A Naturally Occurring Extracellular α–β Clasp Contributes to Stabilization of β3 Integrins in a Bent, Resting Conformation

Anthony N. Vomund,† Sarah Stuhlsatz-Krouper,‡ Julie Dimitry,† Yuhua Song,§ and William A. Frazier‡,*†

Departments of Biochemistry and Molecular Biophysics and Cell Biology, Washington University School of Medicine, 660 South Euclid Avenue, St. Louis, Missouri 63110, and Department of Biomedical Engineering, The University of Alabama at Birmingham, 1825 University Boulevard, Birmingham, Alabama 35294

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ABSTRACT: Control of αIIbβ3 and αvβ3 integrin activation is critical for cardiovascular homeostasis. Mutations that perturb association of integrin α and β subunits in their transmembrane and cytoplasmic regions activate the integrin heterodimer, suggesting that a low-affinity or “off” conformation is the default state, likely corresponding to the bent conformation seen in the crystal structure of αvβ3. In this bent structure, a segment of αv (301–308) and β3 (560–567) are juxtaposed. Here we provide evidence that these regions of αv/αIIb and β3 function as a novel extracellular clasp to restrain activation. Synthetic peptides representing the αIIb and β3 clasp regions promote integrin activation as judged by cell adhesion, cell spreading, and exposure of epitopes for three β3 LIBS antibodies. Mutation of the clasp region of αv or β3 results in a constitutively activated integrin, confirming the role of the extracellular clasp in restraining integrin activation. Molecular dynamics simulations of the αv/β3 structure yield a refined model for the αv/β3 clasp and provide plausible explanations for the effects of the activating mutations.

Control of integrin activity is of crucial importance in platelet aggregation (1), leukocyte adherence and trafficking (2) (3), angiogenesis (4), and apoptosis (5). Crystal structures of integrin α chain domains (2) and the structure of the entire extracellular domain of αvβ3 (6, 7) have provided new hypotheses for integrin regulation. Multiple mechanisms contribute to regulation of integrin activation. This is not unanticipated given the critical role of integrins in so many important biological scenarios and the disastrous consequences of inappropriate activation of platelet or leukocyte integrins. Two general mechanisms regulate the functional state of integrins. Conformational changes of the αβ dimer are clearly involved in transitions from low- to high-affinity states (8), usually judged by the binding of soluble ligands or the exposure of binding sites for mAbs that recognize an activated conformation stabilized by ligands or a “ligand-induced binding site” (anti-LIBS1 mAbs) (9–11). Integrin dimers competent for ligand binding may also be clustered resulting in a high “avidity” state that increases binding to multivalent, usually immobilized ligands (12–14).

Early studies of αIIbβ3 activation suggested that association of juxtamembrane cytoplasmic ion pairs opposite each other in the α and β subunit tails could restrain activation (15) (16). This notion of a cytoplasmic “clasp” of the α and β subunits was strengthened by the effects of additional mutations in the juxtamembrane regions of αIIb and β3 that resulted in constitutive activation (17). Further, the addition of non-native, coiled-coil dimerizing peptides to the cytoplasmic tails of α and β subunits constrained activation (18, 19). A role for the TM domains of α and β subunits in integrin activation has also been proposed based on mutations that constitutively activate αIIbβ3 (17, 20). These data suggest a model in which a specific α–β TM helix interface contributes to stabilizing the off state, likely acting in concert with the juxtamembrane clasp in the cytoplasmic tails.

The publication of the crystal structure of free and RGD-bound extracellular domains of αvβ3 (6, 7) gave rise to an entirely new model for activation. The bent or genuflected integrin seen in the crystal structure suggested that massive conformational changes of the entire αvβ3 extracellular domain must accompany integrin activation, if indeed the fully active integrin were to assume the extended, upright conformation expected from earlier EM studies (21–24). A growing body of data supports the idea that the bent structure seen in the crystals of αvβ3 is likely the physiologically relevant “off” or low-affinity state and an extended, erect integrin represents the fully active state (8, 13, 14, 25, 26). Particularly revealing was an experiment in which non-native Cys residues were inserted in the α and β subunits of αvβ3 (or the homologous position of αIIb) at locations that were within disulfide bond distance in the bent structure but were predicted to be very far apart in an extended structure (αv-G307 > C, and β3-R563 > C). When the mutant α and β subunits were expressed in cells, they formed αβ dimers that were inactive, even in Mn2+, until mild reducing agent was applied, or either one of the mutant α or β subunits was replaced with the wild-type (non-Cys) construct (8). This

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‡ To whom inquiries should be addressed. Phone: 314-362-3348. Fax: 314-362-7183. E-mail: frazier@biochem.wustl.edu.
§ Washington University School of Medicine.
¶ The University of Alabama at Birmingham.
† Abbreviations: 4N1K, thrombospondin-1 peptide with sequence KRFYVVVMWKK; LIBS, ligand-induced binding site; RGD peptide, peptide with sequence GRGDSP; TM, transmembrane; Vn, vitronectin; WT, wild type.

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experiment demonstrates that the bent structure can exist on the cell surface and is inactive. Not addressed is whether these juxtaposed regions of the α (G307) and β (R563) subunits normally have a role in regulating integrin activation by forming an extracellular clasp. Here we test this hypothesis.

EXPERIMENTAL PROCEDURES

Reagents, Cell Lines, and Peptides. Human K562 erythroleukemic cells (ATCC: CCL-243), stably expressing αvβ3 integrin (27), HEK 293 cells (ATCC: CRL1573), and C32 human melanoma cells (ATCC: CRL-1585) were grown as described (28). Ligand-induced binding site (LIBS) antibodies, LIBS1 and LIBS6, were generously provided by Dr. Mark Ginsberg (Scripps Research Institute) (9), and the LIBS antibody D3 was a gift from Dr. Lisa Jennings (The University of Tennessee, Memphis, TN) (11). Peptides were synthesized, purified, and verified by mass spectrometry as previously described (29). All other reagents were purchased from Sigma-Aldrich unless otherwise stated. Adhesion and spreading assays of C32 cells on Vn were performed as described (30). Collection of human blood was performed under an approved protocol of the Washington University School of Medicine Human Studies Committee. Washed platelets were prepared as described (31) and stored on ice for 1–48 h or treated as described below. Binding of anti-β3 mAb AP3 and anti-LIBS mAbs LIBS1, LIBS6, and D3 to cells and platelets under indicated conditions was quantified using a Coulter EPICS flow cytometer. Data were analyzed using WinMDI software.

Preparation and Expression of Mutants of αv and β3. Full-length cDNAs of human αv and β3 integrin subunits were provided by Dr. Scott Blystone. Restriction fragments containing the mutation sites were subcloned into Bluescript BSKS+ for PCR mutagenesis using overlapping primers containing the mutant bases. Details are provided in Supporting Information. After confirmation by DNA sequencing, the restriction fragment containing the desired mutation was reassembled in pCDNA3 for αv (G418 selection) and either pREP10 or pBLY100 for β3 (hygromycin selection). Initial tests of the β3 mutants were performed by transfecting them into human ovarian carcinoma clone OV10 which expresses WT αv (largely as αvβ5) but no β3 (32). For cotransfections of both subunits, HEK 293 cells were used (33) (34). Expression was determined by flow cytometry with mAbs L230 (αv) and AP3 (β3). LIBS binding was determined as above and normalized to AP3 binding or the binding of the LIBS mAb in the presence of excess RGDS peptide and Mn²⁺ to yield an activation index.

Molecular Dynamics Simulations. The reported crystal structure of αvβ3 (PDB ID: 1JV2) (6) was subjected to energy minimization and equilibration using GROMACS version 3.3 (35). Details of the method are provided in Supporting Information.

RESULTS

Clasp Peptides Stimulate Integrin-Dependent Cell Adhesion and Spreading. We first used synthetic peptides to mimic the α and β sides of the clasp. Since αIIb/β3 is likely to be held in the off state more securely than αvβ3, we chose the αIIb clasp sequence for these experiments (Table 1). To evaluate the effects of the putative clasp peptides on αvβ3 activation in live cells, adhesion and spreading assays using C32 melanoma cells were performed. We previously showed that, at relatively low densities of Vn coated on plastic (ca. 0.5 µg/ml), initial adhesion of C32 cells is mediated by αvβ5. Not until αvβ3 is activated, e.g., via Mn²⁺ or CD47 stimulation, does αvβ3-dependent spreading occur (36). The assay was performed in Ca²⁺/Mg²⁺ which supports integrin activity, and we used Mn²⁺ activation of αvβ3 as a positive control (8). Mn²⁺ resulted in increased adhesion to Vn as well as enhanced spreading, and 0.5 mM GRGDSP effectively reduced cell adhesion. As another positive control, we used the C-terminal TSP1 peptide, 4N1K, an agonist of CD47, which, in this assay, signals αvβ3 activation via heterotrimeric Gi (37). C32 cell adhesion increased 5-fold in the presence of 50 µM 4N1K. The addition of 100 µM P2483 (β3 integrin peptide) or P2484 (αIIb integrin peptide) clasp peptides stimulated cell adhesion to an extent comparable to 4N1K (Figure 1). Each of the treatments that increased cell adhesion to Vn also enhanced cell spreading

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* αv pairs with β1, β3, β5, β6, and β8. † α4 and all other integrin α subunits have deletions in the clasp region.
(Figure 1). Mn$^{2+}$ and 4N1K increased the number of spread cells ca. 3-fold and 10-fold, respectively, while addition of GRGDSP, 50 µM 4N1K, 100 µM P2483 (β3 peptide), and 100 µM P2484 (αIIb peptide) increased cell spreading 7-fold. A control peptide of identical net charge was inactive (not shown). Thus αIIb or β3 clasp peptides were able to stimulate cell spreading, a signaling-dependent function, more effectively than Mn$^{2+}$. While these are functions of activated αvβ3, the peptides might act indirectly via other nonintegrin intermediaries to influence integrin-dependent cell adhesion and spreading.

**Clasp Peptides Induce Conformational Changes in β3 Integrins Consistent with Activation.** To more directly monitor changes in integrin conformation, we used antibodies (mAbs) that react with ligand-bound or activated states of β3 integrins. The three LIBS mAbs used here recognize epitopes at three broadly defined but nonoverlapping sites within the stalk region of the β3 subunit that are masked in the “off” state and only become accessible when the integrin is “opened up” in the ligand-binding, activated conformation (38).

When K562 cells transfected to express αvβ3 integrin were incubated with the LIBS1 or LIBS6 mAbs alone (dotted histograms in Figure 2A), little antibody binding above isotype control mAb or secondary mAb alone was detected. D3 mAb appeared to induce some activation of the integrin since its binding to αvβ3 (Figure 2A, dotted line) was severalfold above that of controls (not shown). GRGDSP peptide (dashed histogram, Figure 2A) increased binding of all three LIBS, and this shift was further augmented by the addition of 0.5 mM Mn$^{2+}$ ion. We then determined the effect of the clasp peptides on the binding of each LIBS mAb in the presence of 1 mM RGD peptide, but in the absence of Mn$^{2+}$. Preliminary studies indicated that the peptide effects were maximal between 50 to 100 µM peptide under these conditions (not shown). As seen in Figure 2A, LIBS1 antibod ybinding was dramatically increased by both 100 µM P2483 (β3 clasp peptide, shaded histogram) and P2484 (αIIb clasp peptide, solid line), well beyond the increase in LIBS1 binding due to RGD alone. This was seen in eight experiments with LIBS1 (two of which were performed with OV10 cells transfected to express αvβ3) (39). In six of eight experiments, rightward shifts with the β3 and αIIb peptides were greater than that promoted by RGD plus Mn$^{2+}$ (not shown). The control peptide did not enhance LIBS1 binding, nor did a number of other peptides based on sequences present in TSP1 or CD47 that were selected to have the same net charge as the clasp peptides (data not shown).

In contrast to the results with LIBS1, the clasp peptides induced no additional binding of LIBS6 antibody beyond that achieved with RGD peptide (Figure 2A, representative of three experiments). However, the effect of Mn$^{2+}$ itself was much less marked in the case of LIBS6 binding to αvβ3 (not shown).

In the case of the D3 LIBS mAb, RGD peptide resulted in an increase in D3 binding, and both clasp peptides further increased D3 binding (Figure 2A, representative of three experiments). Here the histogram shift induced by the clasp peptides was of about the same magnitude as that of Mn$^{2+}$ (not shown). As expected from the fact that these three LIBS mAbs bind to different epitopes on β3, the clasp peptides altered the binding of each mAb differently. Importantly, however, for each particular LIBS mAb, the effect of the α and β clasp peptides was comparable, suggesting that both peptides act via the same mechanism, i.e., competition for the endogenous clasp.

**Clasp Peptides Increase LIBS Antibody Binding to Platelet αIIb/β3.** Platelets and megakaryocytes are the only cells to express αIIb/β3 integrin, and thus platelets offer a unique system in which to test the effects of the clasp peptides. Platelets also provide the opportunity to compare the activation response to the clasp peptides of αIIb/β3 vs αvβ3. To eliminate inside-out activation of platelet αIIb/β3, we incubated platelets with PGE1, which elevates cyclic AMP levels, and in some experiments used aprotinin to block activation by leaked ADP (31). We also kept platelets on ice for 24–48 h to ensure metabolic inactivity. To be sure that platelets were metabolically inert, they were challenged with 50 µM ADP or 10 µM thrombin receptor activating peptide (TRAP) in the presence of the three LIBS mAbs. Neither agonist was able to increase LIBS binding to the platelets used in these experiments, indicating that inside-out signaling was effectively disabled.

We first determined that 100 µM RGD was sufficient to increase LIBS1 binding to platelets, and this shift was further magnified with the addition of 0.5 mM Mn$^{2+}$ (not shown). As seen in Figure 2B, the β3 clasp peptide (P2483, shaded histogram) was unable to increase LIBS1 binding to αIIb/β3 and, in some experiments, slightly reduced LIBS1 binding as compared to the effect of RGD alone. However, the αIIb peptide (P2484, solid line) increased LIBS1 binding in the presence of RGD to an extent comparable to the addition of Mn$^{2+}$ (six experiments, two different platelet donors). In all six experiments, the β3 clasp peptide failed to increase LIBS1 binding. This suggests that the binding of the αIIb clasp to the β3 subunit is of substantially greater affinity than the binding of the αv clasp to β3. With LIBS6 antibody, results similar to those with αvβ3 were obtained in that neither clasp peptide increased binding of LIBS6 beyond that seen with RGD alone (Figure 2B). In the case of αIIb/β3, however, LIBS6 binding was not increased by addition of RGD at
this concentration. This suggests that the epitope recognized by LIBS6 is more protected in αIIbβ3 than in αvβ3, likely due to the tighter “off state” of αIIbβ3.

Finally, we tested the effect of the clasp peptides on the binding of D3 LIBS antibody to platelets (Figure 2B). Here, peptide P2483 (β3) marginally stimulated D3 binding, while P2484 (αIIb) produced large rightward shifts in the binding histogram, in this case even greater than those seen with Mn2+ plus RGD (not shown). Thus the αIIb clasp peptide is much more effective at activating αIIbβ3 than the β3 clasp peptide, while the two clasp peptides are more comparable in potency to activate αvβ3, again likely reflecting the difference in affinities of the αv and αIIb clasps for their counterpart in the β3 subunit.

Mutation of αvβ3 Clasp Residues. The data obtained with the peptides suggested that they compete for the endogenous integrin clasp and thus promote integrin activation. To confirm this, we sought to create point mutations in the αv and β3 clasp in the context of the full-length integrin subunits expressed on the cell surface. We turned to the crystal structure of αvβ3 (6) in an effort to identify specific amino acid residues in αv and β3 that might be important in stabilizing the clasp. In the crystal structure, the αv clasp peptide forms a loop that projects from the β-propeller domain (Figure 4). It contains the sequence RGSD (αv 303–306) juxtaposed to the β3 clasp which contains the sequence RTD (β3 563–565), suggesting the possible formation of two R–D ion pairs or salt bridges, a situation analogous to the juxtamembrane intracellular α–β clasp (15) (40) (18). If the oppositely charged pairs do in fact form an extracellular clasp, then swapping the R and D in either αv or β3 should break the clasp since it would create an R–R and a D–D pairing. Further, Springer’s group mutated β3-R563 to insert a non-native Cys which formed a disulfide with the Cys inserted in place of αv-G307 (8), indicating that β3-R563 can make close contact with the αv clasp residues. Therefore, we initially focused on β3-R563 and β3-D565 as candidates for mutation and created a full-length β3 construct with the R and D swapped, creating the clasp sequence TTDTRT (WT = TTRTD). Importantly, this swap mutation leaves unchanged the net charge and amino acid composition of this short segment of the peptide chain. The β3R/D swap mutant was expressed in OV10 cells (which lack β3 expression) where it paired with endogenous WT αv, resulting in cell surface expression of the integrin heterodimer with the β3 R/D swap mutation (confirmed by flow cytometry, not shown). To determine the activation status of the mutant integrin relative to WT αvβ3 expressed in the same cell line, we employed the D3 LIBS mAb and expressed activation as the ratio of D3 bound at each RGD concentration to D3 binding in the presence of maximum RGD peptide and Mn2+. Normalization to binding of the conformationally insensitive anti-β3 mAb AP3 (34) gave the same results. We determined the activation index over a concentration range of GRGDSP to help identify differences in the activation status of the integrin (41). As seen in Figure 3A, the αvWT/β3R/D mutant became activated at a much lower concentration of RGD peptide than WT αvβ3 and achieved a higher activation index at 50 μM GRGDSP. In Figure 3B, data from several experiments were pooled to assess the significance of the activation of the mutant vs WT integrin. Here data are shown for the low concentration range (0–10 μM) of RGD peptide where differences in activation index are most pronounced. A sensitive index of activation is the
level of LIBS antibody binding in the absence of RGD peptide. Figure 3C shows that the binding (accessibility) of D3 to α\textsubscript{v}WTβ\textsubscript{3}R/D in the absence of RGD peptide was about 20 times the D3 binding to WT integrin.

As a positive control for activation, we created the β3-T562N mutant that was identified by Shattil’s group in a screen for activating mutations of αIbβ3 (34). When expressed with WT α\textsubscript{v} in OV10 cells, the β3-T562N mutant was also activated at low concentrations of RGD peptide, similar to our β3R/D swap mutant (Figure 3A).

To test the effect of mutating the α\textsubscript{v} side of the clasp, we next created the α\textsubscript{v}R/D swap mutant (R303D/D306R) and expressed it with WT β3. This required using HEK 293 cells as expression hosts (34), since OV10 cells express high levels of WT α\textsubscript{v} subunit (normally paired with β3 in this cell type) (42). As with the β3 swap mutant, the α\textsubscript{v}R/Dβ\textsubscript{3}WT integrin was activated relative to the WT integrin as judged by the D3 activation index (Figure 3). Since differences in the expression host cell type can affect the activation status of β3 integrins (43), we also transfected HEK 293 cells with plasmids encoding WT α\textsubscript{v} and the β3R/D mutant and found that α\textsubscript{v}WTβ\textsubscript{3}R/D exhibited identical activation behavior in HEK 293 and OV10 cells. In fact, some of the experiments included in Figure 3B were performed with the HEK 293 transfectants and some with the OV10 cells. Thus both the α\textsubscript{v} and β3 R/D swap mutants result in activation, although the α\textsubscript{v}R/Dβ\textsubscript{3}WT integrin did not appear as readily activated as the α\textsubscript{v}WTβ\textsubscript{3}R/D. This is clearly seen in Figure 3C where binding of D3 in the absence of RGD peptide is about half of that seen for α\textsubscript{v}WTβ\textsubscript{3}R/D.

If the main contribution to clasp stability were two R–D ion pairs, then one might expect that a “double swap” integrin, with the R and D residues swapped in both the α\textsubscript{v} and β3 chains, would have activation properties similar to those of WT α\textsubscript{v}β\textsubscript{3}. To test this idea, we coexpressed the α\textsubscript{v}R/D and the β3R/D constructs in HEK 293 cells and determined the D3 activation index as above. While not as activated as the α\textsubscript{v}WTβ\textsubscript{3}R/D integrin, the double swap integrin was activated to about the same extent as α\textsubscript{v}R/Dβ\textsubscript{3}WT (Figure 3). This result suggested that molecular interactions that stabilize the clasp are more complex than simple charge–charge interactions.

**Molecular Modeling of the Clasp Region of α\textsubscript{v}β\textsubscript{3}**. The α\textsubscript{v}β\textsubscript{3} structure was solved at a resolution of 3.1 Å (6), and at this resolution, one cannot determine the precise conformation and interactions of the residues in the clasp. In an attempt to arrive at a plausible structure for the clasp region, we used a molecular dynamics simulation approach to obtain an equilibrated structure for WT α\textsubscript{v}β\textsubscript{3}. The median equilibrated crystal structure is shown in Figure 4 and Figure 1S (structures available in Supporting Information). The clasp region of the structure is magnified in Figure 4B to show the α\textsubscript{v} clasp that resides on a short loop projecting from the β-propeller domain of the α\textsubscript{v} chain (magenta) and the proximity of the β3 clasp (cyan). The α\textsubscript{v} clasp loop is deleted in many other integrin α subunits (Table 1 and Discussion). Since part of the minimization process is the assignment of hydrogen atoms, which are completely absent in the crystal data, the equilibrated structure contains plausible configurations for hydrogens covalently bound to amino acids in the clasp. The derived model reveals a complex interface between the α\textsubscript{v} and β3 chains in the clasp region (Figures

**Figure 3**: Activation status of α\textsubscript{v}β\textsubscript{3} clasp mutants. (A) The activation state of each mutant integrin was determined by the binding of LIBS D3 as a function of GRGDSP concentration with each value normalized to the maximum D3 binding obtained in 1 mM MnCl\textsubscript{2} and 100 μM GRGDSP. Panel A (top) shows a single representative experiment for each integrin. Panel B (middle) presents data pooled from all experiments in the concentration range of 0–10 μM RGD peptide to emphasize the activation of the mutants at very low peptide concentrations. Panel C (bottom) presents pooled data showing the increase in “spontaneous” activation (i.e., binding of D3 with no added RGD peptide) of each mutant and WT integrin. (p values: WT vs α\textsubscript{v}WTβ\textsubscript{3}R/D = 0.0003; WT vs α\textsubscript{v}R/Dβ\textsubscript{3}R/D = 0.015; WT vs α\textsubscript{v}R/Dβ\textsubscript{3}WT = 0.008; α\textsubscript{v}WTβ\textsubscript{3}R/D vs α\textsubscript{v}R/Dβ\textsubscript{3}R/D = 0.036; α\textsubscript{v}WTβ\textsubscript{3}R/D vs α\textsubscript{v}R/Dβ\textsubscript{3}WT = 0.11).
In this equilibrated structure, the distance between the residues of the Rv and yyyyyyyy3 clasp... of Rv-D306 with K308.

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4C, 1S, and 2S). In this equilibrated structure, the distance between the residues of the αv and β3 clasp residues is decreased overall compared to the crystal coordinates. The interface has many van der Waals contacts between side chains of residues as well as a complex network of hydrogen bonds involving serine and threonine residues of the clasp (Figures 2S and 3S).

One of the three mutant integrins created in our studies, αv R/Dβ3wt, was subjected to the same molecular dynamics protocol as WT αvβ3. Both the solvent-accessible surface area of the clasp interface and the distance between α and β subunit clasp residues were significantly (p < 0.05) increased in the mutant integrin (Figures 4S and 5S). These results are consistent with the increased activation state of the mutant relative to the WT heterodimer.

Perhaps the most interesting result from the equilibrated WT structure is that none of the R or D residues in the α or β clasp segments pair in trans with the oppositely charged residues on the other chain. Instead, the β chain R563 and D565 are seen to pair with one another, closing a loop across a turn (Figure 4C). In the α chain, D306 is seen paired in cis with K308, also forming an intrachain loop, while the side chain atoms of R303 make extensive van der Waals and apparent hydrogen-bonded contacts with both cis and trans Thr and Ser residues of the clasp. Thus in both chains of the clasp, the R and D residues may play important roles in organizing the three-dimensional conformation of the clasp, but our simulations, as well as the activation states of the mutants, suggest that they do not form interchain salt bridges as initially hypothesized.

**DISCUSSION**

A body of data has accumulated suggesting that the bent conformation of integrins as seen in the crystal structure of αvβ3 represents a physiologically relevant, low-affinity default state of integrins on the cell surface. The activation status of β2 and β3 integrins must be tightly regulated. Given the disastrous consequences of inappropriate activation, it is perhaps not surprising that multiple mechanisms have evolved to regulate integrin activation. The first of these to be appreciated was the interaction between the α and β subunits at the cytoplasmic juxtamembrane interface. In fact, the α subunit GFFKR sequence is highly conserved in many integrins and its interacting counterparts in β subunits only slightly less so (17) (44). It is now well accepted that rearrangement of the cytoplasmic domains and breaking this juxtamembrane clasp are key steps in activating integrins via inside out signaling (26). An additional role of the GFFKR sequence appears to be promoting formation of the α-β dimer for export to the cell surface (45). Several groups have shown that packing together of the transmembrane helices of the α and β subunits also contributes to stabilization of the off state of integrins (17, 20, 46). While it is more difficult to imagine how this “clasp” might be regulated, perhaps changes in lipid composition or the intrusion of other transmembrane proteins might alter association of the integrin TM segments (47). Lastly, evidence obtained mainly with the use of conformation-sensitive mAbs has suggested that integrin activation involves separation of the extracellular regions of the α and β subunits (2). Further, most of these LIBS antibodies are directed at epitopes on the β subunit stalk region, the area most likely to be shielded by the α.
subunit when the integrin is in the genuflected state (25). While supporting the idea that the α and β subunits separate, these studies could not distinguish cause and effect. By creating integrins locked in the off state, Springer’s group provided evidence that the bent state can exist on the cell surface and that it is, in fact, a functionally “off” state for binding matrix and cell surface ligands (8). However, there has as yet been no demonstration that specific interactions of the extracellular domains of both the α and β subunits actually stabilize the off state.

We report here that amino acid residues of the α and β chains that are juxtaposed in the bent or genuflected state of the αvβ3 heterodimer seen in the crystal structure contribute to stabilization of the low-affinity state of the integrin. The ability of the αIIb and β3 clasp peptides to activate αIIbβ3 indicates that this mechanism applies to αIIbβ3 as well. The enhanced activity of the integrins in the presence of either αIIb or β3 clasp peptides is evidenced in functional assays including cell adhesion and cell spreading. The most compelling evidence that the peptides are able to induce a conformational change in integrin structure is their effect on the binding of three different anti-LIBS antibodies, LIBS1, LIBS6, and D3, to both αvβ3 and αIIbβ3. Interestingly, the effect of the three peptides differs depending on which LIBS antibody is used and whether the integrin in question is αIIbβ3 or αvβ3. These results are expected since the three LIBS mAbs used here bind to broadly defined but distinct epitopes in the β3 stalk region. Furthermore, one expects that αIIbβ3 will be held in check more rigorously than αvβ3, a result further supported by our data.

On the basis of the suggestive results obtained with the peptides, we tested the clasp hypothesis by introducing mutations in the putative clasp regions of αv and β3. The antiparallel orientation of the αv and β3 clasp peptides suggested that the RGSD of αv and the RTD of β3 might form two interchain R–D salt bridges contributing substantial energy to the stabilization of the bent conformation. We thus swapped the R and D residues in αv and in β3 with the aim of creating R–R and D–D repulsive ion pairs when either mutant subunit was expressed with the other WT subunit. The β3 R/D swap expressed with WT αv was the most activated, comparable to the β3-T562N mutation selected for its activation of αIIbβ3 (34). The αv R/D swap expressed with WT β3 was activated relative to WT αv/β3 but appeared less activated than the αvβ3R/D mutant. Rather than reverting the activation state of the integrin to that of WT αv/β3, the double swap integrin (each subunit bearing the R/D swap mutation) behaved much like the αvβ3W WT/WT single mutant. This indicated that clasp stability is not simply dominated by two interchain ion pairs and likely depends on more complex interactions between the α and β subunits. If this is indeed the case, it is likely that mutation of any of the closely apposed amino acids in the clasp region would have an activating effect of variable magnitude.

Because of the low resolution of the crystal structure, we performed molecular dynamics simulations to arrive at an equilibrated structure for WT αv/β3. This exercise revealed a complex interface at the α–β clasp in which hydrogen bonds and intrachain electrostatic interactions appear to play a more important role than simple interchain charge–charge interactions. Particularly striking was the extent of a network of hydrogen bonds involving Ser and Thr residues from both chains. These were side chain to side chain (e.g., αv-S305: β3-T561) as well as side chain to main chain (a side chain N–H of β3-R563 interacting with the main chain amide nitrogen of αv-G307). Our minimized clasp model has the side chain OH of β3-T562 hydrogen bonded to the main chain carboxyl of αv-S305 (Figures 1S and 2S). The β3-activating mutation T562N identified previously introduced a new N-X-T site that was in fact glycosylated (34). Clearly, introduction of an oligosaccharide at β3-T562N would have a strongly disruptive effect on the clasp interface. To destroy the glycosylation site, those authors introduced a second mutation, β3-T564A, changing the N-R-T sequence to N-R-A. Even though the glycosylation site was destroyed, this double mutant was even more activated than the single mutant β3-T564A. While Kashiwagi et al. provided evidence that the region of β that we identify as a clasp was somehow involved in activation of αvβ3 and αIIbβ3, residues of complementary function were not identified in the αv or αIIb subunits. It should be noted that their data were obtained by selecting random variants for activation without the benefit of a three-dimensional structure to guide their interpretation of results. The crystal structure of αvβ3, along with the mapping of Glanzmann’s mutations (48) and data of Kashiwagi et al. (34) and Takagi et al. (8), led us to hypothesize the existence and location of an extracellular, interchain clasp.

The β3 clasp lies in the EGF domain region of the β integrin stalk, long suspected to somehow regulate integrin activation (25, 49–52). Furthermore, two well-described Glanzmann’s mutations have been mapped to the β3 clasp region: C560F and C560R (34, 48). The latter mutation was directly demonstrated, using techniques similar to those employed here, to result in activation of αIIbβ3. Further support for a functional role of the clasp region is found in the integrin structures themselves. Comparison of integrin sequences using BLAST reveals that the clasp regions are not found in all integrins (Table 1). The αv and αIIb clasp regions have homologues only in α5 and α6 (although α6 is very divergent). The clasp region is completely or partially deleted in genes of all other α subunits. Since the αv clasp is formed by a loop projecting from the β-propeller in the αv headpiece, it is amenable to deletion in homologous integrins without compromising the fundamental structure of the propeller domain. The β3 clasp region is not deleted in other β subunits, but it is rather divergent in sequence (Table 1). Only β3 and β2 have a positively charged Arg residue along with one or two negative charges in the clasp between cysteines homologous to C560 and C567 of β3. β1, β5, and β6 have only a single Asp, β7 has two Asps, and β4 and β8 have no charged residues at all. Interestingly, the β4 sequence homologue of the β3 clasp contains a potential glycosylation site (CPLSNATC). While αIIb pairs with only β3, αv pairs with β3, β1, β5, β6, and β8 (53). It seems likely that these divergent β subunit clasp regions will be reflected in different set points for basal affinity and affinity modulation of the different αv-containing heterodimers.

The activity or affinity for ligand of αvβ3, as well as αIIbβ3, can be switched from a low-affinity to a high-affinity state, although the factors that regulate this transition for αvβ3 have not been as thoroughly studied as those regulating αIIbβ3. Further, the activation state of αvβ3 appears to be less rigidly controlled than that of the platelet integrin, such that increases in clustering, perhaps driven by binding to
densely clustered ligands of αvβ3, appear to be able to overcome, for some purposes, the lower affinity of the “resting” αβ dimer for its ligands. While αv is highly homologous to αIIb in amino acid sequence, there are slight differences between them in all three potential clasp regions, the juxtamembrane, the TM, and the extracellular α−β clasp identified here. These differences in sequence are responsible for the extremely tight off state of αIIbβ3 necessary to avoid inappropriate thrombosis. Differences in regulation of the two β3 integrins are also likely due to different interactions of the cytoplasmic domains of αv and αIIb with β3 and with intracellular regulatory proteins such as talin (54), CIB (55), and others. Our results indicate that the extracellular domains of β3 integrins contain a functional clasp that contributes to maintenance of the off state and thus raise the possibility that extracellular interactions with soluble or membrane-associated proteins such as CD47 (29) or CD9 and other 4TM proteins (56) may promote integrin activation via an outside-in mechanism. Such is clearly the case for activating antibodies directed against extracellular epitopes (2). Furthermore, the extracellular integral clasp described here may be a target for a novel class of drugs that directly regulate integrin activity to control thrombosis and inflammation.

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SUPPORTING INFORMATION AVAILABLE

Detailed methods, additional figures, and data supporting the molecular dynamics studies. This material is available free of charge via the Internet at http://pubs.acs.org.

REFERENCES


