

High Affinity Ligand Binding by Integrins Does Not Involve Head Separation*

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Conformational change in the integrin extracellular domain is required for high affinity ligand binding and is also involved in post-ligand binding cellular signaling. Although there is evidence to the contrary, electron microscopic studies showing that ligand binding triggers α - and β -subunit dissociation in the integrin headpiece have gained popularity and support the hypothesis that head separation activates integrins. To test directly the head separation hypothesis, we enforced head association by introducing disulfide bonds across the interface between the α -subunit β -propeller domain and the β -subunit I-like domain. Basal and activation-dependent ligand binding by $\alpha_{\text{IIb}}\beta_3$ and $\alpha_V\beta_3$ was unaffected. The covalent linkage prevented dissociation of $\alpha_{\text{IIb}}\beta_3$ into its subunits on EDTA-treated cells. Whereas EDTA dissociated wild type $\alpha_{\text{IIb}}\beta_3$ on the cell surface, a ligand-mimetic Arg-Gly-Asp peptide did not, as judged by binding of complex-specific antibodies. Finally, a high affinity ligand-mimetic compound stabilized non-covalent association between α_{IIb} and β_3 headpiece fragments in the presence of SDS, indicating that ligand binding actually stabilized subunit association at the head, as opposed to the suggested subunit separation. The mechanisms of conformational regulation of integrin function should therefore be considered in the context of the associated $\alpha\beta$ headpiece.

Integrins are major metazoan adhesion receptors that play a fundamental role in cellular organization. They mediate cell-extracellular matrix as well as cell-cell adhesion, connect extracellular cues to the cytoskeleton, and activate many intracellular signaling pathways (1, 2). One unique aspect of integrins is that the affinity of their extracellular domain for biological ligands can be rapidly up-regulated by signals from within the cell. Rapid and precise control of integrin activation is particularly important for leukocytes and platelets, which circulate in the vascular system, where leukocyte emigration and thrombus formation mediated by integrins must be triggered only at the appropriate location. Integrins compose two noncovalently associated type I transmembrane glycoprotein α - and β -subunits (3). A crystal structure of the extracellular domain of the integrin $\alpha_V\beta_3$ revealed a bent conformation, in which there is an acute angle between the headpiece and tail-

piece (4), and an extensive headpiece-tailpiece interface (5). Recently, we have shown that the bent conformation represents the low affinity receptor and that activation is associated with a switchblade-like motion of the headpiece resulting in a highly extended conformation (5).

The integrin headpiece contains the ligand-binding site. The headpiece contains the α -subunit β -propeller and thigh domains and the β -subunit I-like and hybrid domains (Fig. 1), corresponding approximately to the N-terminal two-thirds of the extracellular domain of each subunit. A crystal structure with a bound ligand-mimetic peptide revealed that ligand binds to an interface formed by the β -subunit I-like domain and β -sheets 2–4 of the α -subunit β -propeller domain (6) (Fig. 1).

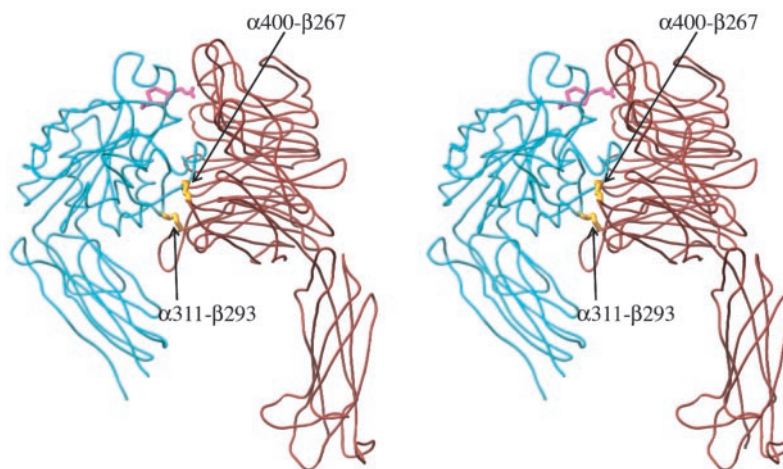
Many experiments support the idea that the inter-subunit association at the cytoplasmic region maintains integrins in low affinity state (7–10). Originally, this notion led to a “hinge hypothesis,” where association/dissociation of the cytoplasmic tails caused hinging between the two subunits, and ultimately changed the conformation of the ligand-binding extracellular segments (11). However, the nature of the conformational change that regulates ligand binding by integrins has been controversial (2, 3, 5, 12–15). Hantgan *et al.* (16, 17), using rotary shadowing EM, reported that binding of RGD peptides induced separation of the headpiece of detergent-solubilized $\alpha_{\text{IIb}}\beta_3$. These images suggested a wide separation in the headpiece, with no interaction remaining between the N-terminal halves of the α - and β -subunits. These observations have been highly influential, in part because they seemed to fit with earlier schematics of integrin activation models where a hinge-like motion at the transmembrane domains is transmitted through rigid α - and β -subunit tailpiece segments to the headpiece, resulting in movement apart of the α - and β -subunits in the headpiece (11, 12, 18). However, recent high resolution negative stain EM studies have shown that the α -subunit leg can exist in two distinct conformations with respect to the headpiece and that the β -subunit leg is highly flexible (5). Existence of multiple modular domains (4) also disfavors rigid movement of the entire stalk region. Because the legs are flexible, it is hard to imagine that information can be transmitted to the headpiece as proposed in the hinge model. Furthermore, high resolution EM studies (5), as well as many other EM studies (19–22), have shown that ligand binding to integrins is not accompanied by head separation.

There are other reasons for the popularity of the head separation model. First, it has been suggested that residues that have been implicated in ligand binding are buried in the headpiece and that separation could expose them, resulting in higher affinity binding (2). Second, there is the mystery of the synergy site in fibronectin type III module 9 of fibronectin, which is distant from the RGD site in module 10. It has been proposed that separation of the headpiece (2, 15) would facilitate simultaneous binding of the α -subunit to the synergy site

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FIG. 1. The $\alpha_V\beta_3$ integrin headpiece and location of introduced disulfide bonds. The stereo view of the $\alpha_V\beta_3$ headpiece is based on the crystal structure of the $\alpha_V\beta_3$ extracellular domain bound to a ligand-mimetic RGD peptide (6). The α_V β -propeller and thigh domains are red; the β_3 I-like and hybrid domains are blue, and engineered disulfide bonds are in gold. The bound RGD peptide is magenta, and its Arg and Asp side chains are shown. Backbones are shown as a worm-like trace. Note that the disulfide bonds introduced by mutations α_V -M400C/ β_3 -Q267C and α_V -E311C/ β_3 -G293C (which corresponds to α_{IIB} -E324C/ β_3 -G293C) are located on the side of the β -propeller and I-like domain interface opposite from the ligand-binding site. Figure was prepared with Ribbons (44).



and the β -subunit to the RGD site (23). However, the liganded $\alpha_V\beta_3$ crystal structure shows that the Arg of RGD binds to the α -subunit and the Asp of RGD binds to the β -subunit (6), strongly suggesting that headpiece separation would disrupt binding to RGD. Third, there are structural homologies between integrins and G proteins (4, 24, 25). By taking this analogy further, it has been suggested that upon integrin activation, the β -propeller and I-like domains might dissociate analogously to the G protein β - and α -subunits (2, 15).

An alternative model for integrin activation has been proposed that is supported by high resolution EM, physicochemical studies, ligand binding assays, introduction of disulfide bonds that lock in the bent conformation, and localization of epitopes that become exposed after integrin activation (5, 26). In this model, activation is regulated by the conformational equilibrium between three states as follows: a bent conformation with low affinity, an extended conformation with a closed headpiece with intermediate affinity, and an extended conformation with an open headpiece with high affinity. Binding of RGD peptide was found not to induce head separation but to induce a dramatic change in angle between the β -subunit I-like and hybrid domains, leading to the swing-out of the hybrid domain away from the α -subunit (5). The prominence of the hybrid domain in the interface between the headpiece and the tailpiece in the bent conformation provides a mechanism for linking the change in angle upon ligand binding to the equilibrium between the bent and extended integrin conformations (5).

Definitive experimental testing of the head separation model is important. Therefore, we have used mutagenesis to introduce disulfide bonds between the α -subunit β -propeller domain and the β -subunit I-like domain, and we have tested the effect of preventing head separation on activation of $\alpha_{IIB}\beta_3$ and $\alpha_V\beta_3$ integrins on the cell surface. Furthermore, we test the effect of ligand-mimetic compounds on the association between the α - and β -subunits in native integrins on the cell surface and in soluble integrin fragments that contain only the headpiece.

EXPERIMENTAL PROCEDURES

Monoclonal Antibodies—Mouse monoclonal antibody (mAb)¹ PT25-2 recognizing human α_{IIB} subunit (27) was a gift from Dr. M. Handa (Keio University, Tokyo, Japan). Mouse mAb 10E5 recognizing the human $\alpha_{IIB}\beta_3$ complex (28) was a gift from Dr. B. S. Collier (Rockefeller University, New York). Mouse anti- β_3 AP3 was from American Type Culture Collection. All other mAbs were obtained from the Fifth International Leukocyte Workshop (29).

Plasmid Construction, Transient Transfection, and Immunoprecipitation—

Plasmids coding for full-length human α_{IIB} , α_V , and β_3 were subcloned into pcDNA3.1/Myc-His(+) or pEF/V5-HisA as described previously (5). Mutants were made using site-directed mutagenesis, and DNA sequences were confirmed before being transfected into 293T cells using calcium phosphate precipitation. Transfected cells were metabolically labeled with [³⁵S]cysteine/methionine as described (5). Labeled cell lysates were immunoprecipitated with anti- β_3 AP3, eluted with 0.5% SDS, and subjected to nonreducing or reducing SDS-7.5% PAGE and fluorography.

Two-color Ligand Binding and Flow Cytometry—Binding of fluorescein-labeled human fibrinogen and fibronectin were performed as previously described (5). To test the effect of EDTA treatment on mAb epitope expression, transiently transfected 293T cells were preincubated in 20 mM Tris-buffered saline, pH 8.4, containing either 1 mM Ca²⁺, 1 mM Mg²⁺, or 5 mM EDTA at 37 °C for 30 min, followed by washing and resuspension in 20 mM Hepes, 150 mM NaCl, 5.5 mM glucose, 1% bovine serum albumin, and 1 mM Ca²⁺, 1 mM Mg²⁺ (HBS). Cells were then incubated with mAbs on ice for 30 min, followed by staining with FITC-conjugated anti-mouse IgG and flow cytometry. To test the effect of RGD peptide on mAb epitope expression, cells in HBS were incubated with 100 μ M GRGDSP peptide at room temperature for 30 min before adding mAbs and staining as above.

Stability of the $\alpha_{IIB}\beta_3$ Headpiece in SDS in the Presence of an RGD-mimetic Compound—An integrin headpiece fragment comprising α_{IIB} residues 1–621 and β_3 residues 1–472 was produced in Chinese hamster ovary Lec 3.2.8.1 cells stably transfected with plasmids coding for each fragment. Acid-base α -helical coiled-coil peptides were fused to the C termini of the α - and β -subunits to increase the stability of the heterodimer. Methods were as described previously (8). A hexahistidine tag was also attached to the C terminus of the β -subunit to facilitate purification by nickel chelate chromatography (8). The purified headpiece fragment was treated with 10 μ g/ml chymotrypsin for 16 h at 25 °C to remove the C-terminal clasp, and incubated with the high affinity RGD-mimetic compound L738,167 (gift from Dr. G. D. Hartman, Merck) (30) at 10 μ M for 30 min at 37 °C. The mixture was cooled to room temperature; an equal volume of sample buffer containing 0.1% SDS was added, and samples were immediately subjected to nonreducing SDS-PAGE on a 4–20% gradient gel.

RESULTS

Introduction of Disulfide Bonds between the β -Propeller and I-like Domains—To make mutant integrins that were unable to undergo head separation, we mutationally introduced cysteine residues at the interface between the α_{IIB} β -propeller and β_3 I-like domains (Fig. 1). Inspection of the structure of $\alpha_V\beta_3$ and a model of $\alpha_{IIB}\beta_3$ made from the $\alpha_V\beta_3$ template identified candidate positions for disulfide bonds where C α distances between β -propeller and I-like domain residues were within 7 Å. Disulfide-forming efficiency was assessed by transient transfection of 293T cells followed by immunoprecipitation and nonreducing SDS-PAGE. Preliminary experiments revealed that the efficiency varied from 20 to 100%, depending on the combination of residues chosen (data not shown). In $\alpha_V\beta_3$, the combination of the α_V -M400C and β_3 -Q267C mutations gave

¹ The abbreviations used are: mAb, monoclonal antibody; FITC, fluorescein isothiocyanate.

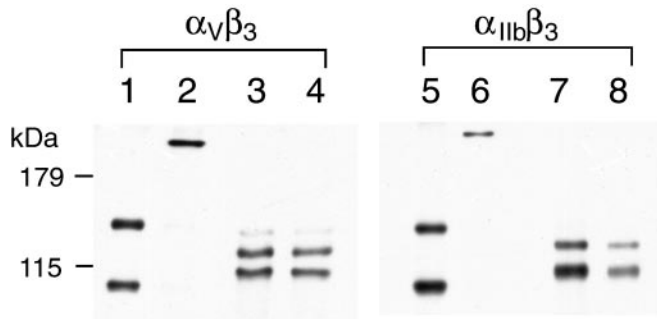


FIG. 2. Formation of intersubunit disulfide bonds in the headpieces of $\alpha_V\beta_3$ and $\alpha_{IIb}\beta_3$. Lysates were prepared from [35 S]methionine- and -cysteine-labeled 293T cells that had been transiently transfected with wild type $\alpha_V\beta_3$ (lanes 1 and 3), α_V -M400C/ β_3 -Q267C (lanes 2 and 4), wild type $\alpha_{IIb}\beta_3$ (lanes 5 and 7), or α_{IIb} -E324C/ β_3 -G293C (lanes 6 and 8), immunoprecipitated with mouse mAb AP3 to human β_3 , and subjected to SDS-7.5% PAGE under nonreducing (lanes 1, 2, 5, and 6) or reducing (lanes 3, 4, 7, and 8) conditions followed by fluorography. Positions of molecular size markers are shown on the left.

~100% formation of an intersubunit disulfide bridge as indicated by the formation of a high molecular weight band in nonreducing SDS-PAGE (Fig. 2, compare wild type in lane 1 and double mutant in lane 2), whereas the α_V - and β_3 -subunits migrated separately in reducing SDS-PAGE (Fig. 2, lanes 3 and 4). The combination of α_V -E311C and β_3 -G293C mutations resulted in slightly less efficient disulfide formation (~70%, data not shown). Since α_{IIb} lacks the long loop containing M400 in α_V , the mutation corresponding to α_V -E311C (α_{IIb} -E324C) was tested and shown to form an efficient disulfide when coupled with the β_3 -G293C mutation (Fig. 2, compare lane 6 with wild type in lane 5). Under reducing conditions, the disulfide-linked complex was reduced into individual α - and β -subunits indistinguishable from the wild type subunits (Fig. 2, lanes 7 and 8). The mutations link β -propeller blade 5 to the connection between blades 6 and 7 to the I-like domain, whereas the ligand-binding interface locates on the opposite side of the interface where blades 2–4 of the β -propeller domain contact the I-like domain (Fig. 1). The mutations should therefore not directly affect the ligand-binding site or disrupt conformational changes in loops near the ligand-binding site. A panel of mAbs directed against the head regions of $\alpha_V\beta_3$ and $\alpha_{IIb}\beta_3$ bound identically to mutant and wild type receptors (data not shown), suggesting that the mutant receptors adopt a native fold.

High affinity binding of $\alpha_V\beta_3$ and $\alpha_{IIb}\beta_3$ transfectants to fluorescent, soluble ligands was measured with simultaneous staining of surface-expressed $\alpha_V\beta_3$ or $\alpha_{IIb}\beta_3$ using two-color flow cytometry (5). As described previously, wild type $\alpha_{IIb}\beta_3$ bound soluble fibrinogen when activated by Mn^{2+} and activating mAb PT25-2 but not in the absence of activation (Fig. 3A). The α_{IIb} -E324C/ β_3 -G293C mutant could not bind soluble fibrinogen in the resting state but showed full activity when treated with Mn^{2+} and PT25-2 (Fig. 3A). Thus, the ligand-binding phenotype of $\alpha_{IIb}\beta_3$ containing a disulfide-linked headpiece is identical to wild type.

Similarly, the mutant $\alpha_V\beta_3$ receptor was tested for activation-dependent binding to soluble fibrinogen (Fig. 3B) and fibronectin (Fig. 3C). As for $\alpha_{IIb}\beta_3$, the ligand binding activities of wild type and mutant $\alpha_V\beta_3$ receptors were indistinguishable. Both showed little or no basal ligand binding and strong binding of both ligands upon activation by Mn^{2+} and activating mAb AP5 (Fig. 3, B and C). These data show that disulfide bond formation between the β -propeller and I-like domains has no effect on high affinity ligand binding by β_3 integrins, strongly arguing against the notion that head separation is required for conversion to high affinity.

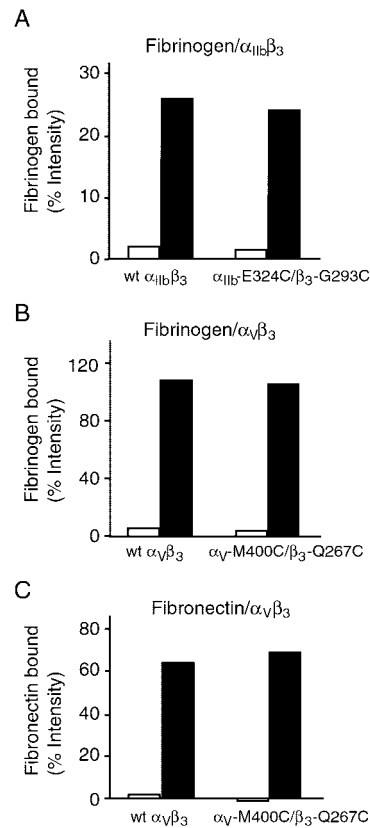


FIG. 3. Ligand binding by disulfide-linked receptors. 293T cells expressing $\alpha_{IIb}\beta_3$ (A) or $\alpha_V\beta_3$ (B and C) integrins were incubated with (filled bars) or without (open bars) 1 mM Mn^{2+} and the activating mAbs PT25-5 for $\alpha_{IIb}\beta_3$ or AP5 for $\alpha_V\beta_3$. Binding of FITC-fibrinogen (A and B) or FITC-fibronectin (C) was determined and expressed as the percentage of mean fluorescence intensity relative to immunofluorescent staining with Cy3-labeled AP3 mAb. wt, wild type.

Introduced Disulfide Bond Blocks EDTA-induced Subunit Dissociation—EDTA treatment at pH 8.4 at 37 °C is known to induce dissociation of the integrin $\alpha_{IIb}\beta_3$ into the α_{IIb} - and β_3 -subunits on the platelet surface (31–33). We confirmed that $\alpha_{IIb}\beta_3$ expressed on 293T transfectants is similarly susceptible to the EDTA-induced subunit dissociation. Incubation of cells expressing wild type $\alpha_{IIb}\beta_3$ with 5 mM EDTA at pH 8.4 completely abolished binding of the $\alpha\beta$ complex-specific mAbs AP2 (34) and 10E5 (34) but had no effect on binding of the β_3 -specific mAb AP3 (Fig. 4A). The complex-specific mAbs require the presence of a complex between α_{IIb} - and β_3 -subunits for recognition, and loss of reactivity is thus an excellent indicator of subunit dissociation. In contrast to the wild type receptor, binding of the mAbs AP2 and 10E5 to the mutant α_{IIb} -E324C/ β_3 -G293C receptor was unaffected by EDTA treatment (Fig. 4B) showing that the disulfide bond conferred resistance to EDTA-induced subunit dissociation. Thus, maintenance of a covalent connection between the β -propeller and I-like domains is sufficient to protect the epitopes of the AP2 and 10E5 mAbs, suggesting that the deleterious effect of EDTA on these epitopes is a consequence of subunit separation, rather than a direct stabilizing effect of divalent cations on residues within the epitopes.

RGD Peptide Binding Does Not Induce Head Separation—We used the AP2 and 10E5 mAbs to determine whether RGD-mimetic peptides, like EDTA, could induce head separation of cell surface $\alpha_{IIb}\beta_3$. Incubation of wild type $\alpha_{IIb}\beta_3$ 293T cell transfectants with 100 μ M GRGDSP peptide resulted in full exposure of cryptic epitopes called ligand-induced binding sites (data not shown), as described previously (35, 36), suggesting

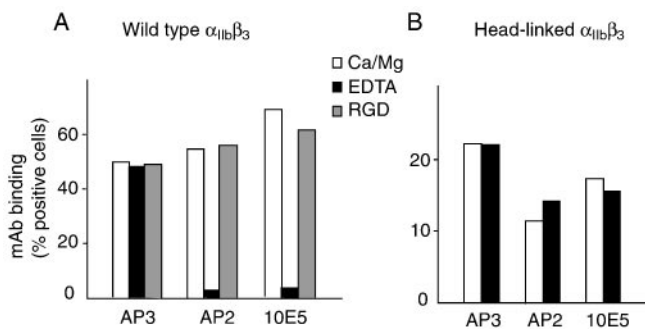


FIG. 4. Effect of EDTA treatment and RGD peptide on the binding of complex-specific $\alpha_{IIb}\beta_3$ mAbs. Transiently transfected 293T cells expressing wild type $\alpha_{IIb}\beta_3$ (A) or mutant $\alpha_{IIb}E324C/\beta_3G293C$ (B) were stained with the indicated mAbs in the presence of 1 mM Ca^{2+}/Mg^{2+} (open bars), 5 mM EDTA (filled bars), or 1 mM Ca^{2+}/Mg^{2+} plus 100 μM GRGDSP peptide (shaded bars, wild type only) as described under "Experimental Procedures." Binding is expressed as the percentage of positive cells after subtraction of background staining by $\times 63$ IgG control.

that all of the $\alpha_{IIb}\beta_3$ on the cell surface bound GRGDSP peptide. However, preincubation with and the continued presence of 100 μM RGD peptide had no effect on binding of AP2 and 10E5 mAbs to $\alpha_{IIb}\beta_3$ (Fig. 4A). This result not only establishes that RGD peptide and these mAbs bind to independent sites on the $\alpha_{IIb}\beta_3$ headpiece but also strongly suggests that the two subunits stay associated in the headpiece when RGD peptide is bound.

RGD-mimetic Compound Stabilizes Rather than Destabilizes Integrin Headpiece Association—High affinity ligand-mimetic compounds can stabilize integrin $\alpha\beta$ complexes and make them resistant to SDS-induced dissociation during gel electrophoresis (37, 38). A wide variety of integrins can be stabilized, including $\alpha_{IIb}\beta_3$ (17, 39, 40). In these studies, the integrins were either native or were recombinant integrins containing the entire extracellular domain. In the bent integrin conformation, there are substantial interactions between the α - and β -subunits in both the headpiece and tailpiece (4, 5). To clarify whether ligand-mimetic peptide binding stabilizes the $\alpha\beta$ interface present within the headpiece, we designed a truncated $\alpha_{IIb}\beta_3$ integrin fragment composed only of headpiece segments. It contains the α_{IIb} β -propeller and thigh domains and the β_3 plexin-semaphorin-integrin (PSI), I-like, hybrid, and the first integrin-epidermal growth factor-1 domains; the only α - β interface present in this fragment is between the β -propeller and I-like domains (Fig. 1). When the purified $\alpha_{IIb}\beta_3$ headpiece fragment was incubated with SDS sample buffer at room temperature and subjected to SDS-PAGE, two distinct bands corresponding to the truncated α_{IIb} - and β_3 -subunits were found (Fig. 5, lane 1). By contrast, when the $\alpha_{IIb}\beta_3$ headpiece fragment was incubated with 10 μM of the RGD-mimetic compound L738,167 prior to SDS-PAGE, a single band with higher molecular mass was found with no free α_{IIb} or β_3 fragments (Fig. 5, lane 2). Thus, the $\alpha_{IIb}\beta_3$ headpiece becomes resistant to dissociation by SDS after incubation with a ligand-mimetic peptide. The ligand-mimetic compound strengthens association between the β -propeller domain of α_{IIb} and the I-like domain of β_3 probably by providing an additional interaction between them, as seen in the crystal structure in which an RGD-mimetic peptide binds to α_V through its Arg and β_3 through its Asp (6). Stabilization by the ligand-mimetic compound of $\alpha\beta$ headpiece association is in strong contradiction to the notion that ligand-mimetic peptides cause dissociation of the α - and β -subunits in the headpiece.

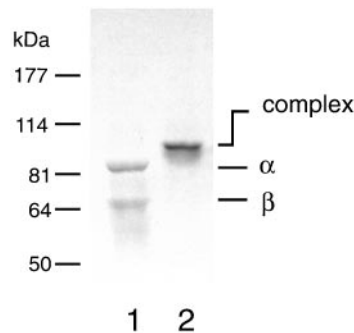


FIG. 5. Ligand-induced stabilization of intersubunit association in the headpiece. The $\alpha_{IIb}\beta_3$ headpiece fragment comprising α_{IIb} residues 1–621 and β_3 residues 1–472 was incubated without (lane 1) or with 10 μM L738,167 (lane 2) and subjected to SDS 4–20% gradient PAGE and Coomassie Blue staining. Positions of molecular size markers are shown on the left.

DISCUSSION

Our study definitively establishes that head separation is not required for activation of ligand binding by integrins, as shown with both $\alpha_{IIb}\beta_3$ and $\alpha_V\beta_3$ by introduction of a covalent linkage between the α -subunit β -propeller domain and the β -subunit I-like domain. Despite the covalent linkage, binding of $\alpha_{IIb}\beta_3$ to fibrinogen, and $\alpha_V\beta_3$ to fibrinogen and fibronectin, was fully activable. We used the AP2 and 10E5 mAbs, which bind to epitopes contained wholly within the $\alpha_{IIb}\beta_3$ headpiece and are specific for the $\alpha_{IIb}\beta_3$ complex, to monitor separation between α_{IIb} and β_3 in the headpiece within intact $\alpha_{IIb}\beta_3$ on the cell surface. EDTA dissociated the $\alpha_{IIb}\beta_3$ headpiece as shown by loss of both epitopes. However, the ligand-mimetic GRGDSP peptide did not dissociate the headpiece, despite saturation binding to $\alpha_{IIb}\beta_3$ as shown by full exposure of ligand-induced binding site epitopes. Finally, a high affinity RGD-mimetic compound was found to stabilize association between α_{IIb} and β_3 in a fragment containing only the headpiece. These results completely contradict the idea that RGD-mimetics induce headpiece separation. A model for I domain-containing integrins, in which separation between the α -subunit β -propeller and the β -subunit I-like domain allows the α -subunit I domain to bind to the β -propeller domain in place of the I-like domain (15), also seems highly unlikely in view of our results, because integrins that contain and lack I domains are activated by similar mechanisms (3).

By contrast, the results are completely consistent with an alternative model of integrin activation, in which the integrin headpiece stays associated and ligand binding affinity is linked to the equilibrium between bent and extended integrin conformations, and between two headpiece conformations that differ in the angle between the I-like and hybrid domains (5). In contrast to the headpiece separation model, the bent-extended model has received experimental support. Disulfide bonds that stabilize the bent conformation inhibit integrin activation (5), and an introduced *N*-glycosylation site designed to wedge open the angle between the I-like and hybrid domains activates ligand binding (36).

The crystal structure of $\alpha_V\beta_3$ bound to an RGD ligand-mimetic peptide was obtained by soaking the peptide into a crystal containing the bent conformer of $\alpha_V\beta_3$ (6). Some movement was seen at the β -propeller interface with the I-like domain upon ligand binding, but its magnitude was small and compatible with the disulfide bonds we have introduced. Thus, the distances between $C\alpha$ atoms before and after ligand binding, respectively, are 6.7 and 6.8 Å for α_V -M400/ β_3 -Q267, and 5.8 and 5.8 Å for α_V -E311/ β_3 -G293. In the absence of restraining crystal lattice contacts, ligand-mimetic peptide binding induces

adoption of the high affinity, extended conformation of $\alpha_V\beta_3$ and a change in angle between the I-like and hybrid domains (5). Our disulfide bond-linked mutants are fully competent for high affinity ligand binding. Therefore, any further change in orientation of the β -propeller domain with respect to the I-like domain between the ligand-bound bent and extended conformations must be relatively small.

Apart from complete head separation, the possibility has been raised of integrin activation by a marked tilt of the I-like domain so that it separates from the β -propeller domain at the ligand binding interface but not on the opposite side of the interface, near where we have introduced disulfide bridges (2, 15). We note that the α_{IB} -E324C/ β_3 -G293C mutation involves residues that are buried in the β -propeller I-like domain interface and that the surrounding region is highly conserved in α_V and α_{IB} . Therefore, pivoting around the introduced disulfide bond to open the ligand-binding site seems unlikely, because on the side of the pivot opposite from the ligand-binding site residues are already closely packed. Thus, β_3 residue Glu-297 would clash with α_V residue Phe-337, and β_3 residues Leu-324 and Pro-326 would clash with α_V residues Lys-308 and Leu-309; all of these α_V residues are highly conserved in α_{IB} . Our results therefore rule out a significant tilting motion between the β -propeller and I-like domains that would open up the ligand binding interface, as well as complete separation of these domains. This conclusion is in accord with high resolution EM projection averages that show no gross change in orientation between the β -propeller and I-like domains upon ligand binding (5). Indeed, the only inter-domain movement observed within the headpiece upon ligand binding is between the I-like and hybrid domains (5). Partial head separation is also inconsistent with the crystal structure of RGD peptide bound to $\alpha_V\beta_3$, because the Arg and Asp side chains of RGD are already extended in opposite directions (Fig. 1) (6), and separation at this interface would abolish one or the other of the highly specific interactions that these side chains make with the α_V - and β_3 -subunits, respectively.

We have suggested that swinging of the hybrid domain pulls down the C-terminal helix of the I-like domain and as a consequence activates the metal ion-dependent adhesion site analogously to I domain activation (3, 36). Since physiological ligands use many residues other than the potential metal ion-dependent adhesion site-coordinating residues to interact with integrins, it is natural to expect that conversion to the high affinity conformation involves rearrangement of residues of the α - β interface outside of the metal ion-dependent adhesion site. In fact, there are activation-reporting mAbs that map to this region (41–43). These rearrangements may involve loop remodeling and some reorientation between the β -propeller and I-like domains, but in a more subtle degree than proposed by head separation/tilting models. Precise determination of the subtle conformational changes responsible for affinity regulation of integrins awaits further study using atomic resolution analysis.

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