The spatial relationship between the binding sites for two cyclic peptides, cyclo(S,S)KYGCRGDWPC (cRGD) and cyclo(S,S)KYGCHarGDWPC (cHarGD), high affinity analogs for the RGD and HLGGAKGADGV peptide ligands, in integrin αIIbβ3 (GPⅡb-Ⅲa) has been characterized.

For this purpose, cRGD and cHarGD were labeled with fluorescein isothiocyanate and tetramethylrhodamine 5-isothiocyanate, respectively. Both cyclic peptides were potent inhibitors of fibrinogen binding to αIIbβ3, particularly in the presence of Mn2⁺; IC50 values for cRGD and cHarGD were 1 and <0.1 nM in the presence of Mn2⁺. Direct binding experiments and fluorescence resonance energy transfer analysis using the purified receptor showed that both peptides interacted simultaneously with distinct sites in αIIbβ3. The distance between these sites was estimated to be 6.1 ± 0.5 nm. Although cRGD bound preferentially to one site and cHarGD to the other, the sites were not fully specific, and each cyclic peptide or its linear counterpart could displace the other to some extent. The binding affinity of the cRGD site was dramatically affected by Mn2⁺; cRGD, but not cHarGD, bound to recombinant β3(95–373) in a cation-dependent manner, indicating that the cRGD site is located entirely within this fragment. With intact platelets, binding of c-RGD and cHarGD to αIIbβ3 resulted in distinct conformational alterations in the receptor as indicated by the differential exposure of ligand-induced binding site epitopes and also induced the opposite on membrane fluidity as shown by electron paramagnetic resonance analyses using 5-doxylstearic acid as a spin probe. These data support the concept that the two peptide ligands bind to distinct sites in αIIbβ3 and initiate different functional consequences within the receptor itself and within platelets.

αIIbβ3 (GPⅡb-Ⅲa) is a member of integrin family of cell adhesion receptors (1–3) and is the most abundant membrane protein on the platelet surface (4). On nonstimulated platelets, αIIbβ3 is incapable of binding most of its soluble macromolecular ligands (5), but after exposure of the cells to appropriate agonists, the receptor undergoes a conformational change (6) as a consequence of signal transmission from inside the cell to the extracellular domain of the receptor (inside-out signaling (7, 8)) and becomes competent to interact with several plasma protein ligands, including fibrinogen, fibronectin, and von Willebrand factor (9–11). Fibrinogen inhibits the binding of other two ligands to αIIbβ3 (10–13), and a common set of monoclonal antibodies (mAbs) to the receptor blocks the interaction of these adhesive ligands with αIIbβ3 (9). Two sets of ligand peptides, γ chain peptides, which correspond to the sequence at the carboxyl-terminal sequence of the fibrinogen γ chain, and RGD(X) peptides, which correspond to sequences present in all three macromolecular ligands, define the recognition specificity of αIIbβ3 for its macromolecular ligands (reviewed in Ref. 14). Both peptide sets inhibit the binding of protein ligands to the receptor, and RGD and γ chain peptides bind directly to αIIbβ3 and interfere with the interaction of each other with the receptor (15–20). Naturally occurring mutations within αIIbβ3 block the binding of both peptides to the receptor (21, 22). Furthermore, both peptides can stimulate a transition of αIIbβ3 from a resting to a ligand-competent state, thereby acting as agonists as well as antagonists (23). The γ chain and RGD peptides also can elicit ligand-induced binding sites, LIBS, epitopes that are expressed primarily by the occupied receptor but not by its resting or active conformers. Such observations suggest that the binding sites recognized by these peptide ligands are overlapping (17) or closely related (24, 25). Nevertheless, other observations suggest that these two peptide ligands interact with distinct sites within the macromolecular ligand binding pocket of αIIbβ3 (20, 26–32). For example, bifunctional reagents cross-link the two peptides to different subunits of the receptor (26, 28, 29), and the peptides differentially inhibit the binding of certain mAbs to αIIbβ3 (33). Furthermore, even though fibrinogen contains two sets of RGD sequences within its dimeric structure, only its γ chain sequences are essential for a productive interaction of the ligand with the receptor (34). Thus, the relationship between the peptide binding subsites within αIIbβ3 and the binding pocket for macromolecular ligands remains uncertain.

A major limitation in the analyses of the interactions of the peptide ligands with αIIbβ3 is their low affinity for the receptor. Fibrinogen itself has a relatively low affinity for αIIbβ3 (Ka = 3 μM⁻¹), and the peptides are estimated to have 30–100-fold lower affinities for the receptor (24, 35). Certain of the disintegrins derived from snake venoms have high affinities for...
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αIIbβ3 but are still relatively large and complex ligands (36). The high affinity of the disintegrins can be attributed to the placement of peptide ligand sequences, RGD or KGD, within a constraining disulfide loop (37). Scarborough et al. (38) synthesized a series of cyclized peptides and demonstrated their high affinity and specificity for αIIbβ3. One of these peptides, cycloS(S)KYCGRGPDWPC (cRGD), was found to be a high affinity surrogate for fibrinogen and its γ chain peptide based upon its reactivity with αIIbβ3 and its sister receptor, αβ6, under different cation conditions (30).

In this study, we have used cHarGD and a second, very closely related cyclic peptide, cycloS(S)KYCGRGDWPC (cRGD), as probes of αIIbβ3 recognition specificity. Both cyclic peptides are shown to react with αIIbβ3 and αβ6 with high affinities. By using fluorescence energy transfer methodology, we demonstrate that these ligands bind to different sites in the ligand binding domain of αIIbβ3, and the distance between these sites is estimated. A specific recombinant fragment of the β3 subunit is shown to contain one of these sites. Furthermore, occupancy of these sites by the cyclic peptide ligands results in distinct conformational alterations of the receptor and its microenvironment as indicated by distinct patterns of exposure of LIBS epitopes and differential effects on membrane fluidity.

EXPERIMENTAL PROCEDURES

Peptide Synthesis—The cyclic peptides, cycloS(S) KYCGRGDWPC (cRGD) and cycloS(S) KYCGRGDWPC (cHarGD), were synthesized as described previously (39) and cyclized with potassium ferricyanide (cRGD) and cyclo(S,S)KYGCHarGDWPC (cHarGD), were synthesized previously (30). The cyclic peptides, cyclo(S,S) KYGGRGDWPC (cRGD), as probes of αIIbβ3 recognition specificity. Both cyclic peptides are shown to react with αIIbβ3 and αβ6 with high affinities. By using fluorescence energy transfer methodology, we demonstrate that these ligands bind to different sites in the ligand binding domain of αIIbβ3, and the distance between these sites is estimated. A specific recombinant fragment of the β3 subunit is shown to contain one of these sites. Furthermore, occupancy of these sites by the cyclic peptide ligands results in distinct conformational alterations of the receptor and its microenvironment as indicated by distinct patterns of exposure of LIBS epitopes and differential effects on membrane fluidity.

Experimental Procedures

Peptide Synthesis—The cyclic peptides, cycloS(S) KYGGRGDWPC (cRGD) and cycloS(S) KYGGRGDWPC (cHarGD), were synthesized as described previously (39) and cyclized with potassium ferricyanide (cRGD) and cyclo(S,S)KYGCHarGDWPC (cHarGD), were synthesized previously (30). The cyclic peptides, cyclo(S,S) KYGGRGDWPC (cRGD), as probes of αIIbβ3 recognition specificity. Both cyclic peptides are shown to react with αIIbβ3 and αβ6 with high affinities. By using fluorescence energy transfer methodology, we demonstrate that these ligands bind to different sites in the ligand binding domain of αIIbβ3, and the distance between these sites is estimated. A specific recombinant fragment of the β3 subunit is shown to contain one of these sites. Furthermore, occupancy of these sites by the cyclic peptide ligands results in distinct conformational alterations of the receptor and its microenvironment as indicated by distinct patterns of exposure of LIBS epitopes and differential effects on membrane fluidity.

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modified Tyrode's buffer containing 0.1% BSA, 1 mM CaCl2 or MnCl2, 10μg/ml prostaglandin I2, and 2μM d-phenylalaninyl-L-prolyl-arginine chloromethylketone and incubated with cRGD (10μM), cHarGD (10μM), or with no additions for 60 min at room temperature. LIBS1 mAb (47), PMI-1 (48), or control mouse IgG was added at a final concentration of 1 μg/ml. After 30 min, the platelets were washed twice by centrifugation in the same buffer, incubated with fluorescent isothiocyanate/goat anti-mouse IgG on ice for 20 min, and then analyzed by flow cytometry. Flow cytometry was performed using a FACScan instrument (Becton Dickinson, San Jose, CA); 10,000 events were recorded; and the data were analyzed using the CellQuest software program (version 1.2).

**Spin Labeling and Electron Spin Resonance (ESR) Measurements**—
Human blood (100 ml) was collected into one-sixth volume of acid/citrate/dextrose from a forearm vein through an 18-gauge needle. Platelets were isolated by differential centrifugation (49), and the resulting platelet pellet was resuspended in the modified Tyrode's buffer (140 mmol/liter NaCl, 10 mmol/liter glucose, and 15 mmol/liter Tris-HCl, pH 7.4), supplemented with apyrase (0.1 g/liter) and prostaglandin E1 (20μg/liter). The specific radioactivity of the radioiodinated protein and peptide were stored at room temperature, and the final platelet count was adjusted to 3 × 108 cells/ml. All steps in platelet isolation were performed in plastic and at room temperature, and the final platelet count was adjusted to 3 × 108 cells/ml. For spin labeling experiments, the washed platelets were incubated with a final concentration of 50μM 5-doxylstearic acid for 30 min at room temperature. Specifically, 5μl of a 100 mM solution of the spin label in dilute ethanol was added to 100μl of platelet suspension (3 × 108 cells/ml), and the suspension was mixed gently. The final ethanol concentration in the platelet suspension did not exceed 0.05% (v/v).

ESR measurements were performed at the ambient temperature (23 ± 1°C) in a Bruker SX-300E spectrometer. For all the ESR spectra shown, the ordinate represents the amplitude of the ESR signal and is expressed in arbitrary units. ESR recordings were routinely recorded as the first derivatives of the absorption spectra. The estimated ratios were calculated from the ESR plots by measuring the amplitudes as the heights of the peaks. The ratio h5/h6 was calculated, where h5 and h6 correspond to heights of low field and middle field lines of the spectra, respectively.

**Statistical Analysis**—The protein content of the platelet membrane preparations was measured by the Lowry procedure. Na125I (specific activity 15–17 mCi of 125I per mg of iodine from Amersham Pharmacia Biotech) was used for radiiodination. Fibrinogen and synthetic peptides were labeled using IODO-GEN (Pierce). The iodinated peptides were separated from free Na125I by gel filtration on Bio-Gel P2 columns (Bio-Rad). The specific radioactivity of the radioiodinated protein and peptides ranged from 0.5 to 1.0 Ci/g. Aliquots of the radioiodinated protein and peptide were stored at –20°C for no longer than 2 weeks before use.

**Results**

**The Cyclic Peptides Are Potent Mimetics of the Linear γ Chain and RGD Peptides**—Previous studies have shown that cHarGD is a potent surrogate of the fibrinogen γ-chain peptide based upon its reactivity with αmβ3 and αvβ3 under different cation conditions (30). Specifically, like the γ-chain peptide, cHarGD reacts with αmβ3 in the presence of Ca2+ and Mn2+, whereas it only binds well to αvβ3 in the presence of Mn2+. cRGD was synthesized as a high affinity mimetic of linear RGD peptides, and the effects of these two cyclic peptides on fibrinogen binding to purified αmβ3 were compared. As shown in Fig. 1, both cyclic peptides effectively inhibited 125I-fibrinogen binding to immobilized αmβ3. After 30 min of incubation, binding of 125I-fibrinogen to immobilized αmβ3 was 99 ± 5% of that measured with control antibodies. In the presence of 1 mM Ca2+, IC50 values for cRGD and cHarGD were 100 ± 10 and 50 ± 4.5 nM, respectively (Fig. 1). When Ca2+ was replaced by 1 mM Mn2+, these values were 100- and 500-fold lower at 1 < 0.07 nM for cRGD and 0.1 ± 0.008 nM for cHarGD. Under similar conditions, IC50 values for representational γ-chain and RGD peptides are 200–1000-fold higher (14). Studies also were conducted to determine the reactivity of the cyclic peptides with αmβ3 as the receptor. While fibrinogen does not bind to αmβ3 in the presence of 1 mM Ca2+, vitronectin does (51). cRGD was a potent inhibitor of 125I-vitronectin binding to immobilized αmβ3 (IC50 = 4.5 ± 2.6 nM; n = 3), whereas, consistent with previous results (30), cHarGD produced minimal inhibition at a 50-fold higher concentration. Thus, the cyclic peptides are potent inhibitors of ligand binding to the β3 integrins under specified cation conditions, and are substantially more active than their linear counterparts, cHarGD and cRGD mimic the behavior of γ-chain and RGD peptides, respectively.

**Interaction of Fluoresceinated cHarGD and cRGD with αmβ3**—The cyclic peptides were labeled with either fluorescein (Flu) or rhodaminyl (Rho) fluorophores. Exclusive modification of the amino-terminal group by the fluorophores was accomplished by selection of the pH of the reaction and the electrophilic reagent used (52). Thus, cRGD was derivatized with an equimolar amount of fluorescein isothiocyanate at pH 9.0 to yield the mono-substituted compound, N′-Flu-cRGD. Reaction of cHarGD with an equimolar quantity of tetramethylrhodamine 5-isothiocyanate gave the amino-terminally labeled N′-Rho-cHarGD. Labeling of these peptides did not affect their functional properties, based upon their IC50 values and the slopes of their inhibition curves in inhibiting fibrinogen binding to αmβ3 under different cations conditions (not shown).

N′-Flu-cRGD and N′-Rho-cHarGD were incubated with purified αmβ3 for 1 h at 22°C, and the complexes were separated from free peptide by rapid gel filtration. Within 3 min of beginning the gel filtration, the fluorescence spectra of αmβ3 complexes with either N′-Flu-cRGD or N′-Rho-cHarGD were initiated. The spectra of either bound N′-Flu-cRGD or N′-Rho-cHarGD showed the same characteristics as those of free fluorescein and rhodamine, respectively. Upon excitation at 494 nm, N′-Flu-cRGD bound to αmβ3 could be detected by its emission at 520 nm, whereas bound N′-Rho-cHarGD yielded an emission peak at 572 nm when excited at 541 nm. As shown in Fig. 2, the specific binding of each cyclic peptide to αmβ3 was saturable. The concentrations of the cyclic peptides required for half-maximal binding were in the 0.5–1.0 μM range, approximately 10-fold higher than their IC50 values for inhibiting
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Fig. 2. Binding of N'-Flu-cRGD and N'-Rho-charGD to αIIbβ3. Purified αIIbβ3 (0.1 μM) was incubated with various concentrations of N'-Flu-cRGD (A) or N'-Rho-charGD (B) for 60 min at room temperature. Total volume of the mixture was 500 μL, and all components were dissolved in 0.02 M HEPES buffer, pH 7.3, containing 0.15 M NaCl, 1 mM CaCl₂, and 25 mM octyl glucoside. Then, αIIbβ3 complexed with the fluorescent probe was separated from free ligand by rapid gel filtration on Sephadex G-25 mini-columns equilibrated and eluted with the same buffer. Binding of fluorescent probes was evaluated by fluorescence measurements at 520 and 572 nm upon excitation at 494 and 541 nm for N'-Flu-cRGD and N'-Rho-charGD, respectively. Data obtained in the presence of 1 mM EDTA (●), the nonspecific binding, were subtracted from total binding (○) to obtain specific binding (■). Data represent the means from three separate binding experiments.

Fibrinogen binding to immobilized receptor. A 10-fold difference also has been observed in the apparent affinity under the following circumstances: (a) charGD binding to immobilized αIIbβ3 as compared with its binding to αIIbβ3 on intact platelets (30), and (b) fibrinogen binding to immobilized αIIbβ3 (53) versus its binding to αIIbβ3 on platelets (10) or in liposomes (54).

Simultaneous Binding of charGD and cRGD to αIIbβ3—αIIbβ3 was incubated with a mixture of the two cyclic peptides under the same conditions and was separated from the free peptides by gel filtration. When excited at 494 nm, the fluorescence emission spectrum of the complexes had two peaks, one at 520 nm and a second at 572 nm (Fig. 3). This pattern was accompanied by a decrease in the N'-Flu-cRGD fluorescence at 520 nm. This spectrum is indicative of considerable energy transfer from the excited fluorescein to the rhodamine, providing evidence that N'-Flu-cRGD and N'-Rho-charGD are bound in close proximity within the receptor. Two experiments were performed to verify that the observed energy resonance transfer involves donors and acceptors associated with the same receptor molecule rather than arising from their binding to different receptor molecules. First, we analyzed the efficiency of energy transfer as a function of αIIbβ3 concentration. The efficiency of energy transfer from donor to acceptor, E, was determined to be 0.24 ± 0.05 and 0.27 ± 0.04 when αIIbβ3 was used at 0.1 and 1.0 μM, respectively. Since our previous studies showed that αIIbβ3 itself, or in complex with peptide ligands, does not form stable aggregates (55), the independence of the efficiency of energy transfer on αIIbβ3 concentration indicates that the fluorochromes must be bound to the same receptor. Second, complexes of αIIbβ3 with N'-Flu-cRGD or N'-Rho-charGD were separated by gel filtration as indicated; these samples were immediately mixed together, and spectral analyses were performed. Under this condition, no energy transfer was observed. This result provides independent evidence that the two ligands must bind to the same receptor for efficient energy transfer. Based upon an efficiency of energy transfer of 0.27 and assuming a Forster critical distance (R₀) range of 5.0–5.2 nm for a donor rhodamine (56), the distance between donor (fluorescein linked to cRGD) and acceptor (rhodamine linked to charGD) was calculated to be 6.13 ± 0.5 nm.

The next series of experiments was designed to test stability and stoichiometry of complexes of αIIbβ3 with the cyclic peptide ligands. αIIbβ3 (0.5 μM) was incubated with N'-Flu-cRGD and N'-Rho-charGD, separated by gel filtration, and used for spectroscopic measurements as described above. Then, after 15 min, the samples were refractionated by gel filtration, and the emission spectra were again recorded. The concentration of each peptide in the αIIbβ3 fraction after the first and second gel filtration was quantitated from their fluorescence intensities using the free cyclic peptides to construct calibration curves. In each, the efficiency of energy transfer was estimated for each sample. The results of these analyses are summarized in Table I. αIIbβ3 purified from the free peptides by the first rapid gel filtration contained 0.45 mol of cRGD and 0.85 mol of charGD per mol of receptor. Thus, under the conditions of the initial spectral analysis, substantial amounts of each peptide were bound to the receptor, and the distance between the two bound peptides allowed for energy transfer with an efficiency of 0.24. After 15 min, the emission spectrum (excitation at 494 nm) was again recorded. The emission peak at 572 nm had almost completely disappeared indicating that the peptides had dissociated from the receptor, and energy transfer no longer occurred. This sample then was subjected to a second gel filtration, which reduced the concentrations of both cyclic peptides significantly (cRGD, 0.06 mol/mol; charGD, 0.18 mol/mol). These data indicate that both cyclic peptides were bound with high stoichiometry and reversibility to αIIbβ3. Corroborating data were obtained by a second experimental approach. A spectrum was taken of the peptide-αIIbβ3 complex after the first gel filtration; then EDTA was added at a final concentration of 1 mM, and the spectrum was analyzed again. EDTA affected the fluorescent characteristics of N'-Flu-cRGD and N'-Rho-charGD only slightly but completely abolished energy transfer (Table I). Thus, EDTA dissociated the bound peptides from αIIbβ3, and the free peptides, although present in the mixture at the same concentration as when bound to αIIbβ3, could not transfer energy.

Cation Dependence and Specificity of Sites for N'-Flu-cRGD and N'-Rho-charGD—The binding of both peptides required the presence of divalent cations, Ca²⁺ or Mn²⁺. When αIIbβ3 was depleted from cations by incubation with EDTA, interaction with N'-Flu-cRGD and N'-Rho-charGD was almost completely abolished (Fig. 4). Substitution of Mn²⁺ for Ca²⁺ resulted in a significant increase in fluorescence emission at 572 nm as indicated by higher ratio of luminescence at 572 nm to that at 520 nm (0.588 and 0.823 in the presence of Ca²⁺ and Mn²⁺, respectively). This increase in fluorescence energy transfer was not associated with a change in the efficiency of energy transfer (E); in the presence of Mn²⁺, E was calculated to be 0.25 ± 0.04. Direct excitation of rhodamine at 541 nm also
and emission was quantified at 520 and 572 nm for citation at 494 and 541 nm. The relative change in fluorescence Mn2+ excess caused a 25 and 85% reduction of fluorescence intensity of fluorescein and rhodamine by the addition of a 100-fold excess of nonlabeled cRGD decreased the and binding of each other.

We also evaluated the capacity of a linear RGD and γ-chain peptide to inhibit the binding of N'-Flu-cRGD and N'-Rho-cHarGD to α1β3. The data shown in Table II indicate that the linear counterparts of the cyclic peptides yielded similar patterns of inhibition. GRGDSP was more effective in inhibiting N'-Flu-cRGD than N'-Rho-cHarGD binding, whereas the γ-chain peptide corresponding to fibrinogen γ(400–411) was more effective in inhibiting N'-Rho-cHarGD than N'-Flu-cRGD binding to α1β3.

N'-Flu-cRGD but Not N'-Rho-cHarGD Binds to β3(95–373)—To characterize the binding sites for the peptide ligands, a recombinant fragment corresponding to β3(95–373) was expressed, renatured, and purified. The fragment preparation used in subsequent ligand binding studies was homogeneous as assessed by polyacrylamide gel electrophoresis in sodium dodecyl sulfate under reducing and nonreducing conditions. This fragment would contain the putative metal ion-dependent adhesion site (MIDAS motif) in the β3 subunit (57, 58). As shown in Fig. 6, β3(95–373) bound fibrinogen in a cation-dependent manner. Similar to α1β3, this binding was inhibited by EDTA and supported by Ca2+ and Mn2+. However, in contrast to the intact cHarGD, higher concentrations of Ca2+ inhibited the binding of fibrinogen to the recombinant fragment; 125I-fibrinogen binding to β3(95–373) was significantly lower in 1 mM Ca2+ than in 0.1 mM Ca2+. A suppressive effect of Ca2+ on fibrinogen binding is observed with α1β3 (51). Binding of 125I-fibrinogen to β3(95–363) was blocked effectively by cRGD (72% inhibition of specific binding at 1 μM) whereas a high concentration (100 μM) of cHarGD only partially inhibited the interaction.

The direct binding of the cyclic peptides to the recombinant fragment was tested in spectroscopic analyses (Fig. 7). The β3(95–373) fragment was incubated with N'-Flu-cRGD and N'-Rho-cHarGD as described for α1β3. As shown in Fig. 7A, when excited at 494 nm, a single emission peak was observed at 520 nm, reflecting the binding of N'-Flu-cRGD to the recombinant fragment. No emission peak was observed at 572 nm, indicating that N'-Rho-cHarGD could not be excited by energy transfer from N'-Flu-cRGD bound to β3(95–373). The lack of N'-Rho-cHarGD binding to the β3 fragment was further demonstrated by the absence of an emission spectrum upon direct excitation of rhodamine at 541 nm (Fig. 7B).

Binding of cRGD or cHarGD to Platelets Induces Different Functional Responses—If cRGD and cHarGD bind to different

| Sample | Time* (min) | cRGD/α1β3 | cHarGD/α1β3 | Energy transfer efficiency |
|--------|-------------|------------|-------------|--------------------------|
| α1β3 | 3 | 0.45 | 0.85 | 0.24 |
| α1β3 (gel filtration I) | 15 | 0.44 | 0.80 | 0.08 |
| α1β3 (gel filtration II) | 18 | 0.06 | 0.18 | 0 |
| α1β3 (gel filtration II) + EDTA (1 mM) | 4 | 0.38 | 0.70 | 0 |

*The time refers to the interval between the end of incubation of the reaction mixtures and the start of the spectral analyses.
sites in \( \alpha_{IIb}\beta_3 \), the two cyclic peptides might initiate distinct functional responses. This possibility was explored initially by assessing the differential induction of LIBS in \( \alpha_{IIb}\beta_3 \). LIBS are the target epitopes of mAbs that react selectively with the occupied receptor (47). Induction of two prototypic LIBS epitopes, one expressed by the \( \beta_3 \) subunit and recognized by mAb LIBS-1 (59) and a second expressed by the \( \alpha_{IIb} \) subunit and recognized by mAb PMI-1 (60), was analyzed upon binding of the cyclic peptides to platelets by fluorescence-activated cell sorter. As summarized in Table III, binding of cRGD and cHarGD to \( \alpha_{IIb}\beta_3 \) on platelets resulted in a differential induction of the two LIBS epitopes. cRGD increased expression of

![Diagram](image1)

**Fig. 4.** Effects of cations on fluorescence emission spectra of cRGD and cHarGD. Binding of fluorescent probes to \( \alpha_{IIb}\beta_3 \) was performed in the presence of \( Ca^{2+} \), \( Mn^{2+} \), or EDTA. Emission spectra were recorded upon excitation at 494 nm (A) or 541 nm (B).

![Diagram](image2)

**Fig. 5.** Displacement of cRGD and cHarGD bound to \( \alpha_{IIb}\beta_3 \) by nonlabeled peptides. Binding of both fluorescent probes to \( \alpha_{IIb}\beta_3 \) was performed in the presence of either nonlabeled cRGD (●) or cHarGD (○). The extent of binding of cRGD and cHarGD was evaluated by measurement of fluorescence intensity at 520 and 572 nm upon excitation at 494 (A) and 541 nm (B), respectively. Each point represents a mean of three separate experiments.

**TABLE II**

| Peptide          | Concentration | N°-Flu-cRGD | N°-Rho-cHarGD |
|------------------|---------------|-------------|---------------|
| GRGDSP           | 0.1 mM       | 12 ± 5      | 55 ± 6        |
|                  | 1.0 mM       | 72 ± 7      | 75 ± 6        |
| Fibrinogen \( \gamma_{400-411} \) | 0.1 mM | 0           | 13 ± 3        |
|                  | 1.0 mM       | 20 ± 4      | 65 ± 7        |

**Fig. 6.** Specificity of the interaction of fibrinogen with \( \beta_3-(95-373) \) fragment. \(^{125}\)I-Fibrinogen (25 nM) was added to microtiter wells coated with \( \beta_3-(95-373) \) in the presence of \( Ca^{2+} \) (1 and 0.1 mM), \( Mn^{2+} \) (0.1 mM), and EDTA (1 mM). The effect of peptides, cRGD (1 mM), cHarGD (1 mM), GRGESP (100 mM), and fibrinogen (3 mM) was tested in the presence of 0.1 mM \( Ca^{2+} \). Values represent the means ± S.D. of three determinations and are expressed as a percent of a control with 0.1 mM \( Ca^{2+} \) lacking inhibitors.
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**TABLE III**

| Epitope | Peptide | Mean fluorescence intensity Ca<sup>2+</sup> | Mean fluorescence intensity Mn<sup>2+</sup> |
|---------|---------|---------------------------------|---------------------------------|
| PMI-1   | cRGD   | 28.78                           | 78.27                           |
|         | cHarGD | 0                               | 16.82                           |
| LIBS1   | cRGD   | 167.52                          | 241.82                          |
|         | cHarGD | 144.71                          | 92.62                           |

Data represent mean fluorescence measured by flow cytometry of platelets incubated with cyclic peptides in the presence of Ca<sup>2+</sup> or Mn<sup>2+</sup> as described under “Experimental Procedures.”

Both the LIBS-1 and PMI-1 epitopes as indicated by the increase in the mean fluorescence intensity of the platelets upon incubation with the two antibodies. The capacity of cRGD to induce both LIBS epitopes was observed in the presence of either Ca<sup>2+</sup> or Mn<sup>2+</sup>. In contrast, whereas cHarGD elicited exposure of LIBS-1, the PMI-1 epitope was minimally induced by this peptide. This differential induction of LIBS epitopes by cHarGD was observed in the presence of either Ca<sup>2+</sup> or Mn<sup>2+</sup>.

As a second functional parameter, the capacity of cHarGD and cRGD to affect differentially the fluidity of the membrane lipid bilayer of platelets was assessed. For this analysis, platelets were labeled with 5-doxylstearic acid, a spin probe that reports fluidity changes proximal to the outer surface of the platelet membrane bilayer (61). ESR spectra were taken in the presence of various concentrations of the two cyclic peptides, and representative spectra are shown in Fig. 8A. The low and middle field maxima are described by their amplitudes, \( h_{0.1} \) and \( h_0 \), respectively, and these values were quantitated. The \( h_{0.1}/h_0 \) ratios derived in the presence of the two cyclic peptides and 0.1 mM Ca<sup>2+</sup> are displayed in Fig. 8B. A substantial reduction of the \( h_{0.1}/h_0 \) ratio was observed upon binding of cRGD to platelets, indicating a significant decrease in the fluidity of lipid bilayer. The effect of cRGD approached that induced by increasing the Ca<sup>2+</sup> concentration from 0.1 to 1 mM. In sharp contrast, cHarGD elevated the \( h_{0.1}/h_0 \) ratios, indicating an increase in membrane lipid fluidity upon binding of this peptide to platelets. This increase was greater than that observed when 0.1 mM Mn<sup>2+</sup> was used instead of 0.1 mM Ca<sup>2+</sup>. Even at the lowest concentration of the peptide ligands tested, 2.5 nM, the differences in membrane fluidity evoked by the two cyclic peptides were significantly different (\( p < 0.001 \)). The effects of each cyclic peptide on membrane fluidity was concentration-dependent. However, the changes were induced by very low concentrations of the cyclic peptides, substantially lower than their \( K_d \) value for α<sub>IIb</sub>β<sub>3</sub>. This was also observed with the linear ligand peptides (31) and indicates that only limited occupancy of α<sub>IIb</sub>β<sub>3</sub> is sufficient to cause changes in membrane fluidity as detectable by ESR.

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![Fig. 7. Binding of N'Flu-cRGD to β<sub>3</sub>- (95–373). The purified β<sub>3</sub>- (95–373) (10 μM) was incubated with N'-Flu-cRGD or N'-Rho-cHarGD in the presence of 0.1 mM Ca<sup>2+</sup> (—) or 0.1 mM Mn<sup>2+</sup> (—). Complexes of β<sub>3</sub> fragment with peptide ligands were purified as described in the legend to Fig. 2 and under “Experimental Procedures.” The extent of binding of N'-Flu-cRGD was evaluated by measurement of fluorescence intensity at 520 nm upon excitation at 494 nm (A). The extent of N'-Rho-cHarGD binding was evaluated by measurement of the fluorescence intensity at 572 nm upon excitation at 541 nm (B).](image-url)

![Fig. 8. Effect of cRGD and cHarGD binding to α<sub>IIb</sub>β<sub>3</sub> on fluidity of lipid bilayer in platelet membranes. Microenvironmental changes in the vicinity of α<sub>IIb</sub>β<sub>3</sub> were analyzed by ESR spectra of 5-doxylstearic acid incorporated into the membrane lipid bilayer of platelets. Platelets labeled with the spin probe were incubated with various concentrations of cyclic peptides in the presence of 0.1 mM CaCl<sub>2</sub>. Typical spectra obtained in the case of control platelets (curve a), platelets with bound c-HarGD (curve b), or cRGD (curve c) are shown in A. B, the \( h_{0.1}/h_0 \) ratios calculated from ESR spectra, where \( h_{0.1} \) and \( h_0 \) correspond to heights of the low field and middle field lines of the spectra, respectively, are presented. Reduction in the \( h_{0.1}/h_0 \) ratio indicates a lower fluidity of the lipid bilayer relative to the 0.1 mM CaCl<sub>2</sub>. The effects of EDTA, 1 mM CaCl<sub>2</sub>, and 0.1 mM MnCl<sub>2</sub> are shown as controls. Data represent mean ± S.D. calculated from five separate experiments each with triplicate measurements.](image-url)
**DISCUSSION**

In this report, we have utilized two structurally similar but nonidentical cyclic peptides to examine the ligand binding properties of \(\alpha_{IIb}\beta_3\). Evidence is developed to indicate that cHarGD and cRGD interact with two distinguishable binding sites within \(\alpha_{IIb}\beta_3\). Moreover, occupancy of these sites by ligand initiates different conformational changes in the receptor which may ultimately lead to distinct functional responses in platelets. These observations shed light on a long-standing question in the platelets. These observations shed light on a long-standing problem regarding the relationship between its peptide ligand-binding sites, may provide insights into how macromolecular ligands may bind to \(\alpha_{IIb}\beta_3\) and may even be relevant to the biological activities of the new class of anti-thrombotic drugs, the GPIIb-IIIa blockers (62).

Fluorescent derivatives of cRGD and cHarGD were developed to aid in our analysis of the interaction of these cyclic peptides with \(\alpha_{IIb}\beta_3\), N\(^*-\)Flu-cRGD and N\(^*-\)Rho-cHarGD were found to have properties functionally analogous to their respective parent cyclic peptides. They bound to \(\alpha_{IIb}\beta_3\) with high affinity in a cation-dependent manner, could be displaced by nonlabeled peptides, and efficiently inhibited fibrinogen binding to \(\alpha_{IIb}\beta_3\). Utilization of these labeled peptide ligands in fluorescence resonance energy transfer experiments provided evidence that both probes could bind to the same \(\alpha_{IIb}\beta_3\) molecules. Since the energy transfer between fluorochromes was largely independent of receptor concentration, and only occurred if the peptides had the opportunity to bind simultaneously to the same receptor, a primary conclusion of this study is that the \(\alpha_{IIb}\beta_3\) expresses two distinct binding sites for these peptide ligands. Based upon the assumptions of the Forster equations, the distance between the two bound fluorophores was estimated to be 6.13 ± 0.5 nm. Molecular models of the two cyclic peptides have been constructed (Insight II, Molecular Simulations, San Diego, CA), and the disulfide bond is predicted to substantially restrict the flexibility of each peptide. The maximum length of the two peptides in these models, from the center of the fluorochrome to the aspartic acid residue is 1.5 ± 0.1 and 0.96 ± 0.017 nm for cHarGD and cRGD, respectively. If the fluorochromes are placed as the most distant residues in the receptor-bound orientation of the two cyclic peptides, overlap between the contact sites would still not be possible.

For the purpose of discussion, these two binding sites for the cyclic peptides are designated as A and B. Site A reacts preferentially with cHarGD, and its affinity for ligand is highly modulated by cations. This site does bind its ligand in the presence of Ca\(^{2+}\), but its affinity for cHarGD was significantly increased by Mn\(^{2+}\). Recombinant \(\beta_3\)(95–373) did not bind cHarGD, suggesting that site A is not reanimated or not fully located with this recombinant fragment. Site B preferentially interacts with cRGD in the presence of either Ca\(^{2+}\) or Mn\(^{2+}\) and is located in recombinant \(\beta_3\)(95–373). Although sites A and B are spatially and functionally distinct, they are not fully independent. Displacement experiments indicate that the two cyclic peptides can mutually inhibit binding of each other to intact receptor. Whether this cross-talk between sites A and B arises from lack of complete specificity or is allosterically induced cannot be distinguished by the present data. A previous study (30) had suggested that cHarGD is a high affinity surrogate for linear \(\gamma\)-chain peptides based upon its differential reactivity with \(\alpha_{IIb}\beta_3\) and \(\alpha_{IIb}\beta_3\) under specific cation conditions. The correspondence between cHarGD and cRGD to linear \(\gamma\)-chain and RGD peptides, respectively, is supported by several additional observations in the present study. First, the two cyclic peptides show the same differential regulation by divalent cations in the reactivity with \(\alpha_{IIb}\beta_3\) and \(\alpha_{IIb}\beta_3\) as observed with the linear peptides, i.e. cHarGD and the \(\gamma\)-chain peptide interact with \(\alpha_{IIb}\beta_3\) in the presence of Ca\(^{2+}\) but Ca\(^{2+}\) inhibits their binding to \(\alpha_{IIb}\beta_3\). This inhibitory effect of Ca\(^{2+}\) is not as marked with cRGD or linear RGD peptides. Second, the binding of each cyclic peptide to \(\alpha_{IIb}\beta_3\) was preferentially inhibited by the corresponding linear peptide. Third, the cyclic peptide and its linear counterpart exerted the same effects on membrane fluidity: cHarGD and the \(\gamma\)-chain peptide increased membrane fluidity, whereas cRGD and the RGD peptide decreased it. Based upon this correspondence, \(\gamma\)-chain peptides would react preferentially with site A and RGD peptides preferentially with site B. The preferential inhibition of cRGD binding by a linear RGD peptide and cHarGD binding by the fibrinogen \(\gamma\)-chain peptide is consistent with this model. A two-site model has been proposed for integrin \(\alpha_{IIb}\beta_3\), which recognizes the RGD and a synergistic sequence in fibronectin (63).

As evidenced by the induction of the LIBS epitopes, LIBS1 and PMI-1, occupancy of either site A or site B induced conformational changes in \(\alpha_{IIb}\beta_3\). However, the pattern of epitope expression was different with the two ligands; binding of cRGD resulted in induction of both LIBS epitopes, whereas binding of cHarGD caused only minimal LIBS1 epitope expression. These results indicate that the occupied receptor can exist in at least two distinct conformational states. Both LIBS epitopes have been at least partially localized: PMI-1 to the carboxyl terminus of the \(\alpha_{IIb}\) heavy chain (60) and LIBS1 to the \(\beta_3\) subunit (47). Thus, occupancy of site B within \(\beta_3\)(95–373) by cRGD appears to transduce conformational changes in both subunits of the receptor. In view of the long range transmission of conformational changes in \(\alpha_{IIb}\beta_3\) induced by ligand occupancy (59), it is not surprising that membrane fluidity might also be influenced by occupancy of sites A and B. However, the two cyclic peptides exerted opposing effects on membrane fluidity as evidenced by their differential alteration of the \(h_{\gamma}\)/\(h_{\alpha}\) ratio obtained with 5-doxylstearic acid as a probe. Interaction of cRGD with platelets reduced the \(h_{\gamma}\)/\(h_{\alpha}\) ratio, indicating a significant rigidification of membrane lipid bilayer, whereas cHarGD increased the \(h_{\gamma}\)/\(h_{\alpha}\) ratio indicating an increase in membrane fluidity. This differential effect is consistent with previous studies in which linear RGD and \(\gamma\)-chain peptides were used in ESR studies (31) and supports the hypothesis that cRGD and cHarGD are the respective high affinity analogs of the linear peptides. With the differential transmission of signals from sites A and B, which are likely to reside in the amino-terminal aspects of \(\alpha_{IIb}\beta_3\) to the membrane proximal LIBS epitopes and then further into the membrane bilayer, it is not unreasonable to suggest that these peptides also would induce different outside-in signaling events. Consistent with this possibility, the linear RGD and \(\gamma\)-chain peptides have been reported to induce different effects on intracellular Ca\(^{2+}\) mobilization (64).

Binding of protein and peptide ligands to integrin receptors is regulated by divalent cations (65–67). Two classes of functionally distinct Ca\(^{2+}\)-binding sites have been identified in \(\beta_3\) integrins; the higher affinity sites can be occupied by Ca\(^{2+}\) and other cations and support ligand binding, whereas the lower affinity sites are Ca\(^{2+}\)-selective and are inhibitory (68). Recombinant \(\beta_3\)(95–373) bound fibrinogen in the presence of Mn\(^{2+}\) and low concentrations of Ca\(^{2+}\), whereas high concentrations of Ca\(^{2+}\) abolished this interaction. Based on these cation effects, it appears that this domain must contain an inhibitory Ca\(^{2+}\)-binding site if not both classes of sites. It should be noted that Gulino et al. (69) concluded that a similar segment of \(\beta_3\) bound fibrinogen in a cation-independent manner. These investigators only employed a high Ca\(^{2+}\) concentration which may have suppressed ligand binding. Thus, our data showing that the
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Therefore, these drugs may very well be totally selective or at least react differently with sites A and B within αIIbβ3. Consequently, the mechanism of antagonism of platelet aggregation by these drugs may be different as may be their induction of LIBS epitopes, platelet microenvironmental changes (membrane fluidity), and intracellular signaling. Indeed, changes in membrane fluidity may occur even at very low levels of αIIbβ3 occupancy. It will be interesting to see if these differences influence the efficacy of these drugs.

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