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Amino Acid Residues in the α_{IIb} Subunit That Are Critical for Ligand Binding to Integrin $\alpha_{IIb}\beta_3$ Are Clustered in the β-Propeller Model*

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Several distinct regions of the integrin α_{IIb} subunit have been implicated in ligand binding. To localize the ligand binding sites in α_{IIb}, we swapped all 27 predicted loops with the corresponding sequences of α_4 or α_5. 19 of the 27 swapping mutations had no effect on binding to both fibrinogen and ligand-mimetic antibodies (*e.g.* LJ-CP3), suggesting that these regions do not contain major ligand binding sites. In contrast, swapping the remaining 8 predicted loops completely blocked ligand binding. Ala scanning mutagenesis of these critical predicted loops identified more than 30 discontinuous residues in repeats 2–4 and at the boundary between repeats 4 and 5 as critical for ligand binding. Interestingly, these residues are clustered in the predicted β-propeller model, consistent with this model. Most of the critical residues are located at the edge of the upper face of the propeller, and several critical residues are located on the side of the propeller domain. None of the predicted loops in repeats 1, 6, and 7, and none of the four putative Ca^{2+}-binding predicted loops on the lower surface of the β-propeller were important for ligand binding. The results map an important ligand binding interface at the edge of the top and on the side of the β-propeller toroid, centering on repeat 3.

The integrin $\alpha_{IIb}\beta_3$ (glycoprotein IIb-IIIa, CD41/CD61) plays a critical role in primary hemostasis by mediating interactions between platelets and fibrinogen (1). Interaction between $\alpha_{IIb}\beta_3$ and fibrinogen is mediated by the C-terminal γ-chain sequence of fibrinogen (2). $\alpha_{IIb}\beta_3$ also binds to von Willebrand factor, vitronectin, and fibronectin through RGD sequences in these ligands (3). The α_{IIb} subunit has seven repeated sequences of ~60–70 residues each in its N-terminal portion. Repeats 4–7 have putative divalent cation binding motifs of the general structure D*X*D*X*DG*XX*D. Although a $\alpha_{IIb}\beta_3$-fibrinogen interaction is a therapeutic target, how ligands interact with $\alpha_{IIb}\beta_3$ has not been established. The second putative cation binding site of α_{IIb} (residues 294–314 in N-terminal repeat 5 of α_{IIb}) can be chemically cross-linked to the fibrinogen γ-chain C-terminal dodecapeptide ($HHLGGAKQAGDV^{400-411}$) (4). This peptide and antibodies against it have been shown to block binding of fibrinogen to $\alpha_{IIb}\beta_3$ (5). Stanley *et al.* (6) proposed that repeats 4–7, which contain these cation binding motifs, are folded as a calmodulin-like EF-hand structure. Consistent with this, recombinant bacterial proteins that consist of repeats 3–7 of α_{IIb} (residues 171–464) or repeats 4–7 or 3–7 of the integrin α_5 subunit (residues 229–448 or 160–448) have been shown to bind to ligand in a cation-dependent manner (7–9).

On the other hand, several lines of evidence suggest that the ligand binding site(s) in α_{IIb} are located in repeats 2–4. The 334 N-terminal residues in α_{IIb} regulate the ligand binding specificity of $\alpha_{IIb}\beta_3$ (10). A recombinant $\alpha_{IIb}\beta_3$ fragment that is composed of residues 1–233 of α_{IIb} and residues 111–318 of β_3 (designated "mini-integrin") has been shown to bind to an RGD-containing peptide (11). Residues that are critical for ligand binding and epitopes for function-blocking monoclonal antibodies (mAbs)[1] have consistently been located in repeats 2–4 of several integrin α subunits, regardless of ligand specificity (for review, see Ref. 12). Epitopes for multiple function-blocking antibodies have been mapped within this region of α_4 (13, 14), α_5 (15), and α_{IIb} (16). Mutating several amino acid residues that are clustered in the predicted loops in repeat 3 of the α subunit, or swapping the predicted loops in repeats 2–4, has been shown to block ligand binding of $\alpha_4\beta_1$, $\alpha_5\beta_1$, and $\alpha_{IIb}\beta_3$ (17, 18). We have recently localized epitopes for ligand-mimetic anti-$\alpha_{IIb}\beta_3$ antibodies (OP-G2 and LJ-CP3) within repeats 2–4 (16). However, these results do not rule out the possibility that ligands bind to other sequences of the α_{IIb} subunit.

In this study, we designed experiments to localize the ligand contact surface in α_{IIb} using loop swapping and site-directed mutagenesis. We systematically swapped all 27 predicted loops, the most likely candidates for ligand binding sites (19), in the α_{IIb} N-terminal sequence repeats with the corresponding regions of α_4 or α_5. We found that ligand binding was not affected by 19 of 27 swapping mutations using fibrinogen and ligand-mimetic mAbs as ligands, effectively ruling out the possibility that a major ligand binding site is located in repeats 1, 6, and 7. In contrast, swapping eight predicted loops in repeats 2–4 and one at the boundary between repeats 4 and 5 of α_{IIb} completely blocked $\alpha_{IIb}\beta_3$ interaction with ligands. We then

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The atomic coordinates and structure factors (code 1JX5) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).

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[1] The abbreviations used are: mAb(s), monoclonal antibody(ies); wt, wild type; CHO, Chinese hamster ovary; FITC, fluorescein-isothiocyanate; PE, phycoerythrin.

TABLE I
Anti-$\alpha_{IIb}\beta_3$ mAbs used in this study

mAbs	Function	Specificity	Epitopes (16)
LJ-CP3 and OP-G2	Ligand-mimetic (the RYD motif in the heavy chain CDR3)	$\alpha_{IIb}\beta_3$	156–162 and 229–230 of α_{IIb}, and 179–183 of β_3
2G12	Inhibitory	$\alpha_{IIb}\beta_3$	1–443 of α_{IIb}
A2A9	Inhibitory	$\alpha_{IIb}\beta_3$	179–183 of β_3
LJ-CP8	Inhibitory	$\alpha_{IIb}\beta_3$	156–159 of α_{IIb}
LJ-P9	Inhibitory	$\alpha_{IIb}\beta_3$	79–84 of α_{IIb}
AP-2	Inhibitory	$\alpha_{IIb}\beta_3$	171–174 of β_3
PT25–2	Activating	$\alpha_{IIb}\beta_3$	335–338 of α_{IIb}
PL98DF6	Nonfunctional	α_{IIb}	Outside the N-terminal 443 residues of α_{IIb}

identified several discontinuous residues within these predicted loops. It has been proposed that the N-terminal seven sequence repeats are folded into a β-propeller domain comprising seven four-stranded β-sheets arranged in a torus around a 7-fold pseudosymmetrical axis (20). We found that most of the residues that are critical for ligand binding are clustered in the proposed β-propeller model. These results predict that the ligand binding interface in $\alpha_{IIb}\beta_3$ localizes on the outer edge of the top and on the side of the β-propeller.

EXPERIMENTAL PROCEDURES

Monoclonal Antibodies and cDNAs

mAb 15 (21) was a kind gift from M. H. Ginsberg (Scripps Research Institute, La Jolla, CA). 2G12 (22) was from V. L. Woods (University of California San Diego). PL98DF6 (23) was from J. Ylänne (University of Helsinki, Finland). A2A9 (24) was from S. J. Shattil (Scripps). OP-G2 (25) was from Y. Tomiyama (Osaka University, Osaka, Japan). LJ-CP3, LJ-CP8 (26), and LJ-P9 (27) were from Z. M. Ruggeri (Scripps). AP-2 (28) was from T. J. Kunicki (Scripps). PT25-2 (29) was from M. Handa and Y. Ikeda (Keio University, Tokyo, Japan). α_{IIb} and β_3 cDNAs were obtained from J. C. Loftus (Scripps). The characteristics of these mAbs are summarized in Table I.

Methods

Construction and Transfection of cDNAs for Human α_{IIb} Swapping Mutants—Wild-type (wt) human α_{IIb} cDNA was subcloned into pBJ-1 vector. The expression vector of each mutant was constructed using overlap extension polymerase chain reaction (30) or site-directed mutagenesis (31). The swapping mutants were named after the predicted β-sheet in which they are located (W1–W7) and the topological position of the loop (4-1, 1-2, 2-3, and 3-4) in each repeat (Fig. 1). The presence of mutation was verified by DNA sequencing.

Transfection of CHO Cells—Twenty μg of wt and mutant α_{IIb} cDNA constructs in pBJ-1 vector were transfected by electroporation into β_3-CHO cells (1×10^7 cells) (18). Transfected cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum at 37 °C in 6% CO_2 for 2 days. Then the cells were detached with 3.5 mM EDTA and used for assays.

Flow Cytometry—Cells were washed once with Dulbecco's modified Eagle's medium and then resuspended in the same medium. Fifty μl of cell suspension was incubated with an equal volume of primary mAb (1:250 dilution of ascites, 10 μg/ml of purified mAb) on ice for 30 min. After washing with Dulbecco's modified Eagle's medium, cells were incubated with fluorescein-isothiocyanate (FITC)-conjugated anti-mouse IgG (BIOSOURCE, Camarillo, CA) for 30 min on ice.

Fibrinogen Binding—Human fibrinogen (Enzyme Research Laboratories, South Bend, IN) was labeled with FITC as described previously (32, 33). Fibrinogen binding to cells transiently expressing $\alpha_{IIb}\beta_3$ was determined as described previously (34) with some modifications. Briefly, cells were first incubated with PL98DF6 followed by phycoerythrin (PE)-conjugated anti-mouse IgG (BIOSOURCE). Cells were washed with modified Tyrode-Hepes buffer (5 mM Hepes, 5 mM glucose, 0.2 mg/ml bovine serum albumin, 1 × Tyrode's solution) supplemented with 2 mM $CaCl_2$ and 2 mM $MgCl_2$. Cells were then incubated with 150 μg/ml FITC-labeled fibrinogen in the presence of 10 μg/ml control mouse IgG or PT25-2 in the same buffer for 30 min. After removing unbound fibrinogen, cells were resuspended in Hepes-buffered saline supplemented with 2 mM $CaCl_2$ and 2 mM $MgCl_2$. Binding of fibrinogen (FITC staining) was analyzed on a gated subset of cells highly positive for $\alpha_{IIb}\beta_3$ expression (PE staining) in FACScan. Relative fibrinogen binding was calculated as $(F_{PT} - F_{mIgG})/(Fw_{PT} - Fw_{mIgG})$, where F_{PT} is the median fluorescence intensity of fibrinogen binding in the presence of PT25-2, F_{mIgG} is the median fluorescence intensity of fibrinogen binding in the presence of normal mouse IgG, Fw_{PT} is the median fluorescence intensity of fibrinogen binding to cells expressing wt $\alpha_{IIb}\beta_3$ in the presence of PT25-2, and Fw_{mIgG} is the median fluorescence intensity of fibrinogen binding to cells expressing wt $\alpha_{IIb}\beta_3$ in the presence of normal mouse IgG. Relative $\alpha_{IIb}\beta_3$ expression is a ratio of the median fluorescence intensity of PL98DF6 binding to the gated population to the median fluorescence intensity of PL98DF6 binding to the gated population expressing wt $\alpha_{IIb}\beta_3$.

α_{IIb} β-Propeller Model—Modeling was done with SegMod (35) of LOOK, version 2.0.5 (Molecular Applications Group, Palo Alto, CA) and MODELLER Release 4 (http://guitar.rockefeller.edu/modeler) (36). Templates were 1tbg, 1gof, and 1kap (http://www.pdb.bnl.gov). A LOOK model was made using the alignment shown in Fig. 1 between α_{IIb} and G protein transducin β subunit (37) (1tbg, gbeta). Additionally, three 3-4 loop templates of W5 of 1gof were used as templates for the 3-4 loops of W5, W6, and W7 as described previously (38). The 1-2 loops were then excised from W4–W7 of this model, and Ca^{2+} binding loops from 1kap were superimposed using four β-strand residues on either side of this loop from 1kap and 1tbg. A final model was made with MODELLER using the entire LOOK model as the initial (.ini) file, and using as templates 1) four different 1kap files containing only the residues shown in Fig. 1 and Ca^{2+} ions (39); 2) the LOOK model of α_{IIb} deleting the residues aligning with the 1kap loops and additionally two residues before and one residue after these loops in W4 and W5, and four residues before and one residue after these loops in W6 and W7; and 3) four circularly permuted, superimposed 1tbg β-propeller domains beginning with residue Thr-86 as shown in Fig. 1 or beginning with residues Glu-130, Thr-173, or Glu-215 (see 20).

RESULTS

Effect of Swapping Predicted Loops of α_{IIb} on Binding of Fibrinogen and Ligand Mimetic mAbs to $\alpha_{IIb}\beta_3$—We generated an α_{IIb} β-propeller model based on the alignment of α_{IIb} with the heterotrimeric G protein β subunit β-propeller domain (Fig. 1). The β-propeller contains seven radially arranged β-sheets, also called "W" because of their W-like topology, with four anti-parallel β-strands and three connecting loops within each sheet. In this model, the 1-2 and 3-4 loops, which connect β-strands 1 and 2 and β-strands 3 and 4, respectively, are located in the lower face of the model. The 4-1 loops, which connect β-strand 4 of one W with β-strand 1 of the next W, and the 2-3 loops, which connect β-strands 2 and 3, are located very close to each other in the upper face of the domain. Fig. 1 shows an alignment of the α_{IIb} sequence with those of integrin α_4 and α_5 subunits and the β-propeller domain of the G protein β subunit. The loops of each W are named after the β-strands they connect.

To identify the α_{IIb} sequences that are critical for ligand binding, we systematically replaced 27 predicted loop structures within residues 1–452 with the corresponding regions of α_4 or α_5 (Fig. 1), which have ligand binding specificities different from that of α_{IIb}. The segments that were swapped are *boxed* in Fig. 1. This strategy is based on the premise that

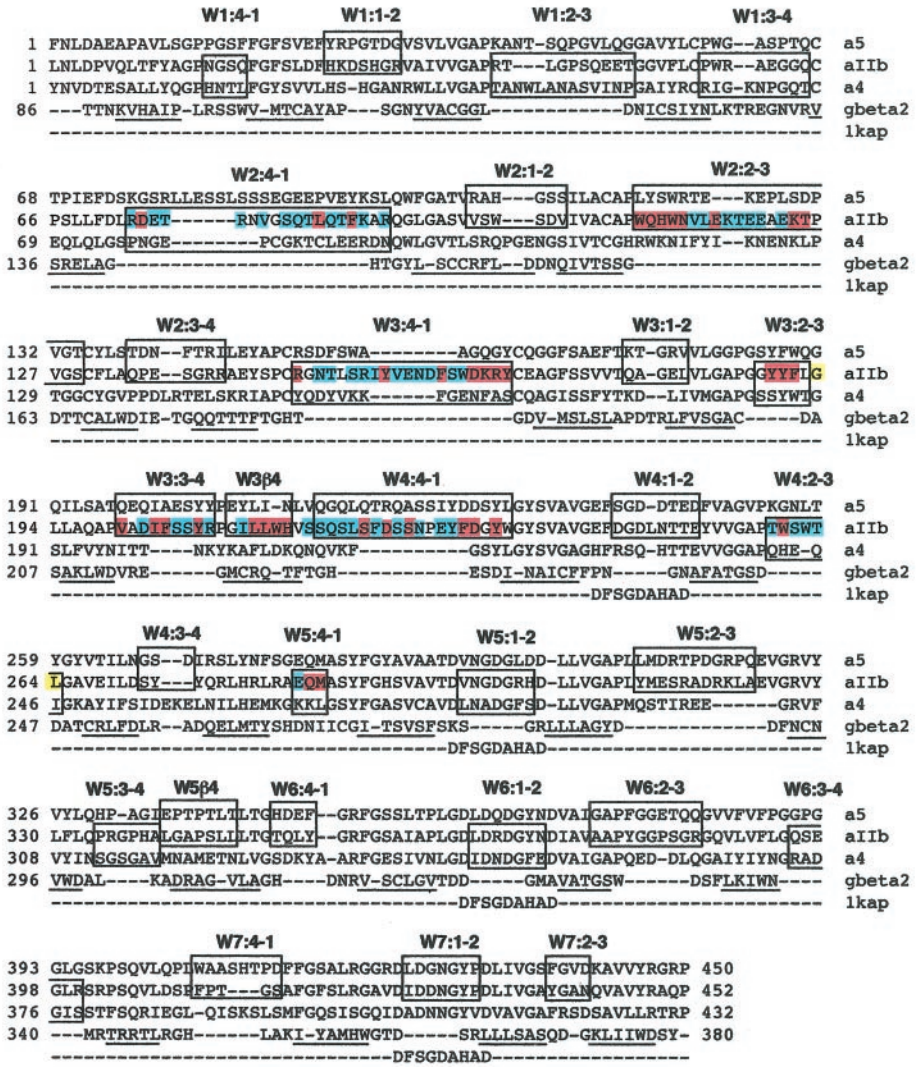

FIG. 1. **The predicted loops of α_{IIb} selected for swapping mutagenesis in this study.** Integrin α subunits have seven repeats of ~60–70 amino acid residues each at their N termini. We swapped all predicted loops of α_{IIb} (*boxed* regions) with the corresponding regions of α_4 or α_5. *W1–W7* represents repeats 1–7. The swapping mutants were named after the repeat in which they are located (W1–W7), and the topological position of the loop (4-1, 1-2, 2-3, and 3-4) in each repeat. The β-strands of gbeta2 are *underlined*. The 4-1, 1-2, 2-3, and 3-4 loops refer to the predicted loops between predicted β-strands 4 and 1, β-strands 1 and 2, β-strands 2 and 3, and β-strands 3 and 4, respectively (20). Amino acid residues that when mutated do and do not affect ligand binding are shown in *red* and in *light blue*, respectively. Amino acid residues that when mutated affect ligand binding and binding of many anti-α_{IIb} antibodies are shown in *yellow*. For details, see Table IV.

swapping homologous residues will block the ligand binding function if the swapped region determines the ligand binding function. This strategy has been used successfully to identify regions that are critical for ligand binding in several integrin α subunits (40, 41). The resulting α_{IIb} swapping mutants were transiently expressed in CHO cells that homogeneously express wt human β_3 (β_3-CHO cells). The 27 swapping mutants were all surface expressed based on flow cytometry of transfected cells. Typically 50–80% of transfected cells were positive with anti-α_{IIb} mAb PL98DF6 (data not shown).

We studied the ability of the α_{IIb} swapping mutants to bind to fibrinogen. Binding of FITC-labeled soluble fibrinogen to CHO cells expressing $\alpha_{IIb}\beta_3$ ($\alpha_{IIb}\beta_3$-CHO) was detected by flow cytometry. The $\alpha_{IIb}\beta_3$ expressed in CHO cells has been reported to be a low affinity form (42). Although $\alpha_{IIb}\beta_3$-CHO cells adhere to immobilized fibrinogen without activation, $\alpha_{IIb}\beta_3$ must be activated with mAb PT25-2 to bind to soluble fibrinogen. The mAb PT25-2 recognizes and activates $\alpha_{IIb}\beta_3$, but not $\alpha_v\beta_3$ (endogenous hamster α_v/exogenous human β_3), indicating that binding of FITC-labeled fibrinogen to $\alpha_{IIb}\beta_3$-CHO cells in the presence of PT25-2 is $\alpha_{IIb}\beta_3$-specific (18). Under the conditions used, parent CHO cells or β_3-CHO cells that express only $\alpha_v\beta_3$ did not bind to fibrinogen. Most of the swapping mutants (19 of 27) bound to fibrinogen upon activation with PT25-2 (Fig. 2), indicating that major ligand binding sites are not present in these predicted loops. In contrast, the W2:4-1 (residues 73–90),

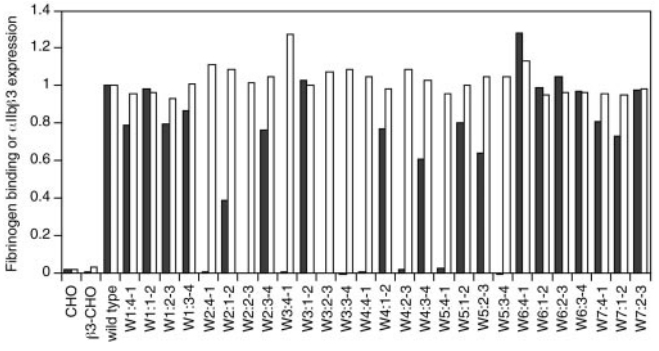

FIG. 2. **Binding of soluble fibrinogen to swapping mutants.** β_3-CHO cells transiently expressing α_{IIb} mutants were stained with mAb PL98DF6 (anti-α_{IIb}) followed by PE-conjugated anti-mouse IgG. After washing, cells were incubated with FITC-labeled soluble fibrinogen in the presence of mAb PT25-2 (anti-$\alpha_{IIb}\beta_3$, activating) or control mouse IgG. Fibrinogen binding to a gated subset of cells expressing $\alpha_{IIb}\beta_3$ at a high level was quantified by flow cytometry. Relative fibrinogen binding (*solid bar*) and relative $\alpha_{IIb}\beta_3$ expression (*open bar*) were obtained as described under "Experimental Procedures." Fibrinogen binding to parent CHO and β_3-CHO are included as controls.

W2:2-3 (residues 110–129), W3:4-1 (residues 147–166), W3:2-3 (residues 188–193), W3:3-4 (residues 200–208), W4:4-1 (residues 217–235), W4:2-3 (residues 259–264), and W5:4-1 (residues 283–285) swapping mutants did not bind to soluble fibrin-

TABLE II

Reactivity of α_{IIb} swapping mutants with anti-$\alpha_{IIb}\beta_3$ mAbs

The α_{IIb} swapping mutants were transiently transfected in β_3-CHO cells. Reactivity of cells with anti-$\alpha_{IIb}\beta_3$ mAbs was determined by flow cytometry. Cells were incubated with primary mAbs followed by FITC-conjugated goat anti-mouse IgG. First, the ratio of the percent mAb binding to the percent PL98DF6 binding was calculated to normalize the mAb reactivity with the $\alpha_{IIb}\beta_3$ expression. This normalized mAb binding for each mutant was further divided by the normalized mAb binding for wt $\alpha_{IIb}\beta_3$ to calculate relative mAb binding. Relative mAb binding is shown as follows: 4+, more than 90% of wt; 3+, 60–90% of wt; 2+, 20–60% of wt; +, 5–20% of wt; and −, 0–5% of wt.

Predicted loops	OP-G2	LJ-CP3	2G12	A2A9	AP-2	LJ-CP8	LJ-P9	PT25–2
W1:4–1	4+	3+	4+	4+	4+	4+	4+	4+
W1:1–2	4+	4+	4+	4+	4+	4+	4+	4+
W1:2–3	4+	3+	3+	4+	4+	4+	4+	4+
W1:3–4	4+	4+	4+	4+	4+	4+	4+	4+
W2:4–1	–	–	+	–	–	–	–	2+
W2:1–2	3+	2+	4+	3+	4+	3+	4+	4+
W2:2–3	–	–	+	–	–	–	–	4+
W2:3–4	4+	4+	4+	4+	4+	4+	4+	4+
W3:4–1	–	–	–	–	–	–	–	3+
W3:1–2	4+	3+	4+	4+	4+	4+	4+	4+
W3:2–3	–	–	+	–	+	–	+	4+
W3:3–4	–	–	–	–	–	–	–	3+
W4:4–1	–	–	–	2+	2+	+	2+	3+
W4:1–2	4+	3+	4+	4+	4+	4+	4+	2+
W4:2–3	4+	3+	4+	3+	4+	4+	3+	4+
W4:3–4	4+	4+	4+	4+	4+	4+	4+	4+
W5:4–1	3+	2+	4+	4+	4+	4+	4+	4+
W5:1–2	4+	4+	4+	4+	4+	4+	4+	2+
W5:2–3	4+	4+	4+	4+	4+	4+	4+	4+
W5:3–4	4+	4+	4+	4+	4+	4+	4+	+
W6:4–1	4+	4+	4+	4+	4+	4+	4+	4+
W6:1–2	4+	3+	4+	4+	4+	4+	4+	4+
W6:2–3	4+	3+	4+	4+	4+	4+	4+	4+
W6:3–4	4+	4+	4+	4+	4+	4+	4+	4+
W7:4–1	4+	3+	4+	4+	4+	4+	4+	4+
W7:1–2	4+	3+	4+	4+	4+	4+	4+	4+
W7:2–3	4+	4+	4+	4+	4+	4+	4+	4+

ogen, although PT25-2 recognized these swapping mutants as shown by flow cytometry (Table II).

The W5:3-4 swapping mutant also abolished fibrinogen binding; however, the reactivity of this swapping mutant with activating mAb PT25-2 was significantly impaired (Table II). The epitope for PT25-2 localizes within 335–338 of α_{IIb} as shown with human-to-mouse α_{IIb} mutants (16). We thus suspected that the W5:3-4 swapping mutant could not bind to fibrinogen because it could not be activated with PT25-2. To test this hypothesis, we expressed the W5:3-4 α_{IIb} swapping mutant together with truncated β_3, which lacks most of the β_3 cytoplasmic domain. It has been reported that truncation of the β_3 cytoplasmic domain constitutively activates $\alpha_{IIb}\beta_3$ and allows fibrinogen binding without further activation (34). In agreement, CHO cells expressing wt or W5:3-4 mutant α_{IIb} together with truncated β_3 bound fibrinogen in the absence of PT25-2 (data not shown). Therefore, it is highly likely that the W5:3-4 swapping mutant has an intact fibrinogen binding site.

The anti-human $\alpha_{IIb}\beta_3$-specific mAbs, OP-G2 (25) and LJ-CP3 (26), have been shown to have a tripeptide RYD sequence that mimics the RGD sequence in the CDR3 region of the heavy chain (43, 44) (Table I). These mAbs inhibit fibrinogen binding to platelets and fibrinogen-dependent aggregation of platelets. Binding of these mAbs is cation-dependent and is completely blocked by RGD-containing peptides. OP-G2 and LJ-CP3 can bind to nonactivated $\alpha_{IIb}\beta_3$, and their binding increases upon activation. The ligand-mimetic properties of these mAbs suggest that they have structural and functional similarities to native ligands (*e.g.* fibrinogen). Structure-function studies of these mAbs indicate that the RYD sequence in their CDR3 in the heavy chain occupies the same space as RGD in conformationally constrained, bioactive $\alpha_{IIb}\beta_3$ ligands (45). We studied the effect of swapping mutations on binding of ligand-mimetic mAbs to $\alpha_{IIb}\beta_3$ using flow cytometry. Of 27 swapping mutants, only the W2:4-1, W2:2-3, W3:4-1, W3:2-3, W3:3-4, and W4:4-1 swapping mutants abolished binding of OP-G2 and LJ-CP3 (Table II). The mutant W2:1-2 and W5:4-1 partially reduced binding. The other 19 swapping mutants bound OP-G2 and LJ-CP3 at levels comparable to that of wt $\alpha_{IIb}\beta_3$ (Table II). These data indicate that swapping the W2:4-1, W2:2-3, W3:4-1, W3:2-3, W3:3-4, and W4:4-1 predicted loops completely blocks binding of ligand-mimetic mAbs.

We studied the reactivity of several function-blocking anti-$\alpha_{IIb}\beta_3$ mAbs to the swapping mutants to establish whether any changes in their adhesive function might result from a major change in their tertiary structure rather than in the region of contact with fibrinogen or ligand-mimetic antibodies (Table I). All mAbs tested bound to the 21 noninhibitory swapping mutants at a level comparable to that of wt $\alpha_{IIb}\beta_3$, indicating that these mutations did not induce gross conformational changes in $\alpha_{IIb}\beta_3$. We found that the W2:4-1, W2:2-3, W3:4-1, W3:2-3, and W3:3-4 swapping mutants do not bind to mAbs 2G12, A2A9, AP-2, LJ-CP8, and LJ-P9 (Table II). The W4:4-1 swapping mutant showed significantly reduced binding to mAbs 2G12 and LJ-CP8. These mAbs are all function-blocking, and several of them have been mapped within or close to the putative ligand binding pocket at the α/β boundary (16). Binding of activating mAb PT25-2, which recognizes the non-ligand binding site of α_{IIb} (16), was not affected by these swapping mutations. Thus it is possible that the W2:4-1, W2:2-3, W3:4-1, W3:2-3, W3:3-4, and W4:4-1 swapping mutations induced local conformational changes within and around the putative ligand binding sites.

Ala Scanning Mutagenesis of the Predicted Loops That Are Critical for Ligand Binding—To identify critical residues for ligand binding, we mutated individual residues within the W2:4-1, W2:2-3, W3:4-1, W3:2-3, W3:3-4, W4:4-1, W4:2-3, and W5:4-1 predicted loops to Ala. We studied the binding of FITC-labeled fibrinogen, or ligand-mimetic mAbs, to CHO cells transiently expressing $\alpha_{IIb}\beta_3$ point mutants (Fig. 3). We found that the D74A, L84A, F87A, W110A, Q111A, H112A, W113A, N114A, E117A, K124A, T125A, R147A, Y155A, F160A, D163A,

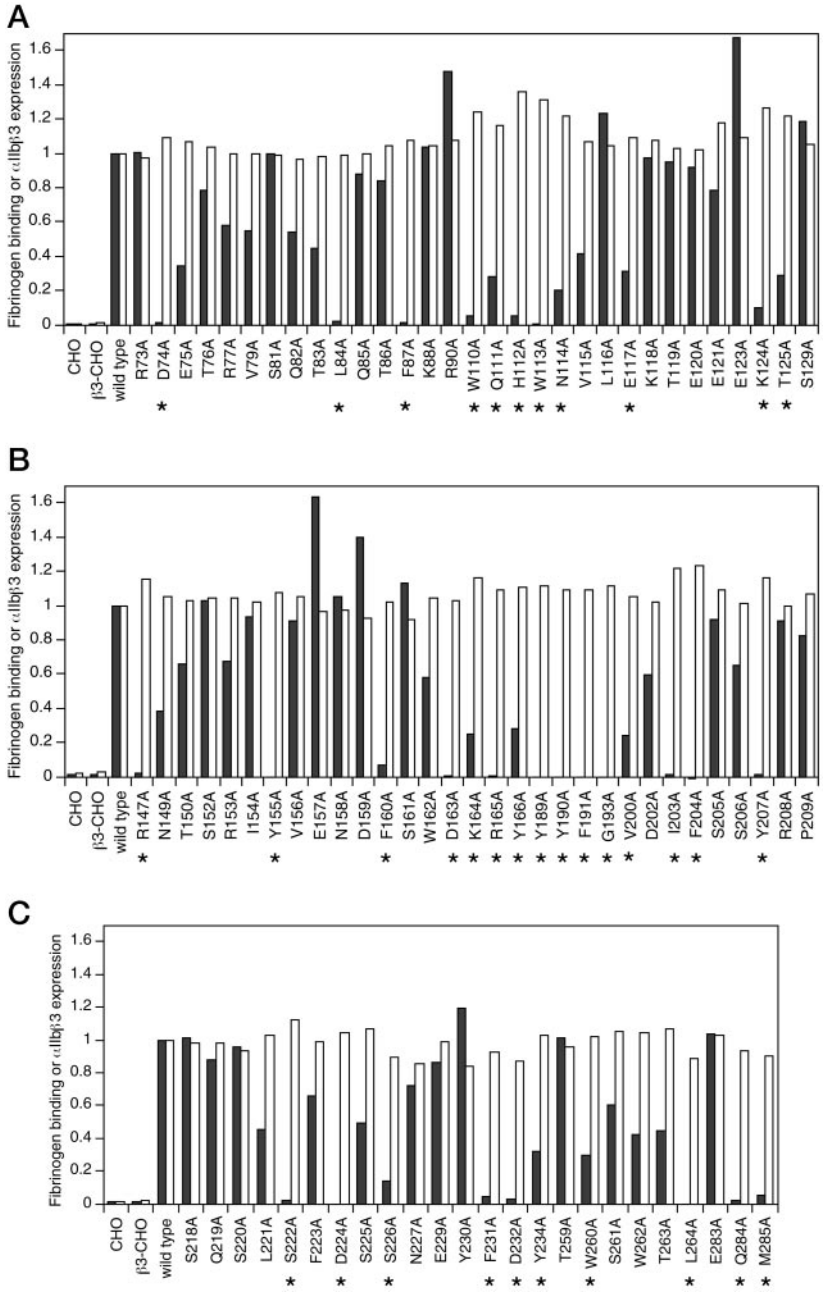

FIG. 3. **Binding of soluble fibrinogen to point mutants.** Individual amino acid residues within the 4-1 loop in repeat 2, the 1-2 loop in repeat 3, and the 2-3 loops in repeats 4 and 5 were mutated to Ala by site-directed mutagenesis. Mutant α_{IIb} cDNA was transiently expressed in β_3-CHO. Cells were first stained with mAb PL98DF6 (anti-α_{IIb}) followed by PE-conjugated anti-mouse IgG. After washing, cells were incubated with FITC-labeled fibrinogen in the presence of mAb PT25-2 (anti-$\alpha_{IIb}\beta_3$, activating) or control mouse IgG. Fibrinogen binding to a gated subset of cells expressing $\alpha_{IIb}\beta_3$ at a high level (PE-positive) was analyzed in flow cytometry. Relative fibrinogen binding (*solid bar*) and relative $\alpha_{IIb}\beta_3$ expression (*open bar*) were calculated as described under "Experimental Procedures." Fibrinogen binding to parent CHO and β_3-CHO are included as controls. Mutants that exhibit fibrinogen binding less than 33% of fibrinogen binding in wt are marked with *asterisks*.

K164A, R165A, Y166A, V200A, I203A, F204A, Y207A, S222A, D224A, S226A, F231A, D232A, Y234, W260, L264A, Q284A, and M285A mutations significantly blocked fibrinogen binding (less than 33% of wt). The previously described Y189A, Y190A, F191A, and G193A mutants (18) used as controls also blocked fibrinogen binding. In addition, mutating several residues surrounding these critical residues also had a moderate blocking effect on fibrinogen binding (Fig. 3). The effect of point mutations on OP-G2 and LJ-CP3 binding was similar to their effect on fibrinogen binding with several exceptions. The F160A, Q284A, and M285A mutations that block fibrinogen binding did not significantly affect OP-G2 and LJ-CP3 binding (Table III). The D159A mutation abolished LJ-CP3 binding, but not fibrinogen binding, to $\alpha_{IIb}\beta_3$; the mutation probably destroyed the LJ-CP3 epitope (residues 156–162 in α_{IIb}) (Table III).

We tested the reactivity of the point mutants to several non-ligand-mimetic anti-$\alpha_{IIb}\beta_3$ mAbs that recognize different epitopes in $\alpha_{IIb}\beta_3$ (Table I) to establish whether the mutations induce gross conformational changes. Most of the fibrinogen binding-defective mutations (*e.g.* F160A, Y190A, D224A, F231A, and D232A) did not affect binding of non-ligand-mimetic mAbs, or they showed only moderately reduced binding to these mAbs (*e.g.* D74A, L84A, F87A, W110A, H112A, W113A, R147A, I203A, and Y207A) (Table III). In contrast, the G193A and L264A mutations completely abolished the binding of most of the mAbs tested, suggesting that these mutations induce gross conformational changes in α_{IIb}. These results suggest that most of point mutations do not induce drastic conformational changes in $\alpha_{IIb}\beta_3$.

Positions of Amino Acid Residues That Are Critical for Binding of Fibrinogen and/or Ligand-mimetic Antibodies in the β-Propeller Model of α_{IIb}—The W3:3-4 loop was the only loop predicted to be on the bottom of the propeller which affected ligand binding in the swap experiments. Because the W4:4-1 loop was also involved in ligand binding, it is possible that the surface-exposed W3 β_4 strand that is located between the two predicted loops may also participate in ligand contact. To test this hypothesis, we generated a swapping mutant in which the

TABLE III

Reactivity of selected α_{IIb} point mutants with anti-$\alpha_{IIb}\beta_3$ mAbs

We mutated the amino acid residues within repeats 2–5 individually to Ala. The α_{IIb} mutants were individually transiently expressed in α_3-CHO cells. D74A represents the Asp-74 to Ala mutation of α_{IIb}. The reactivity of transfected cells with anti-$\alpha_{IIb}\beta_3$ mAbs was determined by flow cytometry. Cells were incubated with primary mAbs followed by FITC-conjugated goat anti-mouse IgG. First, the ratio of the percent mAb binding to the percent mAb PL98DF6 (anti-α_{IIb}) binding was calculated to normalize the mAb reactivity with the $\alpha_{IIb}\beta_3$ expression. This normalized mAb binding obtained with each mutant was divided further by the normalized mAb binding obtained with wt $\alpha_{IIb}\beta_3$ to calculate relative mAb binding. Relative mAb binding is shown as follows; 4+, more than 90% of wt; 3+, 60–90% of wt; 2+, 20–60% of wt; +, 5–20% of wt; and −, 0–5% of wt. Only selected mutants are shown. Other α_{IIb} mutants that are not in this table showed more than 3+ reactivity to the antibodies tested.

Residues	OP-G2	LJ-CP3	2G12	A2A9	AP-2	LJ-CP8	LJ-P9	PT25–2
D74A	3+	2+	3+	3+	3+	3+	3+	4+
T83A	3+	2+	4+	4+	4+	4+	4+	4+
L84A	3+	+	3+	3+	3+	3+	3+	3+
F87A	2+	+	3+	3+	3+	3+	3+	3+
W110A	2+	+	2+	2+	2+	3+	3+	3+
Q111A	4+	3+	4+	4+	4+	4+	4+	4+
H112A	2+	2+	3+	3+	3+	3+	3+	3+
W113A	+	+	2+	2+	2+	2+	2+	3+
N114A	3+	2+	3+	3+	3+	3+	3+	4+
E117A	3+	3+	4+	4+	4+	4+	–	4+
E121A	3+	2+	4+	3+	3+	4+	4+	4+
K124A	2+	2+	3+	3+	3+	3+	3+	3+
T125A	3+	2+	4+	4+	4+	4+	4+	4+
R147A	+	–	2+	2+	2+	2+	2+	3+
Y155A	2+	+	3+	3+	3+	3+	3+	3+
D159A	3+	–	4+	4+	4+	4+	4+	4+
F160A	4+	3+	4+	4+	4+	4+	4+	4+
D163A	–	–	2+	–	2+	–	2+	3+
K164A	4+	3+	4+	4+	4+	4+	4+	4+
R165A	–	–	3+	–	2+	–	2+	3+
Y166A	3+	3+	4+	4+	4+	4+	4+	4+
Y189A	–	–	2+	2+	2+	+	2+	3+
Y190A	–	2+	4+	4+	4+	4+	4+	4+
F191A	–	–	2+	+	2+	+	2+	3+
G193A	–	–	–	–	–	–	–	2+
V200A	3+	3+	4+	4+	4+	4+	4+	4+
I203A	+	+	2+	2+	2+	2+	2+	3+
F204A	+	+	2+	2+	2+	2+	2+	3+
Y207A	2+	+	3+	3+	3+	3+	3+	3+
L221A	3+	2+	3+	3+	3+	3+	3+	3+
S222A	2+	+	3+	2+	3+	2+	3+	3+
D224A	2+	+	4+	4+	4+	4+	4+	4+
S226A	4+	4+	4+	4+	4+	4+	4+	4+
Y230A	4+	2+	4+	4+	4+	4+	4+	4+
F231A	2+	–	4+	4+	4+	4+	4+	4+
D232A	+	–	4+	4+	4+	4+	4+	4+
Y234A	4+	4+	4+	4+	4+	4+	4+	4+
W260A	4+	4+	4+	4+	4+	4+	4+	4+
L264A	–	–	–	–	–	–	–	2+
E283A	4+	4+	4+	4+	4+	4+	4+	4+
Q284A	4+	4+	4+	4+	4+	4+	4+	4+
M285A	4+	4+	4+	4+	4+	4+	4+	4+

W3 β_4 strand spanning amino acid residues 210–215 of α_{IIb} was swapped with the corresponding residues of α_5. The resulting α_{IIb} W3 β_4 mutant was transiently expressed in β_3-CHO, and the ability of this swapping mutant to bind to soluble fibrinogen was tested. We found that the W3 β_4 mutant did not bind to fibrinogen, although it was surface-expressed and bound to mAb PT25-2. Swapping the W5 β_4 strand (residues 340–346) with the corresponding residues of α_5 (the resulting mutant is designated the W5 β_4 mutant) did not block ligand binding (Fig. 4*a*). Ala scanning mutagenesis within the W3 β_3 strand revealed that Leu-212, Leu-213, Trp-214, and His-215 are critical for fibrinogen binding, but nearby Ser-217 is not (Fig. 4*b*). Mutating Ile-211 to Ala markedly increased fibrinogen binding. These results suggest that the W3 β_4 strand may be uniquely involved in ligand binding.

We studied whether our mutagenesis results fit with the β-propeller model of α_{IIb} by plotting critical residues in the model. Amino acid residues that when mutated did or did not affect ligand binding are shown as *black* and *white spheres*, respectively (Fig. 5). Mutations that disrupt ligand binding are clearly clustered to one side of the β-propeller (W2, W3, W4, and W5). Most of the mutations that disrupt ligand binding are on the top of the β-propeller. Some were also present on the side of W3 and on the bottom of W3 in the 3-4 loop. However, mutations that disrupt ligand binding are not associated with the Ca^{2+} binding sites in the 1-2 loops of W4, W5, W6, or W7 (Ca^{2+} ions are shown as *gold spheres*) (Fig. 5).

DISCUSSION

The present study establishes the position of the ligand binding surface in the α_{IIb} subunit using domain swapping and Ala scanning mutagenesis and molecular modeling. Swapping the eight predicted loops W2:4-1, W2:2-3, W3:4-1, W3:2-3, W3:3-4, W4:4-1, W4:2-3, and W5:4-1 blocked binding of fibrinogen and/or ligand-mimetic mAbs. We subsequently identified several point mutations within these predicted loops which block fibrinogen binding (summarized in Table IV). These residues include Asp-74, Leu-84, and Phe-87 in W2:4-1; Trp-110, Gln-111, His-112, Trp-113, Asn-114, Glu-117, Lys-124, and Thr-125 in W2:2-3; Arg-147, Tyr-155, Phe-160, Asp-163, Lys-164, Arg-165, and Tyr-166 in W3:4-1; Val-200, Ile-203, Phe-204, and Tyr-207 in W3:3-4; Ser-222, Asp-224, Ser-226, Phe-231, Asp-232, and Tyr-234 in W4:4-1; Trp-260 and Leu-264 in W4:2-3, Gln-284 and Met-285 in W5:4-1. We determined previously

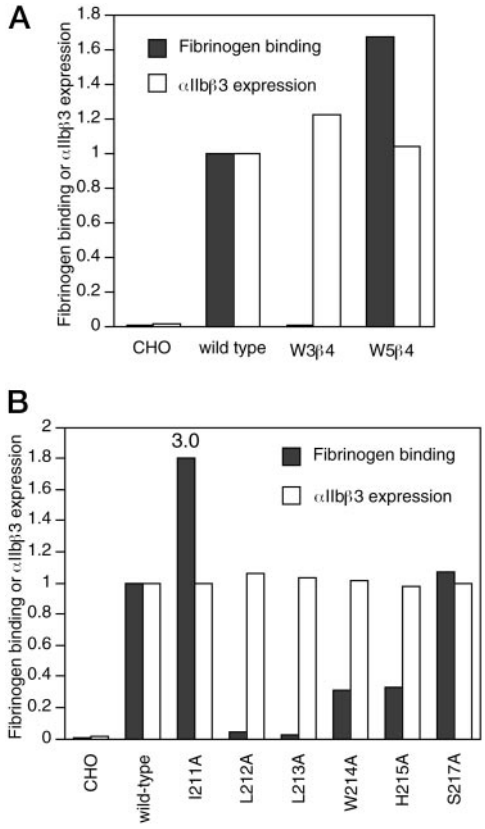

FIG. 4. **Effect of mutations of the β_4 strand residues in repeats 3 and 5 on fibrinogen binding.** *Panel a*, amino acid residues in the predicted β_4 strand in repeats 3 and 5 of α_{IIb} were swapped with the corresponding residues from α_5 (Fig. 1). Individual amino acid residues within this region were mutated to Ala. The resulting α_{IIb} mutants (designated W3β4 and W5β3 mutants, respectively) were transiently expressed in β_3-CHO cells. Fibrinogen binding to cells expressing wt or mutant $\alpha_{IIb}\beta_3$ was examined as described under "Methods." Relative fibrinogen binding (*solid bars*) and relative $\alpha_{IIb}\beta_3$ expression (*open bars*) are shown. The data are shown as fibrinogen binding relative to wt. *Panel b*, individual amino acid residues in the repeat 3 β_4 strand were mutated to Ala, and the capacity of the α_{IIb} mutants to bind to fibrinogen was tested as described above.

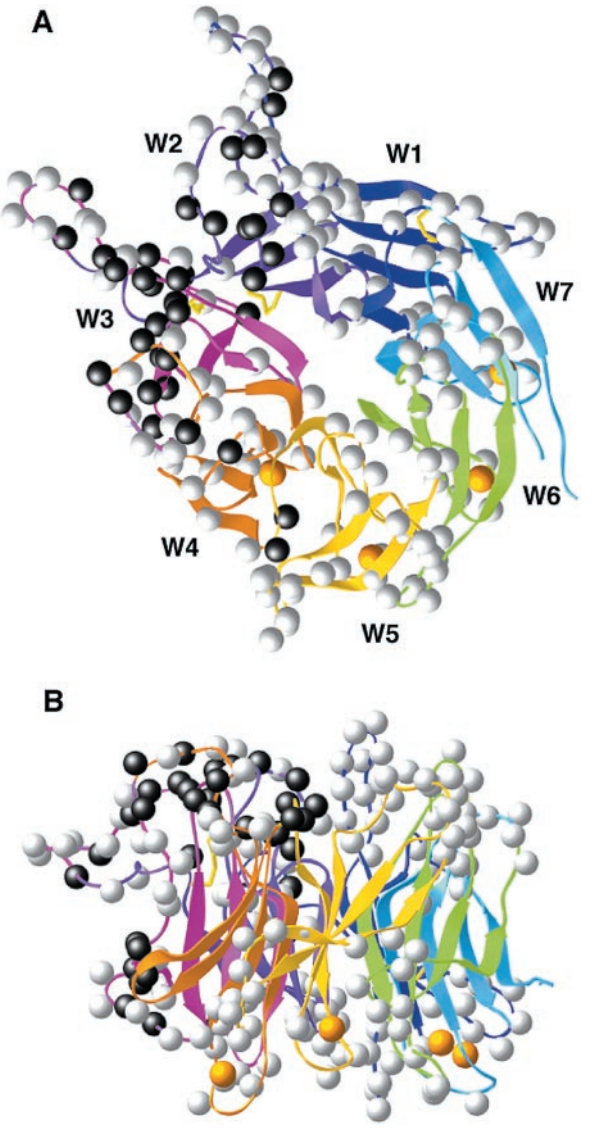

FIG. 5. **Molecular model of the α_{IIb} β-propeller domain.** Molecular modeling of the putative β-propeller domain of the α_{IIb} subunit was carried out as described under "Experimental Procedures." Amino acid residues that when mutated affect or do not affect ligand binding are shown as *black* or *white spheres*, respectively, centered on the Cα atom position. Ca^{2+} ions are shown as *golden spheres*. For loop swaps that did not affect ligand binding activity, only residues that differed between the swapped loops are shown as *white spheres*. Mutations that affect ligand binding cluster to one side of the β-propeller, and are not associated with the Ca^{2+} binding sites. *Panel A*, top view; *panel B*, side view.

that Tyr-189, Tyr-190, Phe-191, and Gly-193 in W3:2-3 are critical for ligand binding (18). In the β-propeller model of α_{IIb}, the predicted loops that are critical for ligand binding (thus the amino acid residues critical for ligand binding within these predicted loops) are clustered (Fig. 5), although these predicted loops are in discontinuous locations in the primary structure (Fig. 1). These amino acid residues that we identified by mutagenesis potentially constitute a ligand binding interface in α_{IIb}. The present β-propeller model, however, does not provide definitive information on whether these critical residues are surface- exposed and on the conformation of the predicted loops. Thus, it is still unclear how and whether these clustered critical residues interact with ligands.

Another important finding in the present study is that 19 of 27 swapping mutations of the predicted loops did not affect the binding of fibrinogen or ligand-mimetic mAbs. These results indicate that the 19 predicted loops do not include major ligand binding sites. A previous report suggests that the second putative divalent cation binding site in α_{IIb} interacts directly with the fibrinogen γ-chain sequence (4). Swapping the predicted loop, including the second cation binding site that corresponds to the 298–304 region (the predicted W5:1-2 loop), did not affect the binding of fibrinogen or ligand-mimetic mAbs in the present study. Our results are consistent with previous studies using peptide-specific antibodies against the divalent cation binding motifs, or α_{IIb}/α_5 swapping mutants (46, 47). However, it is possible that there are allosteric binding sites because the activating anti-$\alpha_{IIb}\beta_3$ mAb PT25-2 recognizes residues 335–338 of α_{IIb}, which is close to the fibrinogen γ-chain sequence cross-linking site in the β-propeller model (Table III and Ref. 16). A recombinant fragment that consists of repeats 3–7 of α_{IIb} (residues 171–464) has also been shown to bind to ligands in a cation-dependent manner (7). The reported α_{IIb} fragment contains several (but not all) of the residues that are critical for ligand binding (*e.g.* Tyr-189, Tyr-190, Phe-191, Ile-203, Phe-204, Tyr-207, Leu-212, Leu-213, Trp-214, His-215, Ser-222, Ser-224, Ser-226, Phe-231, Asp-232, Gln-284, and Met-285). The ability to express this fragment is also inconsistent with the β-propeller model. Thus we will need to study the structure of this fragment and native integrin α_{IIb} subunit in the future experiments to conclude whether the mode of ligand binding to the fragment is similar to that of integrins.

TABLE IV

Effect of mutations in the α_{IIb} subunit on ligand binding to $\alpha_{IIb}\beta_3$

Residues within each predicted loop which are critical for ligand binding are shown. Mutating Gly-193 and Leu-264 in parentheses block binding of antibodies to $\alpha_{IIb}\beta_3$.

The predicted loops that are not critical for ligand binding		W1:4–1, W1:1–2, W1:2–3, W1:3–4, W2:1–2, W2:3–4, W3:1–2, W4:1–2, W4:3–4, W5:1–2, W5:2–3, W5:3–4, W6:4–1, W6:1–2, W6:2–3, W6:3–4, W7:4–1, W7:1–2, W7:2–3
The predicted loops that are critical for ligand binding	W2:4–1	Asp-74, Leu-84, and Phe-87
	W2:2–3	Trp-110, Gln-111, His-112, Trp-113, Asn-114, Glu-117, Lys-124, and Thr-125
	W3:4–1	Arg-147, Tyr-155, Phe-160, Asp-163, Lys-164, Arg-165, and Tyr-166
	W3:2–3	Tyr-189, Tyr-190, Phe-191, and (Gly-193) (18)
	W3:3–4	Val-200, Ile-203, Phe-204, and Tyr-207
	W3 β4	Leu-212, Leu-213, Trp-214, and His-215
	W4:4–1	Ser-222, Asp-224, Ser-226, Phe-231, Asp-232, and Tyr-234
	W4:2–3	Trp-260 and (Leu-264)
	W5:4–1	Gln-284 and Met-285

Ligand binding and enzymatic active sites are usually located in the upper face of the β-propeller domain, but the sides of the β-propeller domains also contribute to ligand binding (48–51). It is thus not surprising that several residues that are critical for ligand binding, including Ile-203, Phe-204, Tyr-207, Leu-212, Leu-213, Trp-214, and His-215, are located on the side of the β-propeller model in α_{IIb}. It is interesting that many amino acid residues that are critically involved in ligand binding are hydrophobic. Repeats 2–4 of α_{IIb} have been predicted to be located at the boundary between the α and β subunits (16). It is thus tempting to speculate that several of these hydrophobic residues critical for ligand binding are also involved in α/β association and that the residues critical for ligand binding are cryptic when the receptor is inactive but are exposed when the receptor is activated. We do not rule out the possibility that several of these critical hydrophobic residues are buried, and mutating these residues affects a local conformation.

Our present and previous mutagenesis results (16, 18) are consistent with the recent genetic analyses of nonfunctional α_{IIb} from patients with variant-type Glanzmann's thrombasthenia, a bleeding disorder that is caused by the expression of nonfunctional $\alpha_{IIb}\beta_3$ in platelets. It has been reported that a Glanzmann's thrombasthenia ligand binding function-defective α_{IIb} has an insertion of two amino acid residues within the predicted W3:4-1 loop (residues 147–166) (52). This mutation blocks ligand binding by affecting the function of this predicted loop without affecting the synthesis or surface expression of $\alpha_{IIb}\beta_3$. Two additional α_{IIb} mutations that block ligand binding have been reported: a Pro-145 to Ala mutation immediately adjacent to the predicted W3:4-1 loop (residues 147–166) (53), and a Leu-183 to Pro mutation immediately adjacent to the predicted W3:2-3 loop (residues 188–193) (54). These last two mutations moderately reduce the level of $\alpha_{IIb}\beta_3$ expression when the mutant α_{IIb} and wt β_3 are coexpressed on mammalian cells, and they eliminate binding to ligands or ligand-mimetic mAbs. These mutations (Pro-145 to Ala, and Leu-183 to Pro) are likely to affect the conformation of the immediately adjacent predicted loops or the entire structure. Another group has found that Asp-224 in the predicted W4:4-1 loop (residues 217–235) is critical for binding of ligand-mimetic mAb by random mutagenesis (55). Consistent with this, we found that Ser-222, Ser-226, Phe-231, and Asp-232, in addition to the reported Asp-224, in this region, are critical for fibrinogen binding in the present study.

The finding that mutating residues Gly-193 and Leu-264 blocks the binding of multiple non-ligand-mimetic mAbs suggests that these mutations induced gross conformational changes of $\alpha_{IIb}\beta_3$. Thus, it is unclear whether these residues are directly involved in ligand binding. The D163A and R165A mutations block binding only of mAbs A2A9 and LJ-CP8, probably because their epitopes are close to the mutations (Table I). The E117A mutation blocked binding of LJ-P9. Although the position of this mutation is not close in the primary structure to the previously reported LJ-P9 epitope (residues 79–93 of α_{IIb}) (16), it is very close in the β-propeller model. This finding is consistent with the β-propeller model. Consistent with the proposed critical function of these residues in ligand binding, most of these critical residues are well conserved among human (56), rat (57), and mouse α_{IIb} (16, 58). The model also predicted that the surface-exposed β_4 strand between the predicted W3:3-4 and W4:4-1 loops may constitute part of the ligand binding interface. We have shown that this is the case: mutating the β_4 strand actually blocked ligand binding. The β-propeller model is thus consistent with the mutagenesis results. Detailed analysis of the function of these residues that are critical for ligand binding requires the real structure of the α_{IIb} subunit.

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REFERENCES

1. Phillips, D. R., Charo, I. F., Parise, L. V., and Fitzgerald, L. A. (1988) *Blood* **71,** 831–843
2. Kloczewiak, M., Timmons, S., and Hawiger, J. (1982) *Biochem. Biophys. Res. Commun.* **107,** 181–187
3. Pytela, R., Pierschbacher, M. D., Ginsberg, M. H., Plow, E. F., and Ruoslahti, E. (1986) *Science* **231,** 1559–1562
4. D'Souza, S., Ginsberg, M. H., Burke, T. A., and Plow, E. F. (1990) *J. Biol. Chem.* **265,** 3440–3446
5. D'Souza, S., Ginsberg, M. H., Matsueda, G. R., and Plow, E. F. (1991) *Nature* **350,** 66–68
6. Stanley, P., Bates, P. A., Harvey, J., Bennet, R. I., and Hogg, N. (1994) *EMBO J.* **13,** 1790–1798
7. Gulino, D., Boudignon, C., Zhang, L. Y., Concord, E., Rabiet, M. J., and Marguerie, G. (1992) *J. Biol. Chem.* **267,** 1001–1007
8. Baneres, J.-L., Roquet, F., Green, M., LeCalvez, H., and Parello, J. (1998) *J. Biol. Chem.* **273,** 24744–24753
9. Baneres, J. L., Roquet, F., Martin, A., and Parello, J. (2000) *J. Biol. Chem.* **275,** 5888–5903
10. Loftus, J. C., Halloran, C. E., Ginsberg, M. H., Feigen, L. P., Zablocki, J. A., and Smith, J. W. (1996) *J. Biol. Chem.* **271,** 2033–2039
11. McKay, B. S., Annis, D. S., Honda, S., Christie, D., and Kunicki, T. J. (1996) *J. Biol. Chem.* **271,** 30544–30547

12. Humphries, M. J. (2000) *Biochem. Soc. Trans.* **28,** 311–339
13. Kamata, T., Puzon, W., and Takada, Y. (1995) *Biochem. J.* **305,** 945–951
14. Schiffer, S. G., Hemler, M. E., Lobb, R. R., Tizard, R., and Osborn, L. (1995) *J. Biol. Chem.* **270,** 14270–14273
15. Mould, A., Askari, J., Aota, S., Yamada, K., Irie, A., Takada, Y., Mardon, H., and Humphries, M. (1997) *J. Biol. Chem.* **272,** 17283–17292
16. Puzon-McLaughlin, W., Kamata, T., and Takada, Y. (2000) *J. Biol. Chem.* **275,** 7795–7802
17. Irie, A., Kamata, T., Puzon-McLaughlin, W., and Takada, Y. (1995) *EMBO J.* **14,** 5542–5549
18. Kamata, T., Irie, A., and Takada, Y. (1996) *J. Biol. Chem.* **271,** 18610–18615
19. Brandon, C., and Tooze, J. (1991) *Introduction to Protein Structure*, Garland Publishing, Inc., New York
20. Springer, T. (1997) *Proc. Natl. Acad. Sci. U. S. A.* **94,** 65–72
21. Frelinger, A. D., Cohen, I., Plow, E., Smit, M., Roberts, J., Lam, S., and Ginsberg, M. (1990) *J. Biol. Chem.* **265,** 6346–6352
22. Woods, V. L. J., Oh, E. H., Mason, D., and McMillan, R. (1984) *Blood* **63,** 368–375
23. Ylanne, J., Hormia, M., Jarvinen, M., Vartio, T., and Virtanen, I. (1988) *Blood* **72,** 1478–1486
24. Bennett, J., Hoxie, J., Leitman, S., Vilaire, G., and Cines, D. (1983) *Proc. Natl. Acad. Sci. U. S. A.* **80,** 2417–2421
25. Tomiyama, Y., Tsubakio, T., Piotrowicz, R. S., Kurata, Y., Loftus, J. C., and Kunicki, T. J. (1992) *Blood* **79,** 2303–2312
26. Niiya, K., Hodson, E., Bader, R., Byers-Ward, V., Koziol, J. A., Plow, E. F., and Ruggeri, Z. M. (1987) *Blood* **70,** 475–483
27. Lombardo, V., Hodson, E., Roberts, J., Kunicki, T., Zimmerman, T., and Ruggeri, Z. (1985) *J. Clin. Invest.* **76,** 1950–1958
28. Pidard, D., Montgomery, R., Bennett, J., and Kunicki, T. (1983) *J. Biol. Chem.* **258,** 12582–12586
29. Tokuhira, M., Handa, M., Kamata, T., Oda, A., Katayama, M., Tomiyama, Y., Murata, M., Kawai, Y., Watanabe, K., and Ikeda, Y. (1996) *Thromb. Haemostasis* **76,** 1038–1046
30. Horton, R. M., and Pease, L. R. (1991) in *Directed Mutagenesis: A Practical Approach* (McPherson, M. J., ed), pp. 217–247, IRL Press, Oxford
31. Deng, W. P., and Nickoloff, J. A. (1992) *Anal. Biochem.* **200,** 81–88
32. Xia, Z., Wong, T., Liu, Q., Kasirer-Friede, A., Brown, E., and Frojmovic, M. M. (1996) *Br. J. Haematol.* **93,** 204–214
33. Goto, S., Salomon, D. R., Ikeda, Y., and Ruggeri, Z. M. (1995) *J. Biol. Chem.* **270,** 23352–23361
34. Hughes, P., O'Toole, T., Ylanne, J., Shattil, S., and Ginsberg, M. (1995) *J. Biol. Chem.* **270,** 12411–12417
35. Levitt, M. (1992) *J. Mol. Biol.* **226,** 507–533
36. Sali, A., and Blundell, T. L. (1993) *J. Mol. Biol.* **234,** 779–815
37. Sondek, J., Bohm, A., Lambright, D. G., Hamm, H. E., and Sigler, P. B. (1996) *Nature* **379,** 369–374
38. Oxvig, C., and Springer, T. A. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95,** 4870–4875
39. Springer, T. A., Jing, H., and Takagi, J. (2000) *Cell* **102,** 275–277
40. Irie, A., Kamata, T., and Takada, Y. (1997) *Proc. Natl. Acad. Sci. U. S. A.* **94,** 7198–7203
41. Zhang, X.-P., Puzon-McLaughlin, W., Irie, A., Kovach, A., Prokopishyn, N. L., Laferté, L., Takeuchi, K.-I., Tsuji, T., and Takada, Y. (1999) *Biochemistry* **43,** 14424-14431
42. O'Toole, T., Loftus, J., Du, X., Glass, A., Ruggeri, Z., Shattil, S., Plow, E., and Ginsberg, M. (1990) *Cell Regul.* **1,** 883–893
43. Taub, R., Gould, R. J., Garsky, V. M., Ciccarone, T. M., Hoxie, J., Friedman, P. A., and Shattil, S. J. (1989) *J. Biol. Chem.* **264,** 259–265
44. Tomiyama, Y., Brojer, E., Ruggeri, Z., Shattil, S., Smiltneck, J., Gorski, J., Kumar, A., Kieber-Emmons, T., and Kunicki, T. (1992) *J. Biol. Chem.* **267,** 18085–18092
45. Prammer, K. V., Boyer, J., Ugen, K., Shattil, S. J., and Kieber-Emmons, T. (1994) *Receptor* **4,** 93–108
46. Niewiarowska, J., Swiderska, M., Majewski, T., and Cierniewski, C. S. (1997) *Thromb. Haemostasis* **78,** 1510–1515
47. Gidwitz, S., Lyman, S., and White, G. C. (2000) *J. Biol. Chem.* **275,** 6680–6688
48. Russell, R. B., Sasieni, P. D., and Sternberg, M. J. (1998) *J. Mol. Biol.* **282,** 903–918
49. Fulop, V., and Jones, D. T. (1999) *Curr. Opin. Struct. Biol.* **9,** 715–721
50. Panchenko, M. P., Saxena, K., Li, Y., Charnecki, S., Sternweis, P. M., Smith, T. F., Gilman, A. G., Kozasa, T., and Neer, E. J. (1998) *J. Biol. Chem.* **273,** 28298–28304
51. ter Haar, E., Harrison, S. C., and Kirchhausen, T. (2000) *Proc. Natl. Acad. Sci. U. S. A.* **97,** 1096–1100
52. Honda, S., Tomiyama, Y., Shiraga, M., Tadokoro, S., Takamatsu, J., Saito, H., Yoshiyuki, K., and Matsuzawa, Y. (1998) *J. Clin. Invest.* **102,** 1183–1192
53. Basani, R. B., French, D. L., Vilaire, G., Brown, D. L., Chen, F., Coller, B. S., Derrick, J. M., Gartner, T. K., Bennett, J. S., and Poncz, M. (2000) *Blood* **95,** 180–188
54. Grimaldi, C. M., Chen, F., Wu, C., Weiss, H. J., Coller, B. S., and French, D. L. (1998) *Blood* **91,** 1562–1571
55. Tozer, E. C., Baker, E. K., Ginsberg, M. H., and Loftus, J. C. (1999) *Blood* **93,** 918–924
56. Poncz, M., Eisman, R., Heidenreich, R., Silver, S. M., Vilaire, G., Surrey, S., Schwartz, E., and Bennett, J. S. (1987) *J. Biol. Chem.* **262,** 8476–8482
57. Poncz, M., and Newman, P. J. (1990) *Blood* **75,** 1282–1289
58. Thornton, M. A., and Poncz, M. (1999) *Blood* **94,** 3947–3950