Critical Residues of Integrin αIIb Subunit for Binding of αIIbβ3 (Glycoprotein IIb-IIIa) to Fibrinogen and Ligand-mimetic Antibodies (PAC-1, OP-G2, and LJ-CP3)∗

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Integrin αIIbβ3 plays a critical role in platelet aggregation through its interaction with fibrinogen. Elucidation of the mechanisms of αIIbβ3-fibrinogen interaction is critical to understanding hemostasis and thrombosis. Here we report that mutations of Gly-184, Tyr-189, Tyr-190, Phe-191, and Gly-193 within the predicted turn structure of the third amino-terminal repeat of αIIb significantly block binding of αIIbβ3 to soluble fibrinogen. These mutations also block binding of αIIbβ3 to ligand-mimetic monoclonal antibodies PAC-1, OP-G2, LJ-CP3, which have an RGD-related RYD sequence in their antigen-binding sites. These mutations do not significantly affect the expression of αIIbβ3, in contrast to most of the natural αIIb mutations occurring in Glanzmann’s thrombasthenic patients. The data suggest that these residues are critically involved in αIIbβ3-ligand interactions.

αIIbβ3-fibrinogen interaction is blocked by synthetic peptides derived from the sequence HHLLGGAKQAGDV at the amino-terminal portion of the extracellular domain contains seven repeats of homologous sequences of about 80 amino acids. The last four repeats each contain a putative divalent cation binding site consisting of the general sequence DXD-DXXD (18, 19). Although several other integrin α subunits (e.g. α2, αL, αM) have an I (inserted) domain of about 200 amino acids which is critical for ligand binding (20–25) between the second and third repeats, αIIb has no such domain. The ligand binding site of the non-1 domain integrin α subunit has not been well characterized. A recombinant αIIb fragment encompassing residues 171–464 has been shown to bind to immobilized fibrinogen (26). The amino-terminal 334 residues of αIIb have recently been shown to be required for ligand binding to αIIbβ3 (27). The γ chain peptide or RGD-containing peptides chemically cross-link to several regions of αIIb, including residues 294–314, that contain the second putative divalent cation binding domain (28), residues 42–73, 672–724, or 752–767 (29). Synthetic peptides derived from residues 294–314 have been shown to bind directly to fibrinogen and inhibit platelet aggregation (30). Also, synthetic peptides derived from residues 656–667 of αIIb have been reported to interact with fibrinogen (31).

We recently localized epitopes of function-blocking monoclonal antibodies (mAbs)1 within residues 108–268 (putative ligand binding sites) of α4 in α4β1, another non-1 domain integrin (32). We also determined that residues 181–190 in the third amino-terminal repeats of α4 and α5 are critical for ligand binding to α4β1 and α5β1, respectively, by introducing multiple mutations into the putative ligand binding sites (33). We hypothesized that the corresponding region of other non-1 domain integrins may be critical for ligand binding. In the present study, we determined whether the corresponding region of the αIIb subunit has a critical role in αIIbβ3-ligand interaction by introducing multiple mutations to the region of αIIb. We determined that the corresponding region in αIIb is critically involved in the binding of fibrinogen and ligand-mimetic anti-αIIbβ3 mAbs (PAC-1, OP-G2, and LJ-CP3). These mutations do not significantly affect expression of αIIbβ3, in contrast to most of the natural αIIb mutations found in Glanzmann’s thrombasthenic patients (2).

EXPERIMENTAL PROCEDURES

Monoclonal Antibodies and cDNAs—mAb 15 (34) was a kind gift from M. H. Ginsberg (The Scripps Research Institute, La Jolla, CA), 2G12 (35) from V. Woods (University of California San Diego, San Diego, CA), PL98DF6 (36) from J. Ylanne (University of Helsinki, Helsinki, Finland), PAC-1 from S. J. Shattil (The Scripps Research Institute, La Jolla, CA), OP-G2 (37) from Y. Tomiyama (Osaka University, Osaka, Japan), LJ-CP3 (38) from Z. M. Ruggeri (The Scripps Research Institute, La Jolla, CA), and PT25–2 from M. Handa and Y. H. 1 The abbreviations used are: mAb, monoclonal antibody; CHO, Chinese hamster ovary; FITC, fluorescein isothiocyanate.
Ikeda (Keio University, Tokyo, Japan). α1b and β3 cDNAs were obtained from J. C. Loftus (The Scripps Research Institute, La Jolla, CA).

Binding of Fibrinogen and mAb PAC-1 to Chinese Hamster Ovary (CHO) Cells—Fibrinogen (Chromogenix, Stockholm, Sweden) was labeled with fluorescein isothiocyanate (FITC) according to Goto et al. (39). Cells were incubated with mouse IgG or PT25–2 at 10 μg/ml for 30 min at 4°C in Dulbecco’s modified Eagle’s medium. Then, FITC-labeled fibrinogen was added at a final concentration of 30 μg/ml, and the mixture was further incubated for 30 min at room temperature. After washing the cells once with phosphate-buffered saline to remove unbound fibrinogen, bound fibrinogen was quantified by flow cytometry in FACSCan (Bedfont-Dickinson).

mAb PAC-1 was labeled with FITC essentially as described (40). Binding of PAC-1 was determined as described above, except that FITC-labeled PAC-1 was used instead of fibrinogen.

Other Methods—Site-directed mutagenesis of α1b cDNA (18) in pBluescript vector (41, 42) was carried out using unique restriction site elimination (43). The presence of mutation was confirmed by DNA sequencing. Transfection of cDNAs into CHO cells by electroporation, selection of transfected cells with G418, immunoprecipitation, and flow cytometry were carried out as described previously (44).

RESULTS

Gly-184, Tyr-189, Tyr-190, Phe-191, and Gly-193 Are Critical for Binding of α1bβ3 to Soluble Fibrinogen—To examine the potential role of residues 184–193 in the third amino-terminal repeat of α1b in integrin α1bβ3-ligand interaction, we introduced multiple mutations within the region. Wild-type or mutant (Gly-184, Tyr-189, Tyr-190, Phe-191, and Gly-193 → Ala) α1b cDNAs were transfected into CHO cells together with wild-type human β3 cDNA and a neomycin-resistant gene. After selection with G418, α1bβ3-positive cells were cloned by cell sorting to obtain cells expressing α1bβ3 at a high level (designated α1bβ3-CHO cells). Fig. 1 shows flow cytometric profiles of CHO cells expressing wild-type or Y190A mutant α1bβ3. The expression levels of α1b and β3 in wild-type and mutant α1bβ3 are comparable (Table I). Similar flow cytometric profiles were obtained with other mutants. Fig. 2 shows immunoprecipitation of wild-type and mutant α1bβ3. Wild-type or mutant α1b with predicted size was expressed on the surface of cells in association with β3, indicating that the mutations did not significantly affect the expression and assembly of α1bβ3 heterodimers.

α1bβ3-CHO cells express an inactive (resting) form of α1bβ3 (45). Although the α1bβ3 on CHO cells adheres to immobilized fibrinogen without activation, α1bβ3 must be activated before it will bind to soluble fibrinogen. We examined the binding function of G184A, Y189A, Y190A, F191A, and G193A mutants to FITC-labeled soluble fibrinogen using a novel α1bβ3 complex-specific activating antibody PT25–2 and flow cytometry (Fig. 3). This PT25–2 antibody recognizes and activates α1bβ3 on CHO cells but not αvβ3 (endogenous hamster αv/endoxogenous human β3). β3-CHO, which expresses αvβ3 (hamster αv/human β3) (46), did not bind to fibrinogen in the presence of PT25–2 (Fig. 3). Binding soluble fibrinogen to wild-type α1bβ3-CHO was dependent on activation with mAb PT25–2 (Fig. 3) and blocked by EDTA and by RGD peptide (data not shown). These data clearly indicate that PT25–2-induced binding of FITC-labeled fibrinogen to α1bβ3-CHO cells is α1bβ3-specific. The G184A, Y189A, Y190A, F191A, and G193A mutants did not bind soluble fibrinogen, even in the presence of PT25–2, although PT25–2 recognized all of the mutants in fluorescence-activated cell sorter analysis (Table I). These data indicate that these mutations block binding of fibrinogen to α1bβ3.

Gly-184, Tyr-189, Tyr-190, Phe-191, and Gly-193 Are Critical for α1bβ3 Binding to Ligand-mimetic Antibodies—Anti-α1bβ3 mAbs PAC-1, OP-G2, and LJ-CP3 are known not only to block fibrinogen binding to α1bβ3 but also to compete with ligand-mimetic antibodies (e.g. RGD and/or γ chain peptides) for binding to α1bβ3. Notably, these ligand-mimetic antibodies have RGD-like RYD sequences in their antigen binding sites (47–49) and are believed to bind to the fibrinogen binding site in α1bβ3. PAC-1 binds only to activated α1bβ3, but OP-G2 and LJ-CP3 bind to resting as well as activated α1bβ3. Since OP-G2 competes with RGD peptide but not with γ chain peptides for binding to immobilized α1bβ3 (48), OP-G2 is considered to bind to the RGD binding site in α1bβ3. We examined by flow cytometry the effects of G184A, Y189A, Y190A, F191A, and G193A mutations on the ability of these ligand-mimetic mAbs to bind to α1bβ3. We also examined the binding of FITC-labeled PAC-1 to these mutants in the presence of activating mAb PT25–2. Fig. 4 and Table I show the binding profiles of FITC-labeled PAC-1 to CHO cells expressing mutant α1bβ3. We did not detect any binding of PAC-1 to these mutants except for the Y190A mutant.

Table I summarizes the binding profiles of mAbs OP-G2 and LJ-CP3 to mutant α1bβ3 on CHO cells in the absence of activating mAb PT25–2. FITC-labeled anti-mouse IgG was used as a secondary antibody to detect binding of OP-G2 and LJ-CP3. OP-G2 and LJ-CP3 bind to wild-type α1bβ3-CHO without activation, but their binding to G184A, Y189A, Y190A, F191A, and G193A mutants was significantly reduced or undetectable. The ligand-mimetic antibodies competed with fibrinogen or RGD peptide for binding to α1bβ3 (data not shown), indicating that the binding of these antibodies is specific. The data indicate that Gly-184, Tyr-189, Tyr-190, Phe-191, and Gly-193 are critical for binding of α1bβ3 to soluble fibrinogen. The expression levels of α1b and β3 in wild-type and mutant α1bβ3 are comparable (Table I). Similar flow cytometric profiles were obtained with other mutants. Fig. 2 shows immunoprecipitation of wild-type and mutant α1bβ3. Wild-type or mutant α1b with predicted size was expressed on the surface of cells in association with β3, indicating that the mutations did not significantly affect the expression and assembly of α1bβ3 heterodimers.
Reactivities of ligand-mimetic antibodies (OP-G2, LJ-CP3, and PAC-1) to αllβ3 mutants

TABLE I

| Antibody | CHO cells | β3 only | Wild-type αll | G184A αll | Y189A αll | Y190A αll | F191A αll | G193A αll |
|----------|-----------|---------|---------------|-----------|-----------|-----------|-----------|-----------|
| PL 98DF6 | 3.7       | 4.1     | 697           | 460       | 466       | 579       | 722       | 389       |
| OP-G2    | 4.4       | 4.4     | 615           | 16        | 11        | 32        | 22        | 5.7       |
| LJ-CP3   | 4.6       | 4.8     | 185           | 8         | 13        | 86        | 20        | 6.3       |
| 2G12     | 3.7       | 4.2     | 1182          | 8         | 13        | 86        | 20        | 6.3       |
| PT25-2   | 5.6       | 6.7     | 1050          | 8         | 13        | 86        | 20        | 6.3       |
| mAb 15   | 4.6       | 115     | 1481          | 8         | 13        | 86        | 20        | 6.3       |
| Mouse IgG| 3.4       | 3.8     | 76            | 3.0       | 6.4       | 41        | 3.7       | 6.2       |
| PAC-1 (+ mouse IgG) | 13.4   | 11.9    | 16.6          | 11.6      | 9.5       | 7.6       | 13.6      | 8.9       |
| PAC-1 (+ PT25-2) | 14.2   | 12.5    | 198.3         | 10.9      | 15.3      | 47.2      | 12.8      | 14.2      |

Fig. 2. Immunoprecipitation of αllβ3 mutants. Lysates of 125I surface-labeled CHO cell expressing human β3, wild-type human αllβ3, or mutant human αllβ3 were immunoprecipitated with anti-αll antibody (PL 98DF6), anti-β3 antibody (mAb 15), or control serum. Samples were analyzed in 7% gel under nonreducing conditions. The data show that wild-type and mutant αll of expected sizes are expressed in association with β3. CHO cells transfected with human β3 cDNA alone (β3 only) expressed human β3 in association with hamster endogenous α subunit, possibly αv.

191, and Gly-193 of αll are critical for binding ligand-mimetic antibodies to αllβ3. The effects of irrelevant mutations in nearby Phe-171 or Tyr-207 to Ala on the binding of LJ-CP3 or OP-G2 were examined. These ligand-mimetic antibodies bound to cells transiently expressing F171A or Y207A mutant αll (data not shown), suggesting that the effects of mutations are site-specific. The binding of function-blocking mAb 2G12 specific to αllβ3 complex was also affected by these mutations (Table I), suggesting that the function-blocking antibody may bind close to the region containing these critical residues.

DISCUSSION

The ligand binding mechanisms of integrin αllβ3 have been extensively studied. Although there are numerous reports concerning the ligand binding sites in the β3 subunit of αllβ3 (Refs. 16, 50 for review), information about the ligand binding sites in αll is limited. The present data establish that 1) the point mutations of αll (G184A, Y189A, Y190A, F191A, and G193A) block binding of soluble fibrinogen; 2) these mutations also affect binding of ligand-mimetic antibodies PAC-1, OP-G2, and LJ-CP3; and 3) these mutations do not affect expression of αllβ3, in contrast to most natural αll mutations in Glanzmann’s thrombasthenic patients (2). These αll mutants will be potentially useful for studying the biological roles of αllβ3-ligand interactions in vivo (e.g. gene knockout in mice) or in vitro.

The amino-terminal region of the integrin α subunit is composed of seven repeats of structurally homologous sequences (51). These critical residues in the third amino-terminal repeat of αll are in the predicted β turn structure, which has been predicted using several secondary structure prediction methods and a large alignment of the seven repeats from 16 integrin sequences (51). We recently reported that the corresponding structures of α4 and α5 (residues 181–190 of α4 and α5) are critical for ligand binding to α4β1 and α5β1, respectively, using alanine-scanning mutagenesis (33). The present study establishes that the conserved amino acid sequences in the corresponding region of αll are also critically involved in αllβ3-ligand interactions. It is difficult to imagine that mutations in the predicted β turn structures affect conformation of the whole molecule. We do not, however, exclude the possibility that...
alterations in this region may affect the cation binding properties of the receptor or cause some changes in the conformation of the receptor. The amino acid sequences of the predicted β-turn structures are relatively conserved among α subunits. Interestingly, there is no divalent cation binding motif in the sequences. Since binding to fibrinogen and to three ligand-mimetic antibodies (all containing RGD-like RYD sequences in the CDR3) was blocked by the mutations in the predicted turn, it is likely that the predicted turn structure may be involved in the recognition of critical components in the ligand (e.g. RGD or γ peptide sequence). It remains to be seen whether the predicted turn structure is involved in the specificity of ligand binding.

The critical region (residues 184–193) of αIIb identified in this study is involved in large chymotryptic or recombinant αIIb fragments that have been reported to interact with ligand. Chymotrypsin digestion of αIIb3 bound to RGD-Sepharose suggests that the ligand binding sites are localized within the amino-terminal 55-kDa fragment of αIIb (52). The recombinant αIIb fragment encompassing residues 171–464, which contains four putative divalent cation binding sites, has been shown to have the ability to bind both Ca\(^{2+}\) and immobilized fibrinogen (26). Recently, using αv/αIIb chimeras, Loftus et al. (27) reported that the ligand recognition sites in αIIb3 are localized within the amino-terminal 334 residues of αIIb that include the second divalent cation binding site. The critical region of αIIb that has been identified in the present study for ligand binding is, however, distinct from the previously reported ligand binding region of αIIb (Fig. 5). Fibrinogen γ chain peptide was shown to chemically cross-link to residues 294–314 of αIIb, which contain a second putative divalent cation binding site (28). Peptide from this region of αIIb was also shown to inhibit fibrinogen binding to platelets (30). However, mutation of critical Asp residues in the homologous region of α4, another non-I domain α subunit, did not critically affect ligand interaction (53). Interestingly, swapping the divalent cation binding domains of αv with those of αIIb alone did not change the ligand binding specificity of αvβ3 (27). Other regions of αIIb spanning residues 42–73, 696–724, or 752–768 were found to be chemically cross-linked by RGD and/or γ peptides (29). Also, syn-

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**Fig. 3.** Binding of labeled fibrinogen to αIIb3 mutants. CHO cells expressing mutant αIIb3 were first incubated with mouse IgG (---) or activating anti-αIIb3 mAb PT25–2 (—) and then with FITC-labeled fibrinogen. FITC-fibrinogen bound to cells was determined by flow cytometry. The data suggest that cells expressing wild-type αIIb3 significantly bind fibrinogen in the presence of PT25–2, but cells expressing the αIIb3 mutants do not.

**Fig. 4.** Binding of ligand-mimetic antibody PAC-1 to αIIb3 mutants. CHO cells expressing αIIb3 mutants were first incubated with mouse IgG (---) or activating anti-αIIb3 mAb PT25–2 (—) and then with FITC-labeled PAC-1. FITC-PAC-1 bound to cells was determined by flow cytometry. The data suggest that PAC-1 binds to wild-type αIIb3, binds only weakly to the Y190A mutant, and does not bind at all to the other mutants.
Fig. 5. Regions of αIIb that are involved in interaction with ligands, ligand-derived peptides, or ligand-mimetic antibodies. Chymotrypsin digestion of αIIb3 bound to RGD-Sepharose suggests that the ligand binding site is localized within the amino-terminal 55-kDa fragment of αIIb (52). The recombinant αIIb fragment encompassing residues 171–464, which contain four putative divalent cation binding sites, has been shown to bind both Ca²⁺ and immobilized fibrinogen (26). The amino-terminal 334 residues of αIIb that include the second divalent cation binding site are involved in binding to PAC-1, OP-G2, and an RGD peptide-mimetic ligand (27). Fibrinogen γ-chain peptide was shown to chemically cross-link to residues 294–314 of αIIb containing a second putative divalent cation binding site (28). Peptide from this region of αIIb was shown to inhibit fibrinogen binding to platelets (30). Other regions of αIIb spanning residues 42–73, 696–724, or 752–768 were found to be chemically cross-linked by RGD and/or γ peptide (29). Synthetic peptides derived from residues 565–667 of αIIb have been reported to interact with fibrinogen (31). More detailed mutagenesis studies will be required to determine whether these regions are involved in the ligand binding of αIIb3.

Two RGD-containing sequences in the α-chain (Aα55–98 and Aα572–579) and one dodecapeptide sequence at the carboxyl terminus of the γ-chain (γ400–411) of fibrinogen have been proposed as binding sites for αIIb3 (3, 4, 54). However, studies using anti-peptide antibodies and recombinant mutant fibrinogen suggest that platelet αIIb3 interacts primarily with the γ-chain sequence rather than with RGD sequences in fibrinogen (55–58). αIIb3 also binds to von Willebrand factor, vitronectin, and fibronectin through RGD sequences. The present data suggest that point mutations in αIIb block binding of both fibrinogen (through γ-chain peptide) and ligand-mimetic antibodies (through RGD-like RGD sequences) to αIIb3. These data support the previous idea that RGD and γ-chain peptide share a common or overlapping binding site, or a common binding mechanism, in αIIb3. Although the binding profiles of fibrinogen and ligand-mimetic antibodies to αIIb mutants are mostly in parallel, there is a slight difference in binding profiles between fibrinogen and PAC-1. We determined that PAC-1 binds to the Y190A mutant of αIIb but that fibrinogen does not. It will be interesting to determine whether this is due to a difference in binding affinity or in mode of recognition between fibrinogen and PAC-1.

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