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RGD-containing Peptides Inhibit Fibrinogen Binding to Platelet $\alpha_{IIb}\beta_3$ by Inducing an Allosteric Change in the Amino-terminal Portion of α_{IIb}*

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To determine the molecular basis for the insensitivity of rat $\alpha_{IIb}\beta_3$ to inhibition by RGD-containing peptides, hybrids of human and rat $\alpha_{IIb}\beta_3$ and chimeras of $\alpha_{IIb}\beta_3$ in which α_{IIb} was composed of portions of human and rat α_{IIb} were expressed in Chinese hamster ovary cells and B lymphocytes, and the ability of the tetrapeptide RGDS to inhibit fibrinogen binding to the various forms of $\alpha_{IIb}\beta_3$ was measured. These measurements indicated that sequences regulating the sensitivity of $\alpha_{IIb}\beta_3$ to RGDS are located in the seven amino-terminal repeats of α_{IIb}. Moreover, replacing the first three or four (but not the first two) repeats of rat α_{IIb} with the corresponding human sequences enhanced sensitivity to RGDS, whereas replacing the first two or three repeats of human α_{IIb} with the corresponding rat sequences had little or no effect. Nevertheless, RGDS bound to Chinese hamster ovary cells expressing $\alpha_{IIb}\beta_3$ regardless whether the α_{IIb} in the heterodimers was human, rat, or a rat-human chimera. These results indicate that the sequences determining the sensitivity of $\alpha_{IIb}\beta_3$ to RGD-containing peptides are located in the third and fourth amino-terminal repeats of α_{IIb}. Because RGDS binds to both human and rat $\alpha_{IIb}\beta_3$, the results suggest that differences in RGDS sensitivity result from differences in the allosteric changes induced in these repeats following RGDS binding.

Ligand binding to integrins initiates intracellular signals that are crucial for cellular growth and differentiation (1). Conversely, many cells regulate the ability of their integrins to recognize ligands. The prototypic example of integrin regulation is the platelet integrin $\alpha_{IIb}\beta_3$ (2). In unstimulated platelets, $\alpha_{IIb}\beta_3$ is inactive; but following platelet stimulation by agonists such as ADP and thrombin, $\alpha_{IIb}\beta_3$ assumes a conformation in which it is able to bind macromolecular ligands such as fibrinogen and von Willebrand factor. Because ligand binding to $\alpha_{IIb}\beta_3$ is a prerequisite for platelet aggregation, regulating the affinity of $\alpha_{IIb}\beta_3$ for ligands assures that only stimulated platelets aggregate.

The major ligand for $\alpha_{IIb}\beta_3$ in plasma is fibrinogen. Three portions of the fibrinogen molecule (the carboxyl terminus of the fibrinogen γ-chain (3) and two Arg-Gly-Asp (RGD) motifs located in the fibrinogen α-chain (4)) have been proposed to be sites that mediate fibrinogen binding to $\alpha_{IIb}\beta_3$. However, ultrastructural examination of fibrinogen bound to $\alpha_{IIb}\beta_3$ (5) and measurements of fibrinogen binding to $\alpha_{IIb}\beta_3$ using fibrinogens containing mutated RGD or γ-chain sequences (6) indicate that it is the γ-chain sequences that mediate fibrinogen binding. Nonetheless, RGD-containing disintegrins and synthetic compounds based on the RGD motif are effective $\alpha_{IIb}\beta_3$ antagonists (7), implying that they either directly or indirectly affect the γ-chain-binding site when they bind to $\alpha_{IIb}\beta_3$.

Ligands appear to bind to $\alpha_{IIb}\beta_3$ by interacting with the amino-terminal portion of β_3 (8), although the specific residues involved are not known. A region of β_3 encoded by the fourth and fifth exons of the β_3 gene that encompasses amino acids 95–223 has been implicated in ligand binding (9). Moreover, chemical cross-linking experiments have suggested that RGD-containing peptides bind to β_3 in the vicinity of amino acids 109–171 (10). It is noteworthy that this region of β_3 contains an array of oxygenated residues whose three-dimensional structure may resemble that of the ligand-binding I domains that are present in several integrin α-subunits (11). In addition, overlapping peptides corresponding to β_3 amino acids 211–222 inhibit fibrinogen binding to purified $\alpha_{IIb}\beta_3$, suggesting that this stretch of residues represents a portion of the fibrinogen-binding site (12, 13). There is also evidence that more distal portions of β_3 may be involved in fibrinogen binding because a naturally occurring $Leu^{262} \rightarrow Pro$ mutation prevents $\alpha_{IIb}\beta_3$ binding to immobilized fibrinogen (14).

Ligand binding to $\alpha_{IIb}\beta_3$ also appears to involve the amino-terminal third of α_{IIb} (15). Chemical cross-linking experiments suggest that the carboxyl terminus of the fibrinogen γ-chain binds to α_{IIb} in the vicinity of amino acids 294–314 (16), a suggestion supported by the ability of a peptide corresponding to α_{IIb} residues 300–312 to inhibit platelet adhesion to fibrinogen (17). In addition, there are a number of reports of naturally occurring and laboratory-induced mutations involving amino acids located between α_{IIb} residues 183 and 224 that impair $\alpha_{IIb}\beta_3$ function, suggesting that this portion of α_{IIb} binds to ligands as well (18–21).

Although fibrinogen binding to $\alpha_{IIb}\beta_3$ on the platelets of all mammalian species is required for platelet aggregation, there are substantial differences in the ability of RGD-containing peptides to inhibit the process. For example, fibrinogen binding to rabbit and rat platelets is relatively insensitive to inhibition by RGD-containing peptides (22, 23). To gain an understanding of the molecular basis for the insensitivity of rat $\alpha_{IIb}\beta_3$ to

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RGD-containing peptides, we measured the effect of the tetrapeptide Arg-Gly-Asp-Ser (RGDS) on fibrinogen binding to chimeric $\alpha_{IIb}\beta_3$ molecules composed of portions of the rat and human proteins. We found that the sequences determining the sensitivity or resistance of $\alpha_{IIb}\beta_3$ to inhibition by RGDS are located in the third and fourth repeats of the amino-terminal portion of α_{IIb}. Moreover, because we also found that RGDS bound to $\alpha_{IIb}\beta_3$ regardless of whether the heterodimer contained human or rat subunits, our results imply that RGDS impairs fibrinogen binding to $\alpha_{IIb}\beta_3$ by inducing an allosteric change in the third and fourth repeats of α_{IIb}. They also suggest that a conformational change in these repeats may be required for the fibrinogen binding to $\alpha_{IIb}\beta_3$ that occurs on agonist-stimulated platelets.

EXPERIMENTAL PROCEDURES

Measurement of Platelet Aggregation—Platelet-rich plasma was prepared from blood anticoagulated with 0.1 volume of 0.13 M sodium citrate, obtained from normal human volunteers by venipuncture and from anesthetized rats by puncture of the exposed abdominal aorta. Platelets were isolated from the platelet-rich plasma by gel filtration on Sepharose 2B (Amersham Pharmacia Biotech) using elution buffer containing 137 mM NaCl, 2.7 mM KCl, 1 mM $MgCl_2$, 5.6 mM glucose, 0.35 mg/ml bovine serum albumin, 3.3 mM NaH_2PO_4, and 4 mM Hepes (pH 7.4) as previously described (24). Turbidometric measurements of ADP-stimulated platelet aggregation were made in a Chrono-Log Lumi dual aggregometer. Platelet suspensions were supplemented with either human or rat fibrinogen (Sigma) at a final concentration of 200 μg/ml, with 1 mM $CaCl_2$, and with various concentrations of RGDS (Sigma) or the less active control tetrapeptide Arg-Gly-Glu-Ser (RGES; Sigma) prior to adding ADP.

Measurement of Fibrinogen Binding to Human and Rat Platelets—Fibrinogen binding to gel-filtered human and rat platelets was measured using ^{125}I-labeled fibrinogen as previously described (24). Briefly, 0.5-ml aliquots of $\approx 5 \times 10^7$ gel-filtered platelets were mixed with 200 μg/ml ^{125}I-fibrinogen (Enzyme Research Laboratories), 0.5 mM $CaCl_2$, and 10 μM ADP. Following a 5-min incubation at 37 °C without stirring, the platelets were sedimented through silicone oil in an Eppendorf centrifuge (Brinkmann Instruments). The tips of the centrifuge tubes containing the pelleted platelets were amputated and counted for ^{125}I. Nonspecific fibrinogen binding was determined by including a 15-fold excess of unlabeled fibrinogen in the assay. The dissociation constants (K_d) for human and rat $\alpha_{IIb}\beta_3$ for fibrinogen were obtained by Scatchard analysis of the fibrinogen binding data.

Construction of Chimeric Human-Rat α_{IIb} Subunits—Full-length cDNAs for human and rat α_{IIb} and a full-length cDNA for human β_3 were used in selected experiments (25–28). A nearly full-length rat β_3 cDNA was completed by inserting the sequences corresponding to the signal peptide and the first 31 amino acids of human β_3 (21, 29). The amino-terminal region of mature β_3 is highly conserved; for example, human and *Xenopus* β_3 cDNAs differ by only nine amino acids in this region (30).

cDNAs encoding chimeras of α_{IIb} in which the amino-terminal halves of human and rat α_{IIb} were exchanged were constructed by swapping homologous *Cla*I/*Nhe*I 5′-fragments of human and rat α_{IIb} cDNAs (28). cDNAs encoding α_{IIb} chimeras containing smaller segments of rat and human α_{IIb} were constructed using a polymerase chain reaction-based site-directed mutagenesis protocol described previously (21). Briefly, using either a human or rat α_{IIb} cDNA template, the 3′-portion of the targeted sequence was amplified using one of the sense primers shown below and the appropriate antisense primer 3′ to the *Cla*I site. Similarly, the 5′-portion was amplified using the appropriate α_{IIb} cDNA template, a primer complementary to one of the sense primers shown below, and a T7 primer. The 5′- and 3′-polymerase chain reaction products were then purified on agarose gels after separation from the templates. A third polymerase chain reaction was performed using the two first-round amplified products, the T7 primer, and the appropriate primer 3′ to the *Cla*I site in the template. The product was double-digested with *Cla*I and *Nhe*I and subcloned into either a human or rat α_{IIb} cDNA that had previously been inserted into the expression plasmid pcDNA3.1 (Invitrogen). Selected clones were sequenced to ensure the fidelity of the desired nucleotide sequence. The nomenclature used to identify the various chimeras is based on the presence of seven tandem repeats in the amino-terminal half of α_{IIb} (31). The sense primers used for the polymerase chain reactions were as follows: R_2-H, GGAGTACTCGGCGCGGCGCCCGCTTTGGAGCTCAGC; R_3-H, GGACACGTGCCACAAAAGGGTACCGGGGCGGTACGT; R_4-H, CTGGTAGTAGGAATCCAAAATTTCCACCGCTCCCAA; H_2-R, GCTGAGCTCCAAAGCGGGCGCCGCGCCGCGTACTCC; H_3-R, ACGTACCGCCCCGGTACCCTTTTGTGGCACGTGTCC; and H_4-R, TTGGGAGCGGTGGAAATTTTGGATTCCTACTACCAG. The sequences of the primers 3′ to the *Cla*I site in human and rat α_{IIb} were GCTGCAGCTCGGCATTTAGG and CTTCAGTGTGGGATTCAG, respectively.

Stable Expression of $\alpha_{IIb}\beta_3$ in Chinese Hamster Ovary (CHO)[1] Cells—CHO cells were cultured in Ham's F-12 medium (Life Technologies, Inc.) supplemented with 10% fetal bovine serum (Hyclone Laboratories). cDNAs encoding human β_3 and either human or rat α_{IIb} were subcloned into pcDNA3.1(+)-Zeo and pcDNA3.1(+)-Neo, respectively, and were introduced into the CHO cells using FUGENE 6 (Roche Molecular Biochemicals) according to the manufacturer's instructions. Two days after transfection, the cells were transferred to selection medium containing 500 μg/ml G418 (Life Technologies, Inc.) and 300 μg/ml Zeocin (Invitrogen). After 3 weeks of selection, $\alpha_{IIb}\beta_3$ expression was assessed by flow cytometry using P34, a mAb that recognizes both rat and human $\alpha_{IIb}\beta_3$ (a gift from Dr. H. Miyazaki, Kirin Brewery, Gunma, Japan). The cells were then sorted by fluorescence-activated cell sorting to obtain cell lines expressing high levels of $\alpha_{IIb}\beta_3$ as previously described (21).

Fibrinogen Binding to CHO Cells Expressing $\alpha_{IIb}\beta_3$—To measure fibrinogen binding to $\alpha_{IIb}\beta_3$ on the transfected CHO cells, purified human fibrinogen was labeled with fluorescein isothiocyanate (FITC) using a Calbiochem FITC labeling kit as described by the manufacturer. Fibrinogen labeled in this manner was monomeric as assessed by gel-filtration chromatography, supported platelet aggregation as well as unlabeled fibrinogen, and was 95% clottable with thrombin (32). 1.5×10^5 CHO cells, suspended in 100 μl of 10 mM sodium phosphate buffer (pH 7.4) containing 137 mM NaCl, 1 mM $CaCl_2$, and 1% bovine serum albumin, were then incubated with 200 μg/ml FITC-fibrinogen and 5 mM dithiothreitol (DTT) for 30 min at 37 °C (33, 34). The cells were washed once with the suspension buffer and fixed with 0.37% formalin. The amount of FITC-fibrinogen bound was determined by flow cytometry as described previously (21). Specific fibrinogen binding represented the difference in fluorescence of transfected and untransfected cells incubated with FITC-fibrinogen in the presence of DTT minus the fluorescence of transfected cells incubated with FITC-fibrinogen in the absence of DTT. The ability of RGDS to inhibit fibrinogen binding was determined by adding increasing concentrations of the tetrapeptide to the 30-min incubation.

Adhesion of B Lymphocytes Expressing $\alpha_{IIb}\beta_3$ to Immobilized Fibrinogen—α_{IIb} and β_3 were expressed in human B lymphocytes as previously described (35). Briefly, pREP vectors containing rat or human α_{IIb} and β_3 cDNAs were introduced into 7.5×10^6 GM1500 B lymphocytes by electroporation (250 V and 960 microfarads). Stable transfectants were selected using G418 and hygromycin, and the amount of $\alpha_{IIb}\beta_3$ on the lymphocyte surface was quantified by flow cytometry using mAb P34. To measure $\alpha_{IIb}\beta_3$-mediated lymphocyte adherence to fibrinogen, the wells of Immulon-2 flat-bottom microtiter plates (Dynatech Laboratories Inc.) were coated with 50 μg/ml purified human fibrinogen in 50 mM $NaHCO_3$ buffer (pH 8.0) containing 150 mM NaCl. Unoccupied protein-binding sites on the wells were blocked with 5 mg/ml bovine serum albumin dissolved in the same buffer. 1.5×10^5 B lymphocytes, metabolically labeled overnight with [^{35}S]methionine, were suspended in 100 μl of 50 mM Tris-HCl buffer (pH 7.4) containing 150 mM NaCl, 0.5 mM $CaCl_2$, 0.1% glucose, and 1% bovine serum albumin and added to the protein-coated wells, in either the presence or absence of 200 ng/ml phorbol myristate acetate. Following incubation at 37 °C for 30 min without agitation, the plates were washed four times with the lymphocyte suspension buffer, and adherent cells were dissolved using 2% SDS. The SDS solutions were then counted for ^{35}S in a liquid scintillation counter. The ability of RGDS to inhibit lymphocyte adhesion to immobilized fibrinogen was determined by adding increasing concentrations of the tetrapeptide to the 30-min incubation.

Induction of mAb Binding to β_3 by RGDS—To measure the RGDS-induced binding of the conformation-specific mAb 10-758 to human β_3 (36), 1.5×10^5 CHO cells expressing human $\alpha_{IIb}\beta_3$ and hybrids of rat α_{IIb} and human β_3 were incubated with 0.3 mM RGDS and a 1:100 dilution of mAb 10-758 for 30 min at 37 °C. The cells were then washed once and incubated with a 1:10 dilution of FITC-labeled goat

[1] The abbreviations used are: CHO, Chinese hamster ovary; mAb, monoclonal antibody; FITC, fluorescein isothiocyanate; DTT, dithiothreitol.

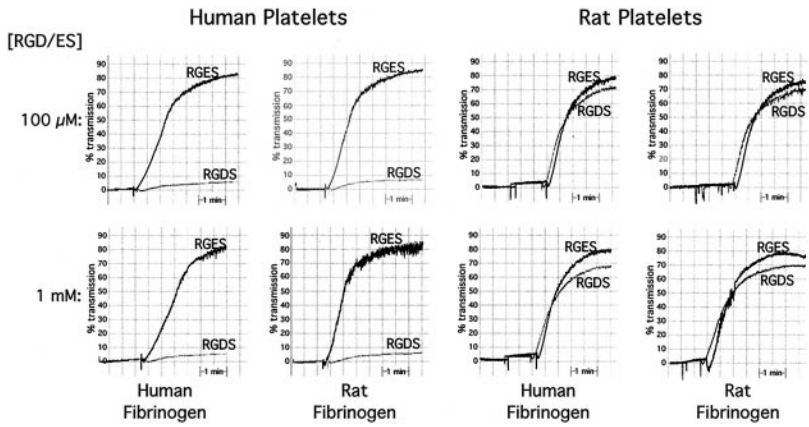

FIG. 1. **Effect of the tetrapeptides RGDS and RGES on the ADP-stimulated aggregation of human and rat platelets.** Gel-filtered human and rat platelets were suspended in buffer containing 1 mM $CaCl_2$ and either 200 μg/ml human or rat fibrinogen. Turbidometric platelet aggregation was stimulated by 20 μM ADP and measured in the presence of 0.1 and 1 mM concentrations of either RGDS or RGES.

anti-mouse IgG for an additional 30 min. Antibody binding was detected using flow cytometry.

RESULTS

Effect of RGDS on the ADP-stimulated Aggregation of Human and Rat Platelets—To confirm the reported insensitivity of rat platelets to the inhibitory effects of RGD-containing peptides (22), we compared the ability of the tetrapeptide RGDS to inhibit the ADP-stimulated aggregation of gel-filtered human and rat platelets. Although neither human nor rat platelets aggregated in the absence of added fibrinogen (data not shown), ADP-stimulated platelets of both species aggregated readily in the presence of either human or rat fibrinogen (Fig. 1). Furthermore, whereas the tetrapeptide RGES had no effect on the aggregation of platelets of either species, the aggregation of human platelets was partially inhibited by 10 μM RGDS and completely inhibited by 100 μM RGDS. In contrast, concentrations of RGDS as great as 1 mM had no effect on the aggregation of rat platelets. Thus, these experiments confirm the difference in sensitivity of human and rat platelets to RGD-containing peptides and indicate that this difference is due to a difference between human and rat platelets and not to a difference between human and rat fibrinogen.

One explanation for the insensitivity of rat platelets to RGDS is simply that the affinity of $\alpha_{IIb}\beta_3$ on rat platelets for fibrinogen is greater than that of $\alpha_{IIb}\beta_3$ on human platelets. To address this possibility, we measured the affinity of $\alpha_{IIb}\beta_3$ on human and rat platelets using ^{125}I-labeled fibrinogen (24). We found that the K_d for fibrinogen binding to $\alpha_{IIb}\beta_3$ on human platelets was $(1.32 \pm 0.12) \times 10^{-7}$ ($n = 21$), compared with a K_d of $(2.31 \pm 0.45) \times 10^{-7}$ ($n = 3$) for fibrinogen binding to $\alpha_{IIb}\beta_3$ on rat platelets. Thus, these measurements indicate that a difference in the affinity of human and rat $\alpha_{IIb}\beta_3$ for fibrinogen cannot account for the difference in sensitivity of human and rat platelets to RGDS.

Effect of RGDS on Fibrinogen Binding to Human-Rat $\alpha_{IIb}\beta_3$ Hybrids Expressed in CHO Cells—We next sought a molecular basis for the difference in sensitivity of human and rat platelets to RGDS by expressing $\alpha_{IIb}\beta_3$ heterodimers composed of human and rat subunits in CHO cells. As shown by the flow cytometry histograms in Fig. 2, comparable amounts of each of the four possible combinations of human and rat α_{IIb} and β_3 were expressed on the CHO cell surface.

Because $\alpha_{IIb}\beta_3$ expressed in CHO cells cannot be activated by cellular agonists, ligand binding is usually induced using "activating" mAbs (37). These antibodies generally do not bind to rat $\alpha_{IIb}\beta_3$. Consequently, we induced fibrinogen binding to $\alpha_{IIb}\beta_3$ in our CHO cell lines by incubating the cells with DTT, based on previous reports that DTT induces fibrinogen binding to $\alpha_{IIb}\beta_3$ on platelets (33, 34). To confirm that the fibrinogen binding induced by DTT is indeed comparable to that induced

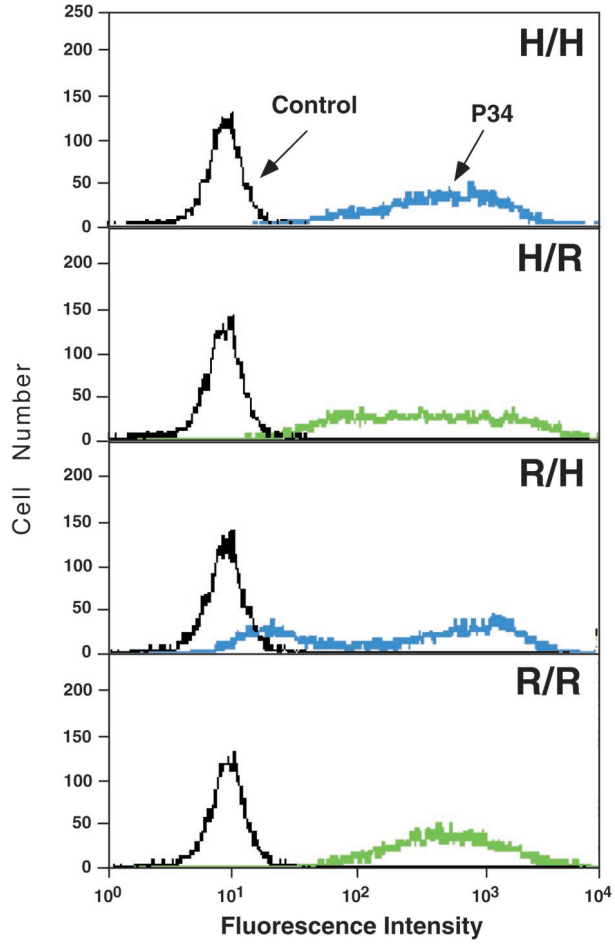

FIG. 2. **Expression of human, rat, and human-rat hybrid $\alpha_{IIb}\beta_3$ on the surface of transfected CHO cells.** CHO cells were cotransfected with plasmids containing cDNAs for either human or rat α_{IIb} and human or rat β_3 as described under "Experimental Procedures." The level of $\alpha_{IIb}\beta_3$ expression by the resulting cells lines was assessed by flow cytometry using P34, a mAb that recognizes both rat and human $\alpha_{IIb}\beta_3$, as well as a class-matched control antibody. *H*/*H*, human α_{IIb}/human β_3; *H*/*R*, human α_{IIb}/rat β_3; *R*/*H*, rat α_{IIb}/human β_3; *R*/*R*, rat α_{IIb}/rat β_3.

by activating mAbs, we incubated CHO cells expressing human $\alpha_{IIb}\beta_3$ with 5 mM DTT and with the activating mAb PT25-2 and measured FITC-fibrinogen binding to the incubated cells using flow cytometry. As shown by the histograms in Fig. 3, fibrinogen binding induced by DTT and mAb PT25-2 was indistinguishable. Moreover, the fibrinogen binding induced by either agent was inhibited by the $\alpha_{IIb}\beta_3$-specific mAb A2A9, confirming that the fibrinogen was bound to $\alpha_{IIb}\beta_3$.

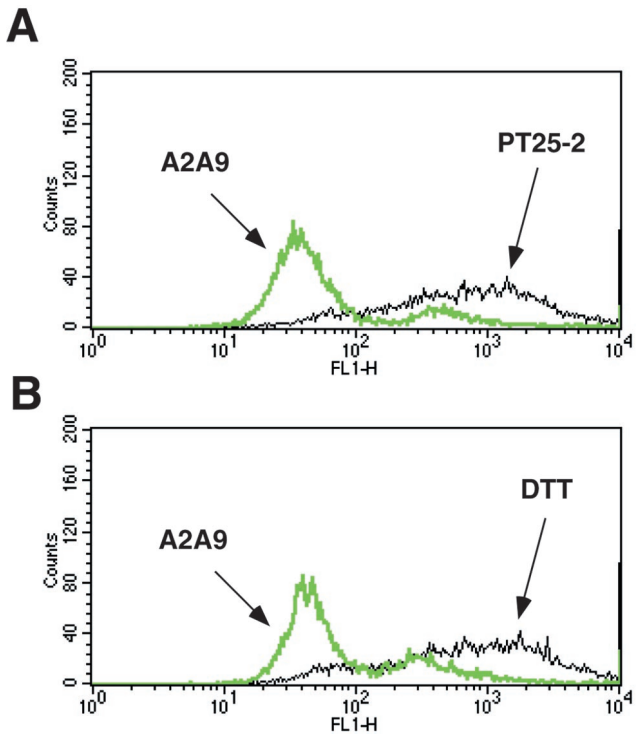

FIG. 3. **Comparison of mAb PT25-2-induced and DTT-induced FITC-fibrinogen binding to CHO cells expressing human $\alpha_{IIb}\beta_3$.** CHO cells stably expressing human $\alpha_{IIb}\beta_3$ were incubated either with the $\alpha_{IIb}\beta_3$-activating mAb PT25-2 at 10 μg/ml (*A*) or with 5 mM DTT (*B*) for 30 min at 37 °C in the presence of 200 μg/ml FITC-fibrinogen and 1 mM $CaCl_2$. The extent of FITC-fibrinogen binding was then measured by flow cytometry. The specificity of fibrinogen binding induced by mAb PT25-2 and DTT was assessed by adding the $\alpha_{IIb}\beta_3$-inhibiting mAb A2A9 to the incubations.

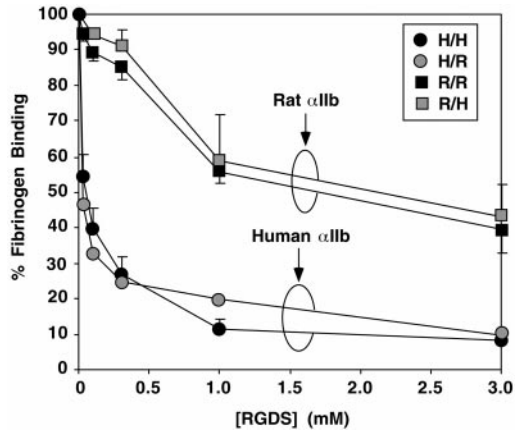

FIG. 4. **Effect of RGDS on DTT-stimulated FITC-fibrinogen binding to CHO cells expressing human and rat $\alpha_{IIb}\beta_3$.** CHO cell lines stably expressing the four possible combinations of human and rat α_{IIb} and β_3 were incubated with 5 mM DTT in the presence of 200 μg/ml FITC-fibrinogen, 1 mM $CaCl_2$, and increasing concentrations of the tetrapeptide RGDS for 30 min at 37 °C. The extent of FITC-fibrinogen binding was measured by flow cytometry. *Solid circles*, human α_{IIb}/human β_3 (*H*/*H*); *shaded circles*, human α_{IIb}/rat β_3 (*H*/*R*); *solid squares*, rat α_{IIb}/rat β_3 (*R*/*R*); *shaded squares*, rat α_{IIb}/human β_3 (*R*/*H*). The data shown are the means ± S.E. of nine (human α_{IIb}/human β_3 and rat α_{IIb}/rat β_3) and three (human α_{IIb}/rat β_3 and rat α_{IIb}/human β_3) experiments.

TABLE I
Inhibition of fibrinogen binding to human $\alpha_{IIb}\beta_3$ and to human-rat chimeras by the tetrapeptide RGDS

$\alpha_{IIb}\beta_3$	IC_{50}	Relative resistance to RGDS
	mM	
H/H	0.04	1
H/R	0.01	0.31
R/H	2.07	49
R/R	1.65	39
R_{1-7}-H/H	0.81	19
H_{1-7}-R/H	0.02	0.48
H_{1-2}-R/H	1.02	24
H_{1-3}-R/H	0.22	5
H_{1-4}-R/H	0.03	1
R_{1-2}-H/H	0.1	2
R_{1-3}-H/H	0.47	11

The effect of RGDS on fibrinogen binding to the cell lines shown in Fig. 2 was studied by adding increasing concentrations of the tetrapeptide to the fibrinogen binding assays. The results of these experiments are shown in Fig. 4. As expected, fibrinogen binding to cells expressing human $\alpha_{IIb}\beta_3$ was relatively sensitive to inhibition by RGDS, whereas fibrinogen binding to cells expressing rat $\alpha_{IIb}\beta_3$ was relatively resistant. However, to our surprise, based on the observation that RGD-containing peptides cross-link to the amino terminus of β_3 (10), we found that fibrinogen binding to cells expressing $\alpha_{IIb}\beta_3$ containing a rat α-subunit and a human β-subunit was resistant to RGDS, whereas fibrinogen binding to cells expressing $\alpha_{IIb}\beta_3$ containing a human α-subunit and a rat β-subunit was sensitive. The IC_{50} values (concentrations of RGDS that inhibited fibrinogen binding by 50%) for RGDS, calculated from semilog plots of the binding data, were 1.65 and 2.07 mM for cells expressing rat $\alpha_{IIb}\beta_3$ and rat α_{IIb}/human β_3, compared with 0.04 and 0.01 mM for cells expressing human $\alpha_{IIb}\beta_3$ and human α_{IIb}/rat β_3 (Table I), respectively.

Effect of RGDS on the Adhesion of B Lymphocytes Expressing $\alpha_{IIb}\beta_3$ to Immobilized Fibrinogen—To rule out the possibility that the observed differences in sensitivity to RGDS were due to differences in the response of human and rat α_{IIb} to DTT, we expressed the four combinations of human and rat α_{IIb} and β_3 in the B lymphocyte cell line GM1500 (35). Flow cytometry of the transfected cells using mAb P34 indicated that each of the combinations of human and rat α_{IIb} and β_3 was expressed to a comparable extent on the lymphocyte surface (data not shown). We then measured the effect of RGDS on phorbol 12-myristate 13-acetate-stimulated lymphocyte adhesion to immobilized fibrinogen. As shown in Fig. 5, we found that lymphocytes expressing $\alpha_{IIb}\beta_3$ heterodimers containing an α-subunit of human origin were ≈20-fold more sensitive to the inhibitory effect of RGDS than lymphocytes expressing heterodimers containing an α-subunit of rat origin. Thus, these experiments confirm that the difference in sensitivity of human and rat $\alpha_{IIb}\beta_3$ to RGD-containing peptides can be attributed to structural differences between human and rat α_{IIb}.

Localization of the α_{IIb} Regions Regulating Sensitivity to RGDS Using Human-Rat α_{IIb} Chimeras—The amino-terminal portion of α_{IIb} consists of seven tandem repeats, each of which contains ~60 amino acids (31). To localize the sites in α_{IIb} that regulate sensitivity to RGDS, we exchanged the amino-terminal repeats of rat α_{IIb} for the human repeats and vice versa, making use of a conserved *Cla*I restriction site. The α_{IIb} chimeras were then coexpressed with human β_3 in CHO cells, and the ability of RGDS to inhibit the binding of FITC-fibrinogen to the chimeras was tested. As shown in Fig. 6, chimeras in which the seven amino-terminal repeats of α_{IIb} were of human origin were sensitive to RGDS, whereas chimeras in which the seven amino-terminal repeats were of rat origin were resistant. Thus, these experiments indicate that the sequences regulating the sensitivity of $\alpha_{IIb}\beta_3$ to RGDS are located in the seven amino-terminal repeats of α_{IIb}.

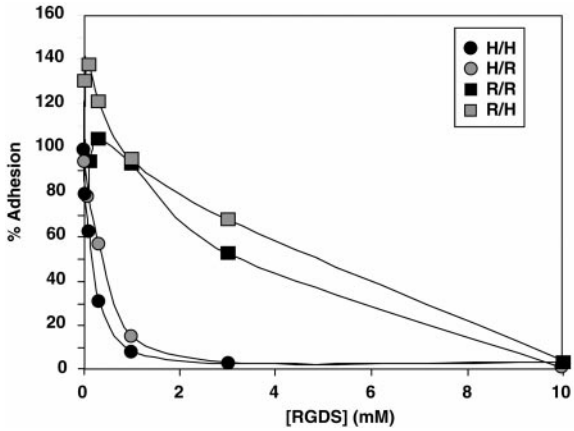

FIG. 5. **Effect of RGDS on the phorbol 12-myristate 13-acetate-stimulated adhesion of B lymphocytes expressing human and rat $\alpha_{IIb}\beta_3$ to immobilized fibrinogen.** 1.5×10^5 GM1500 B lymphocytes stably expressing the four possible combinations of human and rat α_{IIb} and β_3 and metabolically labeled with [^{35}S]methionine were added to the wells of microtiter plates coated with purified human fibrinogen. Following the addition of increasing concentrations of the tetrapeptide RGDS, lymphocyte adhesion to the immobilized fibrinogen was induced by stimulating the cells with 200 ng/ml phorbol 12-myristate 13-acetate, and the extent of cell adhesion was measured as described under "Experimental Procedures." *Solid circles*, human α_{IIb}/human β_3 (*H*/*H*); *shaded circles*, human α_{IIb}/rat β_3 (*H*/*R*); *solid squares*, rat α_{IIb}/rat β_3 (*R*/*R*); *shaded squares*, rat α_{IIb}/human β_3 (*R*/*H*). The data shown are the means of measurements made in triplicate and are representative of three experiments.

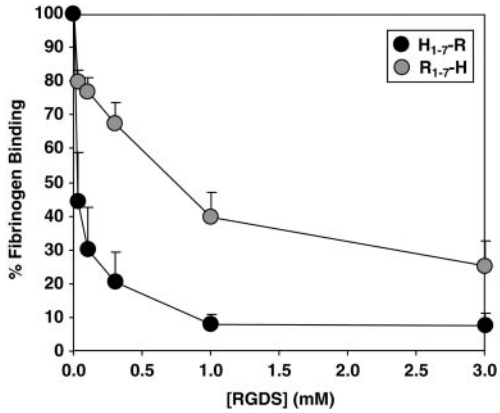

FIG. 6. **Effect of exchanging the amino-terminal halves of human and rat α_{IIb} on the ability of RGDS to inhibit FITC-fibrinogen binding to $\alpha_{IIb}\beta_3$.** CHO cells stably coexpressing human β_3 and chimeras of human and rat α_{IIb} in which the seven amino-terminal repeats had been exchanged were incubated with 5 mM DTT in the presence of 200 μg/ml FITC-fibrinogen, 1 mM $CaCl_2$, and increasing concentrations of the tetrapeptide RGDS for 30 min at 37 °C. The extent of fibrinogen binding was then measured using flow cytometry. *Solid circles*, an α_{IIb} chimera containing the seven amino-terminal repeats of human α_{IIb} (H_{1-7}-*R*); *shaded circles*, an α_{IIb} chimera containing the seven amino-terminal repeats of rat α_{IIb} (R_{1-7}-*H*). The data shown are the means ± S.E. of four (R_{1-7}-*H*) and six (H_{1-7}-*R*) experiments.

Identification of Specific Regions of the Amino Terminus of α_{IIb} That Regulate Sensitivity to RGDS—To further localize the sequences that regulate the sensitivity of $_{\alpha IIb}\beta_3$ to RGDS, we replaced the first two, three, and four amino-terminal repeats of rat α_{IIb} with the corresponding human sequences. The resulting chimeric α-subunits were coexpressed with human β_3 in CHO cells, and the ability of RGDS to inhibit FITC-fibrinogen binding to each cell line was measured. As shown in Fig. 7*A*, when the first four repeats of rat α_{IIb} were replaced by the human sequences, the resulting $\alpha_{IIb}\beta_3$ heterodimer was sensitive to RGDS. In contrast, when only the first two repeats were of human origin, the $\alpha_{IIb}\beta_3$ chimera was resistant. A chimera in

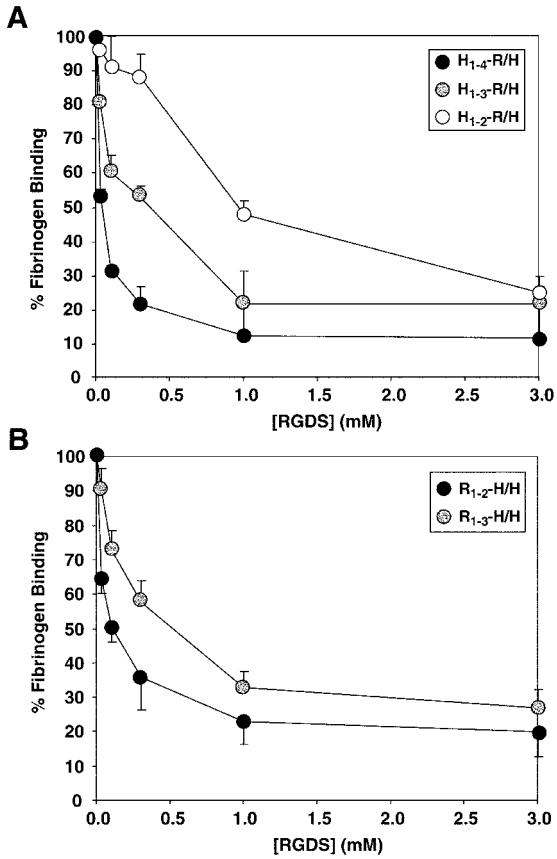

FIG. 7. **Effect of RGDS on DTT-stimulated FITC-fibrinogen binding to $\alpha_{IIb}\beta_3$ composed of human β_3 and chimeras of human and rat α_{IIb}.** Chimeric human and rat α_{IIb} subunits in which the first and second, first through third, and first through fourth amino-terminal repeats had been replaced with those of the other species were stably coexpressed with human β_3 in CHO cells as described under "Experimental Procedures." The effect of RGDS on FITC-fibrinogen binding to the resulting cells lines was measured as described in the legends to Figs. 4 and 6. *A*, the amino-terminal repeats of rat α_{IIb} were replaced by the corresponding human repeats. *Solid circles*, first through fourth repeats (H_{1-4}-*R*/*H*); *shaded circles*, first through third repeats (H_{1-3}-*R*/*H*); *open circles*, first and second repeats (H_{1-2}-*R*/*H*). *B*, the amino-terminal repeats of human α_{IIb} were replaced by the corresponding rat repeats. *Solid circles*, first and second repeats (R_{1-2}-*H*/*H*); *shaded circles*, first through third repeats (R_{1-3}-*H*/*H*). The data shown are the means ± S.E. of three experiments.

which the first three repeats were of human origin was of intermediate sensitivity. Thus, these data indicate that sequences regulating the response of $\alpha_{IIb}\beta_3$ to RGDS are located in the third and fourth amino-terminal repeats of α_{IIb}.

To confirm this conclusion, we made the reciprocal exchanges. However, although human α_{IIb} in which the first and second repeats and the first through third repeats were replaced by the corresponding rat sequences readily coexpressed with human β_3 on the CHO cell surface, α_{IIb} in which the first through fourth repeats were of rat origin was never expressed to a level sufficient to measure fibrinogen binding. Nonetheless, as shown in Fig. 7*B*, replacing the first and second human repeats with the corresponding rat sequence had no effect on the sensitivity of $\alpha_{IIb}\beta_3$ to RGDS, and a chimera in which the first through third repeats were exchanged was only slightly less sensitive. Thus, these results are consistent with those shown in Fig. 7*A*.

The IC_{50} values for RGDS inhibition of fibrinogen binding to the various cell lines, as well as a relative RGDS resistance index derived by normalizing the IC_{50} values to that for human $\alpha_{IIb}\beta_3$, are shown in Table I. This analysis verifies that the locus for sensitivity to RGDS is located in α_{IIb} and that the

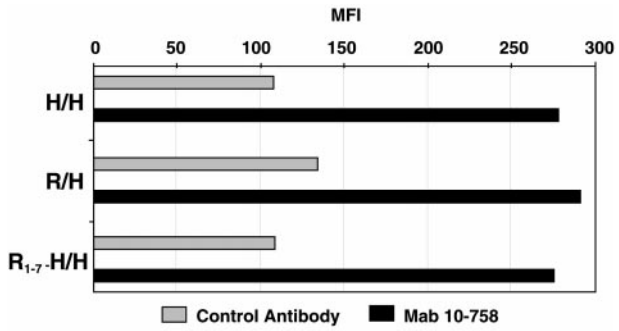

FIG. 8. **RGDS induction of mAb 10-758 binding to $\alpha_{IIb}\beta_3$ containing either a human or rat α-subunit.** 1.5×10^5 CHO cells coexpressing human α_{IIb} with human β_3 (H/H), rat α_{IIb} with human β_3 (R/H), and an α_{IIb} chimera composed of the amino-terminal half of rat α_{IIb} and the carboxyl-terminal half of human α_{IIb} with human β_3 (R_{1-7}-H/H) were incubated with 0.3 mM RGDS and a 1:100 dilution of mAb 10-758 or a class-matched control antibody for 30 min at 37 °C. The cells were then incubated with a 1:10 dilution of FITC-labeled goat anti-mouse IgG for an additional 30 min. Antibody binding was assessed by flow cytometry.

relevant sequences are present in its third and fourth amino-terminal repeats.

Induction of mAb Binding to β_3 by RGDS—Based on these data, there are two possible ways in RGDS could inhibit fibrinogen binding to αIIbβ_3. First, it is possible that RGDS binds to the third and fourth amino-terminal repeats of α_{IIb} and directly competes with fibrinogen for binding to this site. Second, it is possible that RGDS binds elsewhere in $\alpha_{IIb}\beta_3$ and exerts an allosteric effect on the third and fourth amino-terminal repeats of αIIb, thereby inhibiting fibrinogen binding. Binding of RGD-based peptides and peptidomimetics to $\alpha_{IIb}\beta_3$ has been shown to induce the expression of epitopes for a number of anti-α_{IIb} and anti-β_3 mAbs (36). Therefore, to differentiate between the two possibilities discussed above, we measured the ability of RGDS to induce the binding of the human β_3-specific mAb 10-758 to RGDS-sensitive human $\alpha_{IIb}\beta_3$, to RGDS-resistant rat α_{IIb}/human β_3, and to RGDS-resistant $\alpha_{IIb}\beta_3$ in which the amino-terminal half of α_{IIb} was of rat origin. As shown in Fig. 8, 0.3 mM RGDS induced mAb 10-758 binding to each form of $\alpha_{IIb}\beta_3$. We conclude from these data that RGDS bound to each form of $\alpha_{IIb}\beta_3$, a result consistent with the possibility that RGDS inhibits fibrinogen binding to $\alpha_{IIb}\beta_3$ by inducing an allosteric change in the third and fourth amino-terminal α_{IIb} repeats.

DISCUSSION

Although fibrinogen appears to bind to $\alpha_{IIb}\beta_3$ exclusively via sequences located at the carboxyl-terminal end of the fibrinogen γ-chain (5, 6), peptides containing an RGD motif are competitive inhibitors of fibrinogen binding to $\alpha_{IIb}\beta_3$ (4). Moreover, despite chemical cross-linking experiments suggesting that the γ-chain and RGD-containing peptides bind to different subunits of the $\alpha_{IIb}\beta_3$ heterodimer (16, 38), competitive binding measurements indicate that the peptides bind to $\alpha_{IIb}\beta_3$ in a mutually exclusive manner (39), implying either that the peptides bind to same site or that the binding sites interact allosterically. Hu *et al.* (40), using plasmon resonance spectroscopy to study the effect of RGD ligands on fibrinogen binding to $\alpha_{IIb}\beta_3$, concluded that fibrinogen and RGD ligands bind to separate sites on $\alpha_{IIb}\beta_3$, but suggested that there is an allosteric relationship between the two. Using chimeras of RGD-insensitive rat $\alpha_{IIb}\beta_3$ and RGD-sensitive human $\alpha_{IIb}\beta_3$, we found that sensitivity to the inhibitory effects of the tetrapeptide RGDS was determined by the origin of the third and fourth amino-terminal repeats of α_{IIb}. We also found little difference in the affinity of $\alpha_{IIb}\beta_3$ on human and rat platelets for fibrinogen. Thus, our data suggest that rather than directly affecting fibrinogen binding, species differences in the third and fourth α_{IIb} repeats affect an allosteric change that regulates fibrinogen binding to $\alpha_{IIb}\beta_3$.

Ligand binding to $\alpha_{IIb}\beta_3$ is thought to involve regions located in the amino-terminal portions of both α_{IIb} and β_3 (8), although much of this evidence is indirect. The β_3 region encompasses amino acids 95–223 (9) and includes the RGD-cross-linking site located in the vicinity of amino acids 109–171 (38) as well as an array of oxygenated residues whose fold may resemble that of the ligand-binding metal ion-dependent adhesion sites (MIDAS) present in integrin I domains (11). It is noteworthy that the deleterious effect of an $Arg^{214} \rightarrow Trp$ mutation, located in the midst of this sequence, can be reversed by exposing $\alpha_{IIb}\beta_3$ to DTT, suggesting that the presence of Trp at residue 214 does not prevent fibrinogen binding to $\alpha_{IIb}\beta_3$ directly, but rather obscures the fibrinogen-binding site (41).

It is also noteworthy that the location of the binding site for RGD-containing peptides in integrins is uncertain, and there is evidence for binding sites in both α- and β-subunits. For example, proteins corresponding to the fourth through seventh amino-terminal repeats of α_5 and α_{IIb} bind to fibronectin III fragment-(8–10) and to fibrinogen, respectively, in an RGD-dependent manner (42, 43). Conversely, experiments using chemical and photoaffinity cross-linking, site-directed mutagenesis, synthetic integrin and RGD-containing peptides, and mAbs have identified regions in the amino-terminal portion of β_1- and β_3-subunits that recognize the RGD motif (11, 38, 44–46). Based on these observations, one possible explanation for our results is simply that RGDS does not bind to either rat α_{IIb} or rat β_3. However, we found that first, the sensitivity of $\alpha_{IIb}\beta_3$ composed of human subunits or of a human α-subunit and rat β-subunit to RGDS was equivalent, and second, binding of mAb 10-758 to human β_3 was induced by RGDS to an equal extent regardless of whether α_{IIb} was human, rat, or a human-rat chimera. Thus, our data imply that RGDS binds to both human and rat $\alpha_{IIb}\beta_3$ and that differences in its inhibitory potency are due to differences in allosteric events that follow RGDS binding.

The portion of α_{IIb} implicated in ligand binding has also been localized to the amino-terminal third of the molecule (15) and includes the fibrinogen γ-chain peptide-cross-linking site at amino acids 294–314 (16). In addition, a number of naturally occurring and laboratory-induced mutations involving amino acids 145, 183, 184, 189, 190, 191, 193, and 224 have been described that impair $\alpha_{IIb}\beta_3$ function, suggesting that these residues interact with $\alpha_{IIb}\beta_3$ ligands (18–20). Of note, residues 183–224 are located in the third α_{IIb} repeat (25). Because our data suggest that the third repeat is involved in the allosteric regulation of fibrinogen binding to $\alpha_{IIb}\beta_3$, it is possible that mutation of the residues listed above interferes with this allosteric change, rather than directly perturbing the fibrinogen-binding site.

The tertiary structure of integrins has yet to be determined. Based on computer modeling, Springer (31) proposed that the amino-terminal portion of integrin α-subunits folds into a seven-bladed β-propeller configuration, with each of the blades corresponding to a β-sheet formed from four anti-parallel β-strands located within each of the amino-terminal repeats. Loops connecting the β-strands would be located on either the upper or low surface of the proposed propeller such that residues in three loops in human α_{IIb} between Arg^{147} and Tyr^{166}, Val^{182} and Leu^{195}, and His^{215} and Gly^{233}, connecting portions of the third and fourth propeller blades, would be juxtaposed in one quadrant of the upper surface of the propeller (21). Comparison of the amino acid sequence of the loops in human α_{IIb} with that of the analogous portions of rat α_{IIb} (47) indicates

that the putative second loop is fully conserved, whereas the first and third loops would be only 50% homologous. Thus, it is possible that amino acid sequence differences between human and rat α_{IIb} in the putative first and third loops could be responsible for the differences in sensitivity of human and rat $\alpha_{IIb}\beta_3$ to RGD-containing peptides.

Alterations in the tertiary and/or quaternary structure of integrins regulate their affinity, and possibly their avidity, for ligands. Recent nuclear magnetic resonance spectroscopic and x-ray crystallographic studies of the I domain of α_L emphasize the importance of changes in the conformation of the α-subunit amino terminus in integrin function (48, 49). I domains are present in nine integrin α-subunits, where they are inserted between the second and third amino-terminal repeats (49). In α_L and α_M, ligands such as ICAM-1–3 (intercellular adhesion molecule) bind to a divalent cation-containing MIDAS motif on the upper I domain surface (50–53). In the I domain of α_L, residues distal to the MIDAS motif, lining a cleft formed by the seventh α-helix and the central β-sheet, regulate ligand binding to $\alpha_L\beta_2$ allosterically (49) and constitute the binding site for the $\alpha_L\beta_2$ inhibitor lovastatin (48). In addition, mutations in the amino- and carboxyl-terminal linker sequences that connect the I domain to the rest of α_L either activate or inactivate I domain function (49), implying that the changes in I domain conformation that regulate its function are transmitted from the amino-terminal portion of α_L to the I domain via these sequences. In the case of $\alpha_{IIb}\beta_3$, agonist-induced changes in tertiary structure are essential for its function (2). Our results indicate that an allosteric change in the third and fourth amino-terminal repeats of α_{IIb}, a portion of α_{IIb} located immediately downstream from the I domain insertion site in I domain-containing integrins, regulates ligand binding to $\alpha_{IIb}\beta_3$. Thus, by extrapolation, our data suggest that allosteric changes involving the third and fourth α-subunit repeats may be a general mechanism by which ligand binding to integrins is regulated.

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RGD-containing Peptides Inhibit Fibrinogen Binding to Platelet $\alpha_{IIb}\beta_3$ by Inducing an Allosteric Change in the Amino-terminal Portion of α_{IIb}

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