An internal ligand-bound, metastable state of a leukocyte integrin, $\alpha_X\beta_2$

Mehmet Sen,1,4,5 Koichi Yuki,1,3,4 and Timothy A. Springer1,2,4,5

1Program in Cellular and Molecular Medicine, 2Department of Medicine, 3Department of Anesthesiology, 4Children’s Hospital Boston, and 5Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, Boston, MA 02115

How is massive conformational change in integrins achieved on a rapid timescale? We report crystal structures of a metastable, putative transition state of integrin $\alpha_X\beta_2$. The $\alpha_X\beta_2$ ectodomain is bent; however, a lattice contact stabilizes its ligand-binding $\alpha$ domain in a high affinity, open conformation. Much of the $\alpha$ $\alpha_7$ helix unwinds, loses contact with the $\alpha$ domain, and reshapes to form an internal ligand that binds to the interface between the $\beta$ propeller and $\beta$I domains. Lift-off of the $\alpha$ domain above this platform enables a range of extensional and rotational motions without precedent in allosteric machines. Movements of secondary structure elements in the $\beta_2$ $\beta$ domain occur in an order different than in $\beta_3$ integrins, showing that integrin $\beta$ subunits can be specialized to assume different intermediate states between closed and open. Mutations demonstrate that the structure trapped here is metastable and can enable rapid equilibration between bent and extended-open integrin conformations and up-regulation of leukocyte adhesiveness.

Introduction

Integrin $\alpha$ and $\beta$ subunits contain large ectodomains, one transmembrane domain each, and typically short cytoplasmic domains (Fig. 1). The $\alpha$ and $\beta$ subunits come together to form the integrin head, which connects through upper and lower legs in each subunit to the cell membrane. Of 18 integrin $\alpha$ subunits, nine lack and nine contain an $\alpha$ domain. In $\alpha$-less integrins, ligand binds at the interface between the $\alpha$ subunit $\beta$ propeller and $\beta$ subunit $\beta$I domains that form the head. $\alpha$ integrins bind ligand to the $\alpha$I domain, which is inserted in the $\beta$ propeller near its interface with the $\beta$I domain (Luo et al., 2007; Springer and Dustin, 2012).

$\alpha$I and $\beta$I domains are structurally homologous. They have a metal ion-dependent adhesion site (MIDAS) with a Mg$^{2+}$ ion that binds an acidic residue in ligands and two states, closed and open. In the open state, a change in conformation in loops around the MIDAS contact stabilizes its ligand-binding domain in a high affinity, open conformation. Much of the $\alpha$ $\alpha_7$ helix unwinds, loses contact with the $\alpha$ domain, and reshapes to form an internal ligand that binds to the interface between the $\beta$ propeller and $\beta$I domains. Lift-off of the $\alpha$I domain above this platform enables a range of extensional and rotational motions without precedent in allosteric machines. Movements of secondary structure elements in the $\beta_2$ $\beta$ domain occur in an order different than in $\beta_3$ integrins, showing that integrin $\beta$ subunits can be specialized to assume different intermediate states between closed and open. Mutations demonstrate that the structure trapped here is metastable and can enable rapid equilibration between bent and extended-open integrin conformations and up-regulation of leukocyte adhesiveness.
Under resting conditions, the \( \alpha_{\beta 2} \) ectodomain is predominantly bent, and in this state the headpiece is closed. The bent and extended, closed conformations have low affinity, whereas the extended, open conformation has high affinity, as demonstrated for both \( \alpha_{\beta 2} \) and \( \alpha_{\gamma 2} \) (Chen et al., 2010; Schürpf and Springer, 2011).

Thus, opening of the headpieces of \( \alpha_{\beta 2} \) and \( \alpha_{\gamma 2} \) integrins transmits a signal that increases affinities of their \( \alpha \) I domains for ICAM-1 and IC3b, respectively. It has been proposed that in relay the C-terminal portion of the \( \alpha \) I domain \( \alpha 7 \) helix and its following C linker, including an invariant Glu in \( \alpha \) I domains, binds to an interface between the \( \beta \) propeller and \( \beta I \) domains similar to the ligand-binding interface in \( \alpha \) I-less integrins (Alonso et al., 2002; Shimaoka et al., 2002). A bell rope–like pull on the \( \alpha \) I \( \alpha 7 \) helix appears to activate \( \alpha \) I integrins (Yang et al., 2004; Weitz-Schmidt et al., 2011). However, the crystal structure of bent \( \alpha_{\gamma 2} \) with a closed \( \alpha \) I domain showed that the invariant Glu, \( \alpha 5 \) Glu-318, was quite distant from the \( \beta I \) MIDAS, \( \sim 16 \)–19 Å away (Xie et al., 2010). How relay is accomplished remains unknown and is currently the most outstanding unanswered question about allosteric transmission in integrin extracellular domains.

On cell surfaces, integrins likely exist in dynamic equilibrium between all three states (Fig. 1, A–F), with cellular activation and association with the cytoskeleton and ligand regulating this equilibrium temporally and spatially (Springer and Dustin, 2012). How is transition between the states achieved on a timescale rapid enough to regulate leukocyte adhesiveness in the vasculature (Shamri et al., 2005)? Here we describe internal ligand-bound and cocked conformations of \( \beta 2 \) integrins that may function to accelerate equilibration between the bent-closed and extended-open conformational states and reveal how allostery is relayed between \( \alpha I \) and \( \beta I \) domains. Furthermore, we discover unprecedented extensional and rotational flexibility in an allosteric machine and specialization among integrin \( \beta \) subunits in the intermediate states they can assume between closed and open.

**Results**

**Internal ligand-bound, bent \( \alpha_{\gamma 2} \) and its crystal lattice**

Previous \( \alpha_{\gamma 2} \) crystals at 3.5–3.95 Å resolution with metal-chelating salts as precipitants (Xie et al., 2010) lacked metals at the MIDAS and synergistic metal binding site in the \( \beta I \) domain (Fig. 2 A). We deleted seven N-linked glycosylation sites and added a disulfide between the membrane-proximal \( \alpha 5 \) calf-2 and \( \beta 2 \) \( \beta \)-tail domains to obtain greater conformational homogeneity of \( \alpha_{\gamma 2} \). Crystals in PEG 8000 with 0.2 M magnesium acetate and 0.1 M sodium cacodylate, pH 7.2, revealed metals at all three \( \beta I \) domain sites and diffracted at 2.75 or 2.9 Å resolution (Table 1 and Figs. S1 and S2).

The \( \alpha_{\gamma 2} \) ectodomain in the new crystal form was bent; surprisingly, the \( \alpha I \) domain was open (Fig. 2, B and E). This open conformation of the \( \alpha I \) domain was stabilized by the crystal lattice (Fig. 3). In opening, the \( \beta 6-\alpha 7 \) loop reshapes as it moves with the pistoning \( \alpha 7 \) helix (Lee et al., 1995). A lattice contact with the \( \alpha I \) domain \( \beta 6-\alpha 7 \) loop selects the open conformation of this loop; in the closed conformation, the \( \beta 6-\alpha 7 \) loop would extend upward in the orientation shown in Fig. 3 and severely clash with the lattice. Lattice contacts with the opposite side of the \( \alpha I \) domain hold the \( \alpha I \) domain in a specific position in the crystal lattice (Fig. 3). Meanwhile, other lattice contacts surround the headpiece, upper legs, and lower legs of the \( \alpha_{\gamma 2} \) ectodomain and stabilize its bent conformation (Fig. 3).

**The open \( \alpha I \) domain**

The \( \alpha I \) domain in bent, internal ligand-bound \( \alpha_{\gamma 2} \) has all canonical features of open \( \alpha I \) domains. Compared with closed...
αI domains, the Mg2+ ion at the αI MIDAS moves 2.5 Å, breaking and gaining direct coordinations to Asp-240 and Thr-207, respectively (Fig. 4, A and B). Density around the αI MIDAS Mg2+ is strong and includes an additional feature that is modeled as a Cl− ion (Fig. 4 A). The β1-α1 loop bearing the MIDAS-coordinating DXSX5 motif and upper part of the α1 helix shift and the backbone of the β4-α5 loop flips (Fig. 4, A and B). Furthermore, the β6 strand tilts relative to β6 in closed αI domains, and the β6-α7 loop moves toward the C terminus with the pisting α7 helix (Fig. 4 C).

In contrast, the conformation of the α7 helix of the open αI domain in an intact ectodomain differs markedly from that seen in isolated open αI domains (Fig. 4 E). In isolated αI domains, the α7 helix moves as a unit 10 Å in the C-terminal, axial direction between the closed and open conformations (Fig. 4 D). In internally liganded αβ2, the α7 helix retains few helical hydrogen bonds and its C-terminal portion is completely unwound (Fig. 4 C). Stretching is spring-like. The distance moved between the closed and internally liganded αβ2 structures progressively increases from 11 Å at residue 306 to 26 Å at Glu-318 (Fig. 4 C), and the α7 helix elongates to take an overall straight course between the αI domain and the binding interface that includes the βI MIDAS (Fig. 4 E). The open conformation of the αI domain and the binding of the internal ligand to the βI domain are associated with a 70° reorien-
tation of the αI domain relative to the closed-bent conformation (Fig. 2, A and B; and Fig. S2).

αI domain residues following the β6-α7 loop in the internally liganded αβ2 ectodomain have a similar local conformation as in the open isolated αM αI domain, but differ in position by a rigid body movement (Fig. 4, G and H). These segments are Asn-301 to Leu-308 in αM and Asp-299 to Leu-306 in αX. Side chains of αM Asn-301 and αX Asp-299 hydrogen bond to the backbone of the n + 2 residue to form an “N-cap” motif that caps the N-terminal end of the α7 helix (Fig. 4, G and H). Previous αM-isolated αI structures showed Phe-302 was buried in the closed conformation and surprisingly exposed (156 Å2 solvent-accessible surface area) in the open conformation (Fig. 4, D and H). In the open αI domain in intact αMαβ2, the equivalent Phe-300 packs against the side of the domain (Fig. 4, G and H) and has markedly less exposure (65 Å2). In the isolated open αM-αI domain, Leu-305 is partially exposed (15 Å2; Fig. 4 H); the relative rotation and tilt of the remnant α7 helix in intact αMαβ2 completely bury the equivalent αX residue, Leu-303 (0 Å2 exposure; Fig. 4 G). As a result, αX residue Leu-303 occupies the position of a nonequiv-
alent open αM residue, Ile-308 (Fig. 4, G and H).
The internal ligand

The remarkable elongation of the αI domain α7 helix is enforced by internal ligand binding. Residues in the C-terminal portion of the αI α7 helix lose all contact with the αI domain and completely reshape to form an internal ligand (Fig. 2, C–E; and Fig. S3, A and B). At the tip of a loop formed in the internal ligand, the invariant and mutationally important αI Glu-318 side chain (Huth et al., 2000; Alonso et al., 2002; Yang et al., 2004) coordinates the βI MIDAS Mg2+ ion and hydrogen bonds to the backbone of the βI β1-α1 loop (Figs. 2 C and 5 A). The strength of these polar interactions is increased by their burial by hydrophobic internal ligand residues αI Ile-314, Phe-315, and Ile-317 (Fig. 2 C). Furthermore, internal ligand residue Lys-313 helps bury Phe-315 and forms a salt bridge to β propeller residue Glu-331 (Fig. 5 D). Hydrogen bonds involving Thr-320 secure a type II β turn in the internal ligand with Glu-318 at its tip (Fig. 5 E). Another network of hydrogen bonds around Lys-313 and Glu-331 secures the conformation of the segment following the internal ligand, i.e., the C linker, and residues that form the binding pocket in the β propeller domain (Fig. 5 D). The position of the C linker completely reshapes during allostery relay (Fig. 2, D and E) and helps to bury the internal ligand (Fig. 2 C). The internal ligand has low B factors and excellent electron density (Fig. S3 A). The dehydrated structure has an identical internal ligand structure and hydrogen bonds, whereas the C linker differs in conformation at residues 322–324 (Figs. S3 B and S4).

The binding pocket for the internal ligand is formed by hydrophobic residues in the αI β propeller and β2 βI domains (Figs. 2 C and 5 A). The βI domain contributes MIDAS loop residue Tyr-115, synergistic metal ion binding site residue Leu-208, and Pro-168 and Pro-170 of the disulfide-bonded specificity-determining loop (Figs. 2 C and 5 A). The β propeller contributes hydrophobic residues Phe-328 and Met-332 (Figs. 2 C and 5 A).

The binding pocket for the internal ligand (Fig. 5 A) is not unlike that for external ligands in αI-less integrins (Fig. 5, B and C).

The native structure. (B) The dehydrated structure. The single αIβ2 molecule in the asymmetric unit is shown as a Ca trace, colored rainbow from beginning of α (blue) to end of β (red). Residues of surrounding αIβ2 molecules in the lattice within 4 Å are shown as transparent, solvent-accessible surfaces. The external and internal sides of surfaces are white and black, respectively.
Ile-317, Phe-328, and Met-332 are invariantly conserved in all nine αI integrin subunits (Fig. 5 F). Also conserved are Gly-319 and Thr-320, which stabilize the B turn at Glu-318, and Lys-313 and Glu-331, which interact and nucleate many hydrogen bonds at the C linker (Fig. 5 F). These findings suggest that the relay mechanism visualized here in αXβ2 will be general for all αI integrins.

A cocked βI domain

Binding to the internal ligand induces “cocking” of the βI domain (Fig. 6 A). The adjacent to MIDAS (ADMIDAS) metal...
occupy a single hydrophobic ratchet pocket in the cocked con-
formation. Val-124 is buried in the ratchet pocket in closed 
and partially exposed in cocked 
(B
I
) (Fig. 6 A). In contrast, Leu-
127 is substantially solvent exposed in the closed structure and becomes buried in the cocked structure in the ratchet pocket 
vacated by Val-124 (Fig. 6 A). Movement of Leu-127 into the 
ratchet pocket is also associated with nearby backbone move-
ments in the 
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loop that shorten 
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1 and lengthen 
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1
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Val-124, Leu-127, Leu-131, Leu-135, and I-138 are hy-
drophobic residues that anchor the 
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helices in their 
groove; they all move toward the 
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I MIDAS in the cocked con-
formation (Fig. 6 A). Of these, Val-124 and Leu-127 alternate-
ly
ion, 
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1 loop, 
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1 helix, and 
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helix of the 
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I domain all move toward the internal ligand Glu-318 side chain, enabling it to form multiple hydrogen bonds to the 
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1 loop backbone (Figs. 5 A and 6 A). Electron density for the 
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1 helix and 
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1
helix is excellent in both native and dehydrated crystal structures (Fig. S3, C and D).

Val-124, Leu-127, Leu-131, Leu-135, and I-138 are hydrophobic residues that anchor the 
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helices in their groove; they all move toward the 
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I MIDAS in the cocked conformation (Fig. 6 A). Of these, Val-124 and Leu-127 alternately

Figure 5. The internal ligand and its binding site. [A–C] Internal and external ligand binding sites, in identical orientations after superposition on 

propeller and 
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I domains. Structures for 

(B
I; Yu et al., 2012) and 

(Springer et al., 2008) use PDB ID numbers 3V4V and 2VDR, respectively. [D and E] Hydrogen bond networks around the 

and 

propeller proximal ends of the internal ligand and 
C
 linker [D] and in the 

turn in the internal ligand [E]. [F] Sequences of all human 
integrins around the internal ligand. Dots mark decadal residues in 


Figure 6. The cocked and uncocked 

B
2 

B
I domain conformations. Internally liganded, cocked 

(B
2; [C; Zhu et al., 2013]), and open 

(D; Xiao et al., 2004) 

domains [gold] are superimposed on closed counterparts [cyan]. Structurally similar regions are gray and the ligand Asp or Glu residue and hydrogen bonds [dashed] to the 

loop are shown. Mesh shows 2
S
2Fo–Fc density on the

internally liganded 

metal ions.
Table 2. Displacements from closed β1 domains

<table>
<thead>
<tr>
<th>Conformation</th>
<th>β1-α1 loop</th>
<th>α1 helix</th>
<th>α1′ helix</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cocked β2</td>
<td>1.5</td>
<td>2.4</td>
<td>2.9</td>
</tr>
<tr>
<td>Uncocked β2</td>
<td>1.2</td>
<td>0.9</td>
<td>0.6</td>
</tr>
<tr>
<td>Intermediate β1</td>
<td>1.3</td>
<td>0.7</td>
<td>0.2</td>
</tr>
<tr>
<td>Intermediate β3</td>
<td>1.9</td>
<td>1.5</td>
<td>1.5</td>
</tr>
<tr>
<td>Intermediate state 6 β3</td>
<td>1.6</td>
<td>0.8</td>
<td>0.4</td>
</tr>
<tr>
<td>Intermediate state 7 β3</td>
<td>1.6</td>
<td>3.5</td>
<td>1.3</td>
</tr>
<tr>
<td>Final state 8 β3</td>
<td>2.1</td>
<td>4.4</td>
<td>3.8</td>
</tr>
<tr>
<td>Open β3</td>
<td>2.1</td>
<td>4.3</td>
<td>4.0</td>
</tr>
</tbody>
</table>

β1, β2, and β3 β1 domains were superimposed on their closed counterparts. Differences in position were determined for β2 residues Y115-M117 (β1-α1 loop), D120-V124 (α1 helix), and G128-I138 (α1′ helix) and their equivalents in other β1 subunits. Structures use the following PDB ID numbers: intermediate β1 (2VJ3; intermediate β2 (1L5G) and its closed counterpart (1JY2); β1 intermediate state 6 (3ZE2; chain B), state 7 (3ZDZ; chain B), final state 8 (3ZE2; chain D), and open β3 (2VDR) and their closed counterpart (3T3P).

2–6 of β3 integrins obtained after soaking Arg-Gly-Asp mimetics into crystals (Fig. 6 C and Table 2; Xiong et al., 2002; Nagae et al., 2012; Zhu et al., 2013). Furthermore, homologous α1 and α1′ helix hydrophobic residues are buried in closed conformations of β1 and β3 integrins and remain so in intermediate and open conformations (Fig. 6, C and D). As the α1 and α1′ helices join in a single helix in the open state of the β3 subunit, β3 residue Leu-134 moves laterally to occupy a ratchet pocket that is vacated by gatekeeper residue Val-340 in the β6-α7 loop (Fig. 6 D). The semiexposure of homologous β2 residue Leu-127 in the closed state enables it to vault over gatekeeper residue Val-330, achieving substantial α1 and α1′ helix movement without β6-α7 loop movement in cocked αβ2 (Fig. 6 A).

An uncocked β1 domain

Remarkably, dehydration of the αβ2 crystal reversed cocking. A lattice contact with the β1 domain α7 helix inhibits its pistoning and with other lattice contacts prevents swing-out of the hybrid domain (Fig. 3). As the solvent content of crystals decreased from 69 to 64% during dehydration, the lattice contact with the α7 helix expanded to include the adjacent α1′ helix (Fig. 3 B). The dehydrated crystal lattice is incompatible with the position of the cocked α1′ helix in the native crystal. Therefore, it appears that the growth of lattice contacts causes the α1′ helix and in turn the α1 helix to move back toward their positions in the closed conformation and assume a position we term uncocked (Fig. 6 B). Notably, in their cocked positions the β1 domain α1 and α1′ helices have no significant lattice contacts in the native crystal (Fig. 3 A); thus it appears that cocking in the native crystal is a closer approximation of changes that occur in the β1 domain in response to internal ligand binding.

These results demonstrate that cocking is not necessary for internal ligand binding. The conformation of the internal ligand is essentially identical in the two crystal forms (Fig. S4), and neither the internal ligand nor the wound or unwound portions of the α1 domain α7 helix have lattice contacts in the native crystal (Fig. 3 A). Therefore, the energetics of internal ligand binding are sufficient to drive α7 helix unwinding in the absence of cocking. Compared with its position in closed β2, the β1 domain β1-α1 loop moves significantly toward α3 Glu-318 in both the native and dehydrated structures, and the ADMIDAS metal ion moves even more in the dehydrated than native structure (Fig. 6, A and B). Thus, β1 domain β1-α1 loop movement and ADMIDAS movement are closely associated with internal ligand binding. Cocking appears to be a normal consequence of, but is not required for, internal ligand binding.

Mutational evidence for metastability

We tested with mutations the functional relevance of the internal ligand and the hypothesis that the internally liganded state and the cocked state are metastable intermediates in the process of integrin activation on cell surfaces. CR4 function was measured using rosetting with iC3b-sensitized erythrocytes (Fig. 7 A), which has previously been shown to require the open headpiece conformation of αβ2 with hybrid domain swing-out (Chen et al., 2010). We also measured the effect of mutations on activation epitope exposure. Binding of KIM127 antibody to a β2 leg epitope has been shown to require extension (Nishida et al., 2006), and binding of m24 and MEM148 antibodies to β1 and hybrid domain epitopes has been shown to measure headpiece opening (epitopes are marked in Fig. 1 F; Chen et al., 2010).

Internal ligand substitutions αK K313I, F315E, and I317H, predicted to destabilize internal ligand binding and hence allosteric relay, all abolished Mn2+-dependent rosetting (Fig. 7, A and B). Mutation of Glu-318 to Ala, Lys, or Met abolished rosetting and mutation to Asp greatly diminished rosetting (Fig. 7 B). Internal ligand substitutions were also inhibitory using αβ2 activated by replacement of the human β2 subunit with chicken β2 (Fig. 7 C; Bilsland et al., 1994). These results establish the physiological importance of internal ligand residues to iC3b rosetting. The same mutations abolished, or diminished in the case of G319P, Mn2+-dependent exposure of the KIM127 epitope (Fig. 7 D), demonstrating that on cell surfaces binding of the internal ligand to its pocket is required for and associated with extension of αβ2. Mutation of the same residues also diminished, but to a lesser extent, Mn2+-dependent exposure of m24 and MEM148 epitopes (Fig. 7, E and F). Thus, allosteric relay is also associated with headpiece opening on cell surfaces.

If an open α1 domain associated with a bent ectodomain is metastable, then substitutions designed to stabilize the internal ligand-bound, open α1 conformation should stabilize extension and headpiece opening rather than the bent, closed headpiece conformation crystallized here. αK α1 domain residues F300, I306, and I314 are more buried in the β6-α7 loop and α7 helix in bent, closed αβ2 than in bent, cocked αβ2. Mutations of these residues induced iC3b rosetting (Fig. 7, A and B), αβ2 integrin extension (Fig. 7 D), and headpiece opening in Mg2+/Ca2+ (Fig. 7, E and F). These findings suggest that the open α1 domain on cell surfaces is normally associated with the extended, open headpiece rather than the bent, closed conformation of αβ2. In summary, these mutations were designed to stabilize internally liganded, bent αβ2 with a closed headpiece.
and an open αI domain, yet stabilized extension and an open headpiece, demonstrating metastability.

The metastability of the cocked state of the βI domain was independently examined by mutating βI domain ratchet residues implicated in cocking. In cocking, Leu-127 displaces Val-124 from the ratchet pocket and Val-124 becomes partially solvent exposed; therefore, mutation of Val-124 to Ala should stabilize the cocked conformation. However, V124A mutant αβ2 did not remain bent because in Mg\(^{2+}/Ca^{2+}\) the KIM127 epitope in the β2 leg became exposed, demonstrating extension (Fig. 7 D). Furthermore, V124A mutation induced m24 and MEM148 epitope exposure (Fig. 7, E and F). MEM148 binds far from the α1 and α1' helices, to the inner face of the βI-hybrid interface (Fig. 1 F), and demonstrates that the V124A mutation induces hybrid domain swing-out. Moreover, V124A mutation strongly activated ligand binding by αβ2 in Mg\(^{2+}/Ca^{2+}\) (Fig. 7 B). These findings demonstrate that the cocked conformation of the βI domain is metastable; stabilizing cocking induces on cell surfaces the extended-open headpiece conformation rather than the bent, closed headpiece conformation seen here in crystals. The other ratchet residue, Leu-127, is buried in the pocket in the cocked conformation and partially exposed in the closed conformation; therefore, the L127A mutation should inhibit cocking. L127A mutation inhibited Mn\(^{2+}\)-stimulated CR4 function and KIM127, m24, and MEM148 exposure (Fig. 7). These results are consistent with the cocked conformation of the βI domain resembling, and being on pathway to, the active, high affinity state of αβ2. Thus, although lattice contacts with the αI domain stabilize its open conformation, other lattice contacts with the body of the ectodomain stabilize the bent conformation with a swung-in hybrid domain.

**Discussion**

We have described a novel bent, internally liganded conformation of integrin αβ2 with an open αI domain. Internal ligand binding induced a cocked conformation of the βI domain; however, internal ligand binding did not require cocking. The protein construct differed from one that previously yielded crystals of a bent αβ2 with a closed αI domain (Xie et al., 2010). However, the removal of seven N-linked glycosylation sites and introduction of a C-terminal disulfide are unrelated to internal ligand binding and cocking; instead, the open conformation of the αI domain is stabilized by a crystal lattice contact with the αI domain β6-α7 loop.

The internal ligand-bound, cocked and internal ligand-bound, uncocked states seen here appear to be at local energy minima between the bent-closed and the extended-open conformations. However, they are metastable in the sense that they may be higher energy than either the bent-closed or extended-open conformations and thus not at a global energy minimum. A precedent for stabilization by a crystal lattice of two alternative conformational states comes from integrin αMβ2, i.e., CR3. αMβ2 and αβ2 are sister integrins with unusually high sequence identity among α subunits (60%; Yu et al., 2012). The isolated αM αI domain was found to be in the closed conformation in
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A7 helix conformation, is canonical for the open conformation (Shimaoka et al., 2003b). Nonetheless, the amount of A7 helix extension seen here was surprising. It demonstrates that the interaction between the internal ligand and its binding pocket is quite strong and provides enough energy to break nine A-helical hydrogen bonds in the A7 helix. Notably, there are no lattice contacts with the A7 helix itself (Fig. 3), showing that its highly elongated conformation is only a function of connecting to the A1 domain B6-A7 loop at one end and the internal ligand at the other end. Indeed, the largely unwound A7 helix takes a more or less direct, overall straight path between these points. In the dehydrated crystal form, the orientation between the A1 domain and the body of the ectodomain differs, and the unwound A7 helix adjusts to again take a direct path between these points (Fig. 4 F and Fig. S2).

Flexibility between our two internally liganded Axb2 structures is quantitated in Fig. 4 (I and J). Flexibility in the N linker largely occurs between Cys-126, which is disulfide bonded to the propeller domain, and Glu-130, which is integrated into the A1 domain B6-a7 loop at one end and the internal ligand at the other end. Indeed, the largely unwound A7 helix takes a more or less direct, overall straight path between these points. In the dehydrated crystal form, the orientation between the A1 domain and the body of the ectodomain differs, and the unwound A7 helix adjusts to again take a direct path between these points (Fig. 4 F and Fig. S2).

Although it is often convenient to think of conformational pathways as flowing in one direction, in general they are reversible. We may think of the crystal lattice as inducing the open A1 domain state seen here. However, it is equally appropriate to think of the Axb2 ectodomain as having multiple conformational states in solution. We may therefore think of the bent, internally liganded, cocked conformation as one of the biological conformations of Axb2, which enabled crystallization in the particular lattice seen here.

The highly elongated A1 domain A7 helix seen here enables much greater flexibility of the A1 domain when bound to external ligand than had previously been imagined. Previous studies had shown that B6-a7 loop conformation, but not A7 helix conformation, is canonical for the open conformation (Shimaoka et al., 2003b). Nonetheless, the amount of A7 helix extension seen here was surprising. It demonstrates that the interaction between the internal ligand and its binding pocket is quite strong and provides enough energy to break nine a-helical hydrogen bonds in the a7 helix. Notably, there are no lattice contacts with the a7 helix itself (Fig. 3), showing that its highly elongated conformation is only a function of connecting to the A1 domain B6-a7 loop at one end and the internal ligand at the other end. Indeed, the largely unwound A7 helix takes a more or less direct, overall straight path between these points. In the dehydrated crystal form, the orientation between the A1 domain and the body of the ectodomain differs, and the unwound A7 helix adjusts to again take a direct path between these points (Fig. 4 F and Fig. S2).

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Flexibility between our two internally liganded Axb2 structures is quantitated in Fig. 4 (I and J). Flexibility in the N linker largely occurs between Cys-126, which is disulfide bonded to the propeller domain, and Glu-130, which is integrated into the A1 domain B6-a7 loop at one end and the internal ligand at the other end. Indeed, the largely unwound A7 helix takes a more or less direct, overall straight path between these points. In the dehydrated crystal form, the orientation between the A1 domain and the body of the ectodomain differs, and the unwound A7 helix adjusts to again take a direct path between these points (Fig. 4 F and Fig. S2).

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when α3β2 engages physiological ligands on cell surfaces. Adhesion and binding to iC3b are strongly associated with extension and require the open headpiece conformation of α3β2 (Chen et al., 2010, 2012). Our internally liganded, bent structures already reveal the open αI domain and how the internal ligand binds at the β propeller–βI interface. All that remains to make a model of extended-open α3β2 using bent, internally liganded α3β2 is to swing out the hybrid domain, using the open headpiece of αmβ3 as the model, and to extend the α and β subunits at their knees (Fig. 8). Integrins predominantly bind to the cytoskeleton through their β subunit cytoplasmic domains (Gahmberg et al., 2009). Elongational force exerted by the cytoskeleton and resisted by an ionized particle bound to the α3β2 αI domain will tend to straighten all the intervening domain–domain orientations. The resulting orientation between the αI and βI domains, and the alignment of their axes, is very similar to that crystallized here (and distinct from that in bent-closed α3β2 [Fig. 2 A]); a line drawn through the αI and βI MIDAS Mg2+ ions also passes through the βI–hybrid interface and continues in a similar direction as the hybrid domain would assume when swung out (Figs. 1 F, 2 B, and 8 C). Furthermore, the high affinity state of the αI domain is more extended between the MIDAS and Glu-318 than the closed state, and the open headpiece is more extended between the βI MIDAS and the end of the hybrid domain than the closed state (Astrof et al., 2006). Force times the difference in distance between conformational states gives a difference in energy. Therefore, cytoskeletal force applied across the integrin–ligand complex will stabilize the open, high affinity state over the closed, low affinity state, and help to offset the tendency of force to rupture adhesive bonds.

Despite these constraints that favor parallel, aligned αI and βI domain axes, there is likely to be considerable latitude for variation in αI MIDAS–βI MIDAS distance and for rotation of the αI domain about the αI MIDAS–βI MIDAS axis. The unwound α7 helix and N linker each have highly extended conformations in the bent, internally liganded structures (Figs. 2 E, 3, and 4 [E and F]). A more compact conformation of the N linker, and less unwinding of the α7 helix, should enable shortening by ~10 Å of the αI MIDAS–βI MIDAS distance. Lengthening also seems possible with untwisting (see next paragraph). Thus, substantially greater variation in αI MIDAS–βI MIDAS distance may occur than the increase from 63 Å in the native crystal to 65 Å in the dehydrated crystal seen here.

Looking down on the αI MIDAS, both connections of the αI domain to the ectodomain body are twisted ~180° in the counterclockwise direction in bent, internally liganded α3β2 (Figs. 2 B and 3), whereas there is essentially no twisting in closed α3β2 (Fig. 2 A). Because the N linker and unwound α7 helix are each extended, further counterclockwise twisting, as well as clockwise untwisting, of internally liganded α3β2 is easily attainable without creating clashes between the αI domain and the remainder of the integrin headpiece.

Complexes of integrin α3β2, ectodomain with the C3c moiety of its ligand iC3b (Chen et al., 2012) are suggestive of such αI domain rotation (Fig. 8, D and E). Negative stain electron microscopy shows two classes of C3c α3β2 complexes with indistinguishable orientations of α3β2 but orientations of C3c that are flipped 180° (Fig. 8, D and E). The orientation of the integrin αI domain is not discernable. However, the orientation of the remainder of α3β2 and three bound Fab fragments is quite clear (Fig. 8, D and E, schematics). Similarly, the orientation of C3c, with its distinctive head-like C345C knob, is quite clear. The lack of significant interaction between the body of the open αI domain and the headpiece in the internal ligand-bound α3β2 crystal structures now allow us to interpret these images. It is unlikely that such a large rotation could occur at the ligand–receptor interface between C3c and the αI domain. In contrast, our structures suggest that rotation can readily occur between the αI domain and the headpiece, and the images in Fig. 8 (D and E) suggest that a rotation at least of 180° is readily obtainable in α3β2.

The ability of the αI domain to bind ligand at a range of distances and rotations relative to the reminder of the integrin endows αI integrins with great versatility. Although αI-less integrins are present in all metazoans, from sponges to vertebrates, αI integrins are known only in chordates (Whittaker and Hynes, 2002). αI subunits expanded in vertebrates to comprise half of all integrin α subunits. The flexibility of αI domains, both in the closed state as shown previously (Xie et al., 2010) and in the open state shown here, may give αI integrins an advantage over αI-less integrins for recognition of certain types of ligands. These may include ligands in less accessible environments, including collagens in bundles (integrins αIβ1, αIβ3, αIβ6, and αIβ7), adhesion molecules on cell surfaces (integrins αIβ2 and αIβ1), and foci of complement activation on pathogen surfaces (integrins αIβ2 and αIβ3).

In cocking, the βI domain αI’ helix moves more than in any previously described intermediate state of βI or βI integrins (Fig. 6 and Table 2; Xiong et al., 2002; Nagae et al., 2012; Zhu et al., 2013). We therefore examined βI for specializations that could support the cocked conformation. The αI and αI’ helices lie in a groove formed by the α7 helix on one side and the α2 helix on the other (Fig. 9 A). Any features that facilitate helix sliding in this groove toward the MIDAS would promote formation of the cocked state. One such feature is a wider groove in βI that is lined with smaller side chains (Fig. 9 A).

In the most striking specialization of βI for cocking, residue Leu-127 in the βI domain αI’ helix is precocked in the closed conformation. Residues corresponding to βI Val-124, Leu-127, Leu-131, Leu-135, and Ile-138 are conserved as hydrophobic in all eight vertebrate integrin β subunits (Fig. 9). The corresponding residues in βI and βI are completely buried in the αI/αI’ groove (Fig. 9, B and D). However, ratchet residue Leu-127 in βI is exceptional; as described in Results, it is not buried (Fig. 9 C). Additionally, although αI’ helix residue Leu-131 is buried in βI as in other integrin β subunits, it adopts a different rotamer that places its center 3 Å closer to the β1-αI loop (Fig. 9). The Leu-135 residue in βI also is more aligned with and forward in the groove than the equivalent Met residue in βI and βI.

The residue equivalent to βI Val-330 in the β6-α7 loop points toward the αI’ helix and may be considered a gatekeeper for βI domain shape shifting (Fig. 9 A). The partially exposed, relatively high position of Leu-127 in the groove in the precocked state of βI enables it during cocking to pass Val-330 by vaulting over the gate.
The order of secondary structure movements thus differs in intermediate states of βI and β3 integrins. In β2, large movements of the α1 and α1′ helices occur with no movement of gatekeeper residue Val-330 in the cocked conformation. In β3, movement of the gatekeeper Val-340 residue precedes α1′ helix sliding (Zhu et al., 2013).

Do other integrin β subunits have a precocked configuration of βI domain α1 and α1′ helix hydrophobic residues, suggesting they might also undergo cocking? The β1 and β2 subunits are particularly interesting because they can associate with both α1 and α1′-less integrin α subunits (β2 is unique in only associating with α1 subunits). In β1, the first four hydrophobic α1 and α1′ helix residues are chemically identical to those in β3, and their side chains are in essentially identical positions. In contrast, in β7, Leu-155 has a solvent-exposed rotamer identical to that of key β2 ratchet residue Leu-127 (Fig. 9 E). β7, like β2 also has Leu in the fourth hydrophobic position, whereas β1, β3, and β6 all have a Met residue at this position (Fig. 9 F). These comparisons suggest that a cocked conformation might also be found for the leukocyte integrins αβ2 and αβ7.

Cocking thus has been found for β2 integrins and is predicted for β3 integrins, which are unique among integrins in only being expressed on leukocytes. β2 has higher sequence identity to β2 than to any other integrin β subunit: 46% in the ectodomain and 67% in the βI domain, whereas the values range from 31 to 42% and 43 to 54%, respectively, for the other six integrin β subunits (Fig. S5). This level of sequence identity is consistent with similar regulatory specializations in β2 and β3 integrins.

Previously, the process of α1 integrin activation has been presumed to require transitions between five states, a bent conformation with separated lower legs (Fig. 1 B), an extended conformation with a closed βI domain (Fig. 1 C), an extended conformation with an open βI domain (Fig. 1 D), and an extended conformation with open βI and α domains (Fig. 1 F). With the requirement for four transition states, each with an energy barrier that must be traversed before the next state is reached, activation could be slow. The metastable internal ligand-bound and cocked states defined here have several important implications for speeding conformational transitions in β2 integrins and possibly in β3 integrins. They directly connect the bent conformation to the extended conformation with open βI and α domains (Fig. 1 E). Hybrid domain swing-out is unimpeded in the bent conformation (Takagi et al., 2002) and would bring with it the lower β leg, thus inducing lower leg separation and extension as well as headpiece opening in one fell swoop (Fig. 1, E and F). Therefore, all key conformational transitions could be accomplished with a single intermediate, internal ligand-bound state. The internal ligand-bound state of the βI domain found here lowers the energy barrier of the transition state for conformational change by enabling the βI domain to activate the αI domain before βI opening and integrin extension. αI domain opening may thus occur before βI domain opening (Fig. 1 E), instead of in the reverse order (Fig. 1 D). The cocked conformation of the βI domain must lower the energy of the internal ligand-bound state even further because it is found in the absence of countervailing lattice interactions. In terms of α1 and α1′ helix movement, the cocked conformation is more than halfway to the open conformation (Table 2). The internal ligand-bound, cocked, bent conformation is thus clearly on the pathway to the extended conformation with open αI and βI domains (Fig. 1, E and F).

The importance of the bent, internal ligand-bound metastable state in integrin extension and headpiece opening postulated here is supported by previous work on LFA-1 (integrin αβ2; Salas et al., 2004). This work showed that αI Glu-310 (equivalent to α5 Glu-318) is important for inducing LFA-1 extension, consistent with passage of β3 integrins through an intermediate similar to that shown in Fig. 1 E on the pathway to extension. Furthermore, the experimental requirement of αI Glu-310 for leukocyte rolling and Mn2+-induced β2 integrin extension (Salas et al., 2004) is inconsistent with the pathway for extension shown in Fig. 1 (A–C). Like αIβ2, αIβ3 extension on cell surfaces is facilitated by the internal ligand, as shown here with inhibition by four different Glu-318 mutations of Mn2+-dependent KIM127...
cocked structure enables rapid equilibration between bent and extended states in other allosteric machines. The metastable, internally liganded, cocked structure opens and that “the life of the Glu of the C-terminal linker region” and “interaction of the C-terminal linker region with the Glu of the C-terminal linker is an important topic for further research.

Yet the internally liganded and cocked states yield further unexpected discoveries. The surprising lift-off of the αβ domain over the β propeller and βI domain platform enables a range of extensional and rotational motions that are without precedent in other allosteric machines. The metastable, internally liganded, cocked structure enables rapid equilibration between bent and extended-open integrin structures and an explicit mechanism for linking integrin extension and headpiece opening. Finally, the difference in order of secondary structure movements in opening of β2 and β3, βI domains and the cocked conformational changes of β2 demonstrate that integrin β subunits can be specialized not only to modulate ligand recognition but also to assume different intermediate states between closed and open, with important consequences for signal transmission in integrin ectodomains.
or horizontally on the page. Figures were prepared with PyMol. Detailed comparisons of the closed β2/β domain use a structure of the α2β2 head-piece, which has metal ions at all three sites in the β domain, has better density for the β domain, and is at higher resolution (2.5 Å) than the α2β2 ectodomain (Xie et al., 2010).

Mutations
RosettaFixed backbone design (Kuhlman et al., 2003) was used to predict mutations that differentially destabilized the previous (Xie et al., 2010; PDB Rosetta-fixed backbone design (Kuhlman et al., 2003) was used to predict Mutations


Online supplemental material
Fig. S1 shows purification, PAGE, and crystals of αβ2. Fig. S2 shows differences in interdomain angles among different αβ2 structures. Fig. S3 shows simulated annealing composite omit map density for the internal ligand and the β domain α1α1 helices. Fig. S4 compares the internal ligand and C linker in native and dehydrated structures. Fig. S5 shows sequence relationships among integrin β subunits. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.201308083/DC1.

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References


**Figure S1.** Purification and crystallization of α₂β₂ ectodomain. (A) S200-Superdex gel filtration of purified α₂β₂ with reducing SDS 7.5% PAGE of fractions superimposed. (B) Native PAGE of purified α₂β₂. (C) Rod-like α₂β₂ crystals.

**Figure S2.** Comparisons of domain-domain orientations in α₂β₂ in different lattices. (A) Differences in interdomain angles between the cocked α₂β₂ 2.75 Å structure and closed α₂β₂ (10 molecules; Xie et al., 2010). The mean and SD (or difference from the mean for the two αI/β propeller comparisons) are shown above the plots. (B) Differences between the current 2.75 Å and dehydrated 2.9 Å structures.
Figure S3. **Density for the internal ligand and the α1 helix of the β1 domain.** Stereoviews show simulated-annealing composite omit 2Fo–Fc density in mesh contoured at 1σ around models in stick. (A and B) Internal ligand residues L310 to T320. (C and D) β1 domain α1 and α1' helix residues D120 to D130. Structures are of cocked (A and C) and uncocked (B and D) αβ2.

Figure S4. **Internal ligand and C linker comparison in native (gold) and dehydrated (cyan) structures.** Superposition is on the β propeller and β1 domains. The cocked and dehydrated structures differ in C linker region 322–324 while having essentially identical internal ligand orientations.
Figure S5. **Sequence relationships among the eight human integrin β subunits.** Sequence identities (identities/length of the alignment) are shown for mature β subunit ectodomains (A) and βI domains (B). The results for both the ectodomains and the βI domains show that the β2 and β7 subunits form a distinct subfamily because they are on the same family branch in 99 or 97% of replicate trees in A and B, respectively. The divergence distances (the number of base substitutions per site) were computed using the maximum composite likelihood method using MegAlign (Lasergene Core Suite 10.1.2; DNASTAR). Phylogenetic trees are shown to scale, with branch lengths in the same units as those of the divergence distances used to infer the phylogenetic tree. Bootstrap values were calculated with 10,000 replicates and 1,000 seeds. Bootstrapping compares the trees generated from similar alignment and counts the number of times a specific branching pattern occurs, shown in percentage on the branches. When no value is available, NA appears on the branch node instead of a numerical value. This occurrence is related to the fact that MegAlign always generates a rooted phylogenetic tree; however, the Clustal algorithms produce unrooted trees. In order for MegAlign to display the Clustal tree, it must first root the tree, which involves introducing a new node. When the tree is then rerooted, if the new node lands on a branch within the rooted tree, it does not have a bootstrapping value because it was not included in the analysis.

Reference