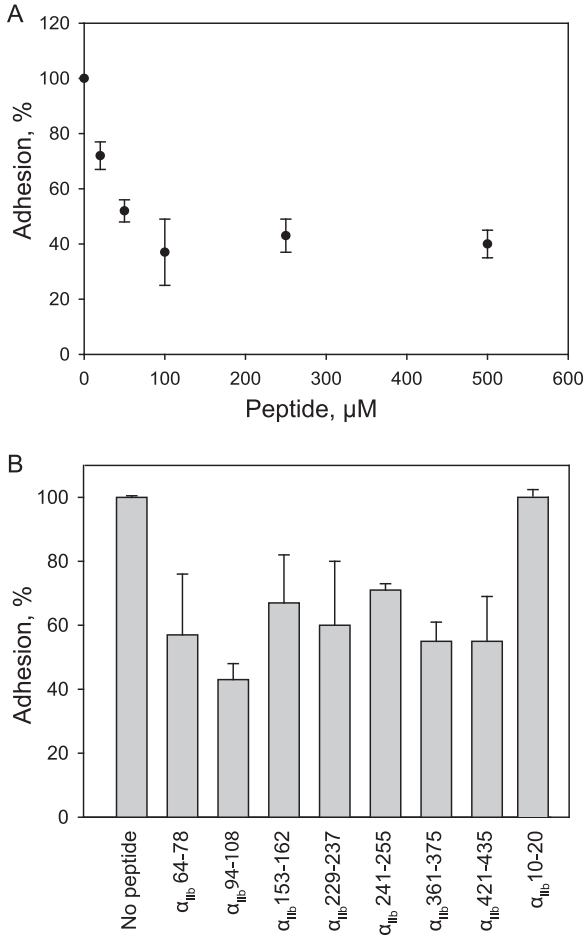

FIGURE 5. **Effect of the α_{IIb} β-propeller-derived peptides on platelet adhesion.** Microtiter wells were coated with 10 μg/ml D98 fragment and postcoated with 1% BSA. 50-μl aliquots containing different concentrations of the α_{IIb}-derived peptides in isotonic HEPES buffer were added to the wells for 15 min at 37 °C followed by suspensions of calcein-labeled platelets (1×10^7/50 μl). After 30 min at 37 °C, nonadherent cells were removed, and adhesion was determined. *A*, a dose-dependent inhibition of adhesion by α_{IIb} 94–108 is shown. *B*, inhibition of platelet adhesion by each peptide used at 250 μM is shown. The data are expressed as the percentage of adhesion in the absence of peptides. *Error bars* represent S.D. The results shown are the average of triplicate measurements at each experimental point and are representative of three to five experiments.

TABLE 2
Densitometry analyses of P3 binding to the substitutional peptide libraries derived from the active α_{IIb} β-propeller clusters in which each residue was mutated to Ala

Amino acid	Reactivity[a]
	%
Leu[68]	50
Leu[69]	**81**
Phe[70]	**89**
Asp[71]	**94**
Asp[74]	**80**
Glu[75]	**85**
Trp[100]	**89**
Asp[102]	**95**
Glu[157]	**92**
Asp[159]	**93**
Trp[162]	64
Tyr[166]	40
Glu[168]	**90**
Asp[224]	40
Glu[229]	67
Tyr[230]	36
Phe[231]	41
Trp[235]	**72**
Tyr[237]	43
Val[241]	31
Glu[243]	**89**
Asp[247]	**70**
Glu[252]	**89**
Tyr [253]	**70**
Asp[365]	52
Asp[367]	53
Asp[369]	**82**
Tyr[371]	43
Asp[373]	65
Gly[423]	43
Asp[426]	**75**
Asp[428]	**70**
Asp[429]	**73**

[a] The loss of reactivity is shown as a percentage of binding to wild-type peptides. Mutations that resulted in the loss of ≥70% binding are in boldface.

$\alpha_M\beta_2$. The deletion of the α_MI domain generates the I-less integrin, which supports ~10–15% adhesion to fibrinogen compared with wild-type $\alpha_M\beta_2$ (37). Because of the considerable homology between the two propellers, substitutions of individual residues in α_{IIb} propeller with corresponding α_M residues are not expected to alter the conformation of the mutant receptor. The sequence alignment of two domains (supplemental Fig. 7S) revealed that many residues identified as candidates for P3 binding, especially those that are negatively charged, are not conserved between the two domains. For example, the ^{69}LFDLRDE^{75} sequence (cluster 1; critical residues are underlined) in the α_{IIb} β-propeller is replaced with RLQVPVE in the α_M β-propeller. Thus, Leu[69], Asp[70], and Asp[74] were substituted with homologous residues in α_M. Glu[75], which is identical in both propellers, remained unchanged. In another example, Trp and Asp in α_{IIb} ^{100}WSD^{102} were replaced with Ser and Pro present in the homologous α_M SPP sequence. Residues Asp[224], Glu[229], and Phe[231] in cluster 4 were excluded from the analyses because Asp[224] and Phe[231] coordinate γLys[406] in soluble fibrinogen (6); therefore, it is unlikely that residues in this segment bind P3. In addition, in view of high homology between the α_{IIb} residues in cluster 7 and those in α_M, they were left unchanged. The residues that have been mutated are boxed in supplemental Fig. 7S. Wild-type and mutant α_{IIb} subunits were transfected into the HEK293 cells together with wild-type β_3 subunit, and stable cell lines carrying mutant integrins were established. Heterodimer association of mutants was evaluated by immunoprecipitation of detergent-lysed surface-labeled cells, and mutant cell lines were sorted to select the expressors with similar levels of integrins (supplemental Fig. 8S).

The effect of point mutations on the $\alpha_{IIb}\beta_3$ function was explored using adhesion and clot retraction assays. Fig. 6*A* shows adhesion of wild-type $\alpha_{IIb}\beta_3$-expressing HEK293 cells and W100S/D102P mutant as an example to the increasing concentrations of D98. To exclude the potential effect of $\alpha_v\beta_3$ on cell adhesion to D98 (38), cells were preincubated with anti-$\alpha_v\beta_3$ mAb LM609. Adhesion of wild-type and mutants cells reached a maximal level at 10 μg/ml D98, and the effect of mutations was expressed as a percentage of maximal adhesion attained with wild-type expressing cells. As shown in Fig. 6*B*, adhesion of cells expressing W100S/D102P, E157A/D159S/W162F, W235L, E243L/D247A/E252S, and D248T/D429M mutants was reduced by ~40–70% of WT cells. Adhesion of cells expressing the L69R/D71Q/D74V triple mutant was not impaired (not shown), and that of cells expressing the quadru-

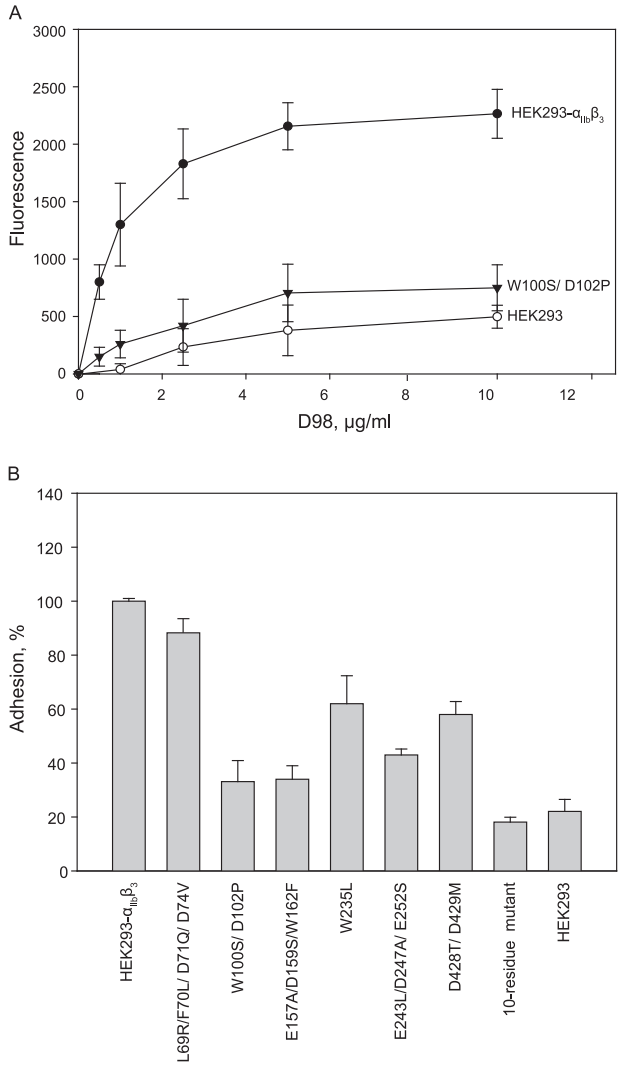

FIGURE 6. **Adhesion of HEK293 cells expressing wild-type and mutant $\alpha_{IIb}\beta_3$ receptors.** *A*, adhesion of HEK293 cells expressing the α_{IIb} β-propeller double point mutant W100S/D102P is shown. Microtiter wells were coated with different concentrations of the D98 fragment (0–10 μg/ml). Calcein-labeled HEK293 cells expressing WT (●) or mutant (W100S/D102P) integrin $\alpha_{IIb}\beta_3$ (▼) were added to the wells (5×10^4 cells/well) and allowed to adhere for 30 min at 37 °C. After washing of non-adherent cells, cell adhesion was assessed by measuring fluorescence. Adhesion on WT HEK293 cells (○) was also determined. *B*, adhesion of each mutant to different concentrations of D98 was performed as described in *A*. Adhesion of each mutant reached the maximal level at 10 μg/ml D98 and is shown as a percentage of maximal adhesion attained with HEK293 cells expressing WT $\alpha_{IIb}\beta_3$. The data are means ± S.D. (*error bars*) of triplicate determinations at experimental data point and are representative of 3–5 experiments.

ple L69R/F70L/D71Q/D74V mutant was reduced by ~20%. A mutant in which L69R, D71Q, D74V, W100S, D102P, W235L, E243L, E252S, D428T, and D429M (10-residue mutant) were simultaneously mutated supported ~20% adhesion (Fig. 6*B*).

Further evidence for the role of selected residues in $\alpha_{IIb}\beta_3$ function was obtained in clot retraction experiments. These analyses were performed in the presence of mAb LM609 to block $\alpha_v\beta_3$, which is known to support clot retraction of HEK293 cells transfected with the β_3 integrin subunit (30). While cells expressing wild-type $\alpha_{IIb}\beta_3$ supported clot retraction, retraction of cells expressing the 10-residue mutant was significantly delayed. Compared with cells bearing wild-type integrins that began to retract clots after ~38 min, the lag phase for mutant cells was prolonged to ~100 min (Fig. 7). Furthermore, the final clot size retracted by mutant cells after 4 h was ~70% compared with 45% retracted by cells expressing wild-type $\alpha_{IIb}\beta_3$. Clot retraction of cells expressing E157A/D159S/W162F, E243L/D247A/E252S, and D428T/D429M mutants was also delayed (Table 3); however, final clot size was not significantly different from that retracted by cells expressing wild-type receptor. No significant change in clot retraction by the L69R/F70L/D71Q/D74V mutant cell line was detected, suggesting that the loss of one site may be compensated by another site(s). The activity of the W100S/D102P cell line was not tested. These data indicate that mutations of selected negatively charged and aromatic residues in $\alpha_{IIb}\beta_3$ impair the interaction of the receptor with fibrin required for the development of contractile force during clot retraction.

DISCUSSION

In this study, we characterized the interaction of integrin $\alpha_{IIb}\beta_3$ with the fibrin-specific peptide P3 (γ^{370}-ATWKTRWYSMKK^{381}) and identified residues critical for P3 binding in the α_{IIb} β-propeller domain of the receptor. The analyses of the binding data obtained by SPR indicate that P3 can bind to multiple sites in $\alpha_{IIb}\beta_3$. In agreement with this finding, P3 bound to various peptides in the peptide library spanning the sequence of the α_{IIb} β-propeller. The peptides duplicating the P3-binding sequences inhibit clot retraction and platelet adhesion but not platelet aggregation. A common feature of the peptides is their enrichment with negatively charged and aromatic residues. Indeed, substitutions of Leu ^{69}, Phe^{70}, Asp^{71}, Asp^{74}, Trp^{100}, Asp^{102}, Glu^{157}, Asp^{159}, Trp^{162}, Trp^{235}, Glu^{243}, Asp^{247}, Glu^{252}, Asp^{428}, and Asp^{429} in the α_{IIb} β-propeller blocked adhesion and clot retraction mediated by HEK293 cells expressing mutant receptors. These amino acid residues potentially represent the sites through which $\alpha_{IIb}\beta_3$ contacts fibrin fibers during clot retraction.

The amino acid residues identified as critical for P3 binding in the α_{IIb} β-propeller are largely different from those that coordinate the fibrinogen recognition peptide γ^{404}-GAKQAGDV^{411} and situated at the interface between the α_{IIb} β-propeller and β_3 I domains (6). One notable exception is α_{IIb} Asp^{224} and α_{IIb} Phe^{231}, mutation of which in peptides constituting the substitutional peptide library (supplemental Fig. 6S) modestly reduced P3 binding. However, the fact that synthetic peptide ^{229}EYFDGYWGY^{237}, which contains Phe^{231}, does not inhibit platelet aggregation, although it efficiently blocks clot retraction and platelet adhesion, suggests that its activity depends on other residues. Indeed, mutations of Glu^{229} and Trp^{235} strongly reduced P3 binding and decreased adhesion of HEK293 cells expressing $\alpha_{IIb}\beta_3$ carrying the W235L mutation. The P3-binding residues are also distinct from 40 discontinuous residues in the α_{IIb} β-propeller identified by Takada and co-workers (32, 33) as critical for binding of soluble fibrinogen. Those residues have been mapped to the loops in repeats 2–4 and at the boundary between repeats 4 and 5 of the α_{IIb} β-propeller. The crystal structure of $\alpha_{IIb}\beta_3$ in complex with the γC peptide (6) has subsequently revealed that among these residues not only are those that coordinate γC but many residues

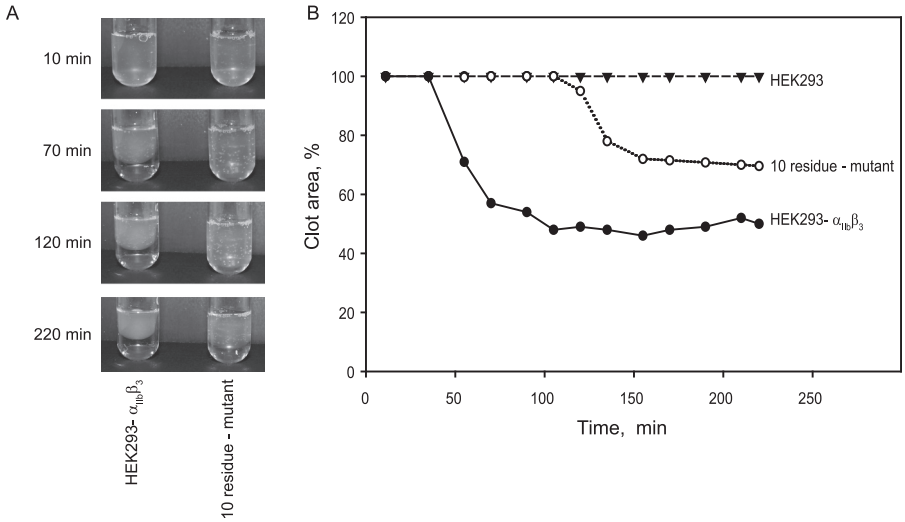

FIGURE 7. **Clot retraction mediated by HEK293 cells expressing mutant $\alpha_{IIb}\beta_3$.** *A*, aliquots (2×10^6 cells/ml) of HEK293 cells stably expressing WT (*left panel*) or mutant $\alpha_{IIb}\beta_3$ (*right panel*)in Tyrode's/HEPES buffer were incubated with 10 mM tranexamic acid, 0.25 mg/ml fibrinogen, and 2 mM $CaCl_2$ in siliconized glass tubes for 5 min at 37 °C. mAb LM609 (10 μg/ml) was added to block $\alpha_v\beta_3$-mediated clot retraction. Fibrin polymerization and clot retraction were initiated by adding 1 unit/ml thrombin to cell suspensions, and fibrin gels were incubated at 37 °C for the next 220 min. Clot retraction was monitored by taking digital photographs. Representative photographs of retracted gels are shown. *B*, kinetic curves of retraction by WT $\alpha_{IIb}\beta_3$ (●) or mutant receptor carrying 10 point mutations (○) were generated by plotting clot areas *versus* time. HEK293 cells (▼) do not support clot retraction. The data are expressed as percentage of clot retraction as described under "Experimental Procedures." Results are representative of three separate experiments.

TABLE 3

HEK293 cells expressing mutant $\alpha_{IIb}\beta_3$ receptors support delayed clot retraction

Cell line	Lag phase	Final clot volume
	min	*%*
WT $\alpha_{IIb}\beta_3$	38 ± 3	42 ± 6
E157A/D159S/W162F	50 ± 2	43 ± 3
E243L/D247A/E252S	90 ± 5	50 ± 3
D428T/D429M	42 ± 3	46 ± 5
10-Residue mutant	110 ± 8	70 ± 4

that form the α_{IIb} cap subdomain, the region of the α_{IIb} β-propeller where epitopes for several function-blocking antibodies were identified (33, 39). In the three-dimensional structure of the α_{IIb} β-propeller, the P3-binding residues Leu^{69}, Phe^{70}, Asp^{71}, Asp^{74}, Trp^{100}, and Asp^{102} surround Insert 1 of the cap subdomain; Glu^{157}, Asp^{159}, and Trp^{162} are present within Insert 3 of the cap subdomain; and Trp^{235} is adjacent to Insert 4 of the cap (Fig. 8). Other residues, including Glu^{243}, Glu^{252}, Asp^{428}, and Asp^{429}, are found within or in the vicinity of the Ca^{2+}-binding sites located in blades W4 and W6 of the β-propeller.

Identification of distinct binding sites for the γC peptide, whole fibrinogen, and the P3 peptide is consistent with a mechanism where $\alpha_{IIb}\beta_3$ exhibits differential recognition specificity for soluble fibrinogen and the insoluble fibrin matrix in platelet aggregation and clot retraction. The binding of soluble fibrinogen to $\alpha_{IIb}\beta_3$ during platelet aggregation was described as a two-step process with potential engagement of different amino acid residues at each step. Accordingly, the binding of soluble fibrinogen to agonist-activated platelets results in the formation of platelet aggregates that can dissociate under certain conditions (10, 40). The underlying mechanism for this phenomenon appears to be a reversible binding of fibrinogen to platelets (40). That initial binding of fibrinogen to $\alpha_{IIb}\beta_3$ is mediated by γC ^{408}AGDV^{411} has been documented in numerous studies that showed that recombinant fibrinogen lacking this sequence does not support platelet aggregation (9, 14) and was recently confirmed with isolated receptor (41). Reversible platelet aggregation is followed by an irreversible step that was proposed to result from the progressive stabilization of the complex between $\alpha_{IIb}\beta_3$ and fibrinogen (10, 11). Indeed, biophysical studies revealed the two-step binding mechanism of fibrinogen binding to isolated $\alpha_{IIb}\beta_3$ in which fast weak binding is followed by slow strong complex formation (42, 43). Although conclusive data are not available and other mechanisms may account for the irreversible step, it is reasonable to assume that stabilization of the complex between $\alpha_{IIb}\beta_3$ and fibrinogen involves the amino acid residues in the α_{IIb} β-propeller cap domain (33, 39). These residues together with those that coordinate the γC sequences may constitute the complete integrin-fibrinogen binding interface formed during platelet aggregation.

In contrast to platelet aggregation, the interaction of $\alpha_{IIb}\beta_3$ with fibrin is strongly associated with the development of platelet contractile activity. A requirement for clot retraction is the spatially even distribution of platelets within fibrin because preaggregated platelets do not retract clots (44). On the basis of electron microscopic studies of clots retracting under isometric tension, it has been proposed that close apposition of platelet pseudopods to long fibrin fibers is the major mechanism for the transmission of contractile force (45). As pseudopods crawl and pull on bound fibrin, the fibers become stretched and aligned in the direction of tension. The contacts between the platelet surface and fibrin strands in retracting clots were observed across a ~15-nm space established by the structures that initially were called "stubs" and presumably represent integrins (46, 47). In contrast, the interplatelet bridges in aggregating platelets span the space of ~50 nm (48, 49). Another distinction between platelet-fibrin and platelet-fibrinogen interactions is their sensitivity to EDTA: *i.e.* although EDTA-treated and then washed platelets do not aggregate in response to agonists, they support normal clot retraction (50). From the comparison of these char-

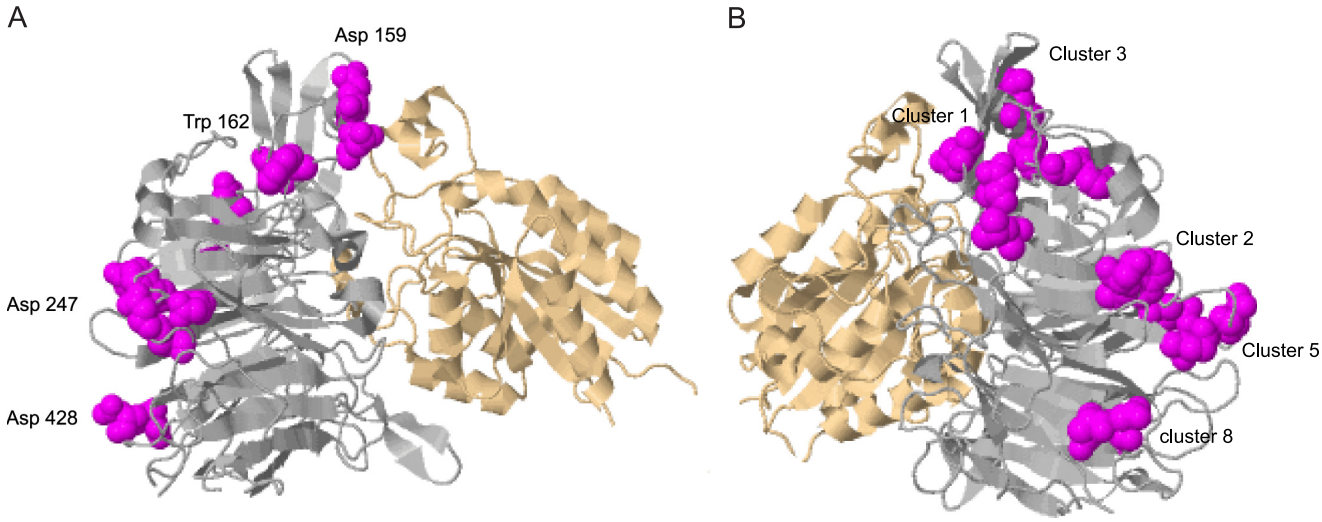

FIGURE 8. **The ribbon model of the $\alpha_{IIb}\beta_3$ headpiece based on the crystal structure (Protein Data Bank code 2VDO).** The α_{IIb} subunit is shown in *gray*, and β_3 is shown in *tan*. Amino acid residues identified as critical for the binding of fibrin-specific peptide P3 in the α_{IIb} β-propeller are shown in *magenta* (selected residues are labeled). Two views (*A* and *B*) are rotated relative to each other by 180° about the vertical axis.

acteristics of platelet aggregation and clot retraction, it is clear that $\alpha_{IIb}\beta_3$ is involved in two separable processes utilizing different mechanisms. Therefore, as a receptor for polymerizing fibrin, $\alpha_{IIb}\beta_3$ may engage the binding sites that are different from those required for platelet aggregation. Indeed, clot retraction does not absolutely depend on the γC-binding site because recombinant fibrinogen with γ^{408}AGDV^{411} missing or fibrinogen from mice in which the γC domain was targeted to delete γ^{407}QAGDV^{411} supports substantial clot retraction (16, 20). The proposal that P3 serves as the binding site for $\alpha_{IIb}\beta_3$ in fibrin was put forward based upon the ability of mAb 2G5 directed against P3 and synthetic peptides duplicating the P3 sequence to inhibit platelet-mediated clot retraction (15). It has also been shown that natural and recombinant fibrinogens with mutations in the P3 sequence exhibit delayed clot retraction (25). The direct interaction between P3 and $\alpha_{IIb}\beta_3$ was confirmed by affinity chromatography using a P3-agarose affinity matrix (15). Further evidence that $\alpha_{IIb}\beta_3$ utilizes distinct recognition specificity toward fibrin comes from the fact that P3 is poorly exposed in soluble fibrinogen and becomes available after its conversion to fibrin (12). Because during clot retraction platelet-fibrin clumps stretch and align fibrin in the direction of tensile force, it is tempting to speculate that P3 may become fully exposed under tension. It should be noted that even though the γC sequence may be exposed on the surface of fibrin fibers it is unlikely that $\alpha_{IIb}\beta_3$ is capable of utilizing it. In fibrin, γLys^{406}, one of the amino acid residues that coordinate several residues in α_{IIb} (6), is cross-linked by Factor XIIIa to γGln$^{399/398}$ on the neighboring molecule and may not be available for $\alpha_{IIb}\beta_3$ binding.

The identification of several P3-binding segments in the α_{IIb} β-propeller suggests a model in which a single receptor may form multiple low affinity contacts with a fibrin polymer. The P3-$\alpha_{IIb}\beta_3$ interactions are largely electrostatic; *i.e.* positively charged P3 binds negatively charged amino acid residues in the α_{IIb} β-propeller, although aromatic residues are also involved. The relatively limited requirements for P3 recognition by the α_{IIb} β-propeller domain imply that other sequences in fibrin(ogen) that contain analogous combinations of amino acid residues or have similar physicochemical properties could function as the $\alpha_{IIb}\beta_3$-binding sites in fibrin. For example, the second homologous domain in the D region of fibrinogen, βC, contains a sequence highly homologous to P3 (β^{438}MNWKGSWYSMRK^{449}). In contrast to platelet aggregation where each $\alpha_{IIb}\beta_3$ binds a single fibrinogen molecule, during clot retraction, the receptor makes contacts with already preformed fibrin. In fibrin polymer, the formation of fibers through the lateral association of protofibrils may cluster the P3 and β438–449 sequences in the interacting γC and βC domains. Although the lateral association of protofibrils that gives rise to mature fibrin has not been defined at the level of atomic resolution, the packing of human and chicken fibrinogens in crystals revealed several interactions that have been proposed to be good candidates for those that occur in fiber formation, including the γC-γC and βC-βC interfaces (51, 52). It is not impossible that each $\alpha_{IIb}\beta_3$ molecule may establish multiple contacts with P3 and/or the βC sequences brought together in fibrin. However, an alternative possibility is that P3 and P3-like sequences may indiscriminately engage one of the negatively charged clusters in α_{IIb} β-propeller.

Among seven P3-binding sites, three have been identified in the segments that span the Ca^{2+}-binding sites in blades W4, W6, and W7. Within these peptides, for example in α_{IIb} 241–255 that spans Ca^{2+}-binding site 1 in W4 and includes flanking residues, mutations of several negatively charged residues, including Glu^{243}, Asp^{247}, and Glu^{252}, resulted in the loss of P3 binding (Table 2 and supplemental Fig. 6S). Among these residues, Glu^{243} and Asp^{247} provide metal-coordinating side-chain oxygen atoms, whereas Glu^{252} is outside of the 9-residue Ca^{2+}-coordinating segment (Ref. 53 and references therein). Likewise, although mutations of both Asp^{428} and Asp^{429} in site 4 reduced P3 binding, Asp^{429} is not involved in metal coordination. Mutations of these residues introduced into the whole receptors reduced cell adhesion and clot retraction (Fig. 6 and Table 3). At first glance, this finding might seem to indicate that the loss of Ca^{2+} resulted in the alteration of the receptor con-

formation, providing support for previous reports that Ca^{2+}-binding sites are essential for α_{IIb} folding and $\alpha_{IIb}\beta_3$ heterodimer formation (53). However, receptors carrying triple and double mutations in the Ca^{2+}-binding sites were assembled normally and expressed on the cell surface at levels comparable with that of WT integrin, suggesting that even though the local conformation may be distorted it does not hamper the biogenesis of integrin. Furthermore, clot retraction is only marginally sensitive to Ca^{2+} (50).[4] Although the dependence on the α_{IIb} negatively charged residues is compatible with the overall P3 cationic nature, other residues in the vicinity of or within the Ca^{2+}-binding sites, including Tyr^{253}, Tyr^{371}, and Gly^{423}, may contribute to binding (supplemental Fig. 6S). Thus, although coordination of calcium by oxygen atoms from side chains of Asp and Glu reduces the negative surface electrostatic potential of this region, other negatively charged and aromatic residues in the vicinity may be involved in P3 binding. One puzzling observation is that no binding of either P3 or the DD fragment was detected to peptides duplicating or overlapping Ca^{2+}-binding site 2 in W5. The lack of DD binding to this region is consistent with previous studies (33) showing that swapping the Ca^{2+}-binding loop W5:1–2 did not affect the binding of soluble fibrinogen; however, the reason for the absence of P3 binding is not clear. Like others, the Ca^{2+}-binding site in W5 contains negatively charged residues forming the consensus motif. The only difference between the residues that constitute this Ca^{2+}-binding site is the absence of aromatic residues present in sites 1, 3, and 4 that may impart additional specificity to P3 recognition. Previous studies showed that the β-propeller Ca^{2+}-binding sites are involved in $\alpha_{IIb}\beta_3$-fibrinogen interactions and in binding $\alpha_4\beta_1$ to several ligands (54, 55). Clarifying whether the Ca^{2+}-binding sites also contribute to fibrin recognition and the role of Ca^{2+} in this process will require further efforts.

It has long been proposed that inhibition of platelet interactions with fibrin may be a necessary and important property of $\alpha_{IIb}\beta_3$ antagonists (5). Recent studies using intravital confocal microscopy as well as traditional histological methods have demonstrated the presence of fibrin in early thrombi (1–3), suggesting that $\alpha_{IIb}\beta_3$ may interact with fibrin not only in retracting clots that contain large masses of fibrin but also during platelet aggregation. The role of $\alpha_{IIb}\beta_3$-fibrin interactions in platelet aggregation is consistent with the finding of the unique GPIb-thrombin pathway that does not depend on the binding of fibrinogen to platelets but instead requires fibrin (56). The relatively simple complementarity between the cationic P3 site and negatively charged residues in the α_{IIb} β-propeller may explain a well known ability of various positively charged compounds, including natural polyamines, to interact with platelets and modulate their responses (57–59). Likewise, negatively charged compounds may affect the $\alpha_{IIb}\beta_3$-fibrin interactions. Consistent with this proposal, polyphosphates released from activated platelets (35) inhibit clot retraction.[5] Further studies may help to define the reagents that specifically target platelet-fibrin bonds in thrombus formation.

[4] N. P. Podolnikova, unpublished data.
[5] N. P. Podolnikova, T. P. Ugarova, and J. Morrissey, unpublished data.

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