Here, we report cryo–electron microscopy structures of native human $\alpha_5\beta_1$ opening by fibronectin ligand

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Integrin $\alpha_5\beta_1$ is a major fibronectin receptor critical for cell migration. Upon complex formation, fibronectin and $\alpha_5\beta_1$ undergo conformational changes. While this is key for cell-tissue connections, its mechanism is unknown. Here, we report cryo–electron microscopy structures of native human $\alpha_5\beta_1$ with fibronectin to 3.1-angstrom resolution, and in its resting state to 4.6-angstrom resolution. The $\alpha_5\beta_1$-fibronectin complex revealed simultaneous interactions at the arginine-glycine-aspartate loop, the synergy site, and a newly identified binding site proximal to adjacent to metal ion–dependent adhesion site, inducing the translocation of helix $\alpha 1$ to secure integrin opening. Resting $\alpha_5\beta_1$ adopts an incompletely bent conformation, challenging the model of integrin sharp bending inhibiting ligand binding. Our biochemical and structural analyses showed that affinity of $\alpha_5\beta_1$ for fibronectin is increased with manganese ions ($\text{Mn}^{2+}$) while adopting the half-bent conformation, indicating that ligand-binding affinity does not depend on conformation, and $\alpha_5\beta_1$ opening is induced by ligand-binding.

INTRODUCTION

Integrin $\alpha_5\beta_1$ is the primary fibronectin (FN) receptor (1), responsible for cell migration and adhesion. Integrin $\alpha_5\beta_1$-FN interactions are of particular interest as both proteins are ubiquitously expressed in various cell and tissue types to maintain the communication between cells and the extracellular matrix (ECM). Furthermore, $\alpha_5\beta_1$ represents an integrin prototype that is fundamental to cellular processes, yet the conformational change through its molecular interaction with FN, a major component of the ECM, is not well understood.

Serving as a bidirectional link between the cell and its environment, integrin $\alpha_5\beta_1$ functions as a control hub for the intracellular focal adhesion machinery. Focal adhesion consists of hundreds of signaling and mechanosensing factors, transmitting extracellular signals to downstream players, including the actin cytoskeleton (2–4). The resulting remodeling and changes in the dynamic behavior of the actin cytoskeleton direct cell shape formation and cell motility, hence, dictating cell fate (2). Integrin $\alpha_5\beta_1$ connects to the FN ligand, a major component of the ECM, through its extracellular domains and to the actin cytoskeleton through its cytoplasmic tail domains in combination with accessory factors (5). Failure of integrin function and corresponding impairment of the focal adhesion machinery is related to numerous diseases (6), including aggressive cancer formation due to uncoordinated migration. Integrin $\alpha_5\beta_1$ particularly contributes to cancer metastasis, reflected by its up-regulation in invasive cancer cells (7, 8).

FN, the binding partner of integrin $\alpha_5\beta_1$, is a fibrous dimer acting as a core component of the meshwork formation of the ECM. FN is a large protein that comprises repetitive modules of type I, II, and III domains, which show a “beads on a string” morphology (9, 10). FN is recognized by the large N-terminal extracellular domain of integrin $\alpha_5\beta_1$, termed ectodomain, via the common integrin ligand motif “arginine-glycine-aspartate” (RGD) found in the FN fragment III containing domain 10 (FN10). During cell movement, as bound integrin is exposed to mechanical force, it grips FN through its so-called synergy site with the sequence DRVPHPSRN in FN9 (11, 12) and anchors cells with an unknown mechanism. Thus, how FN engages integrin $\alpha_5\beta_1$ and activates cells remains enigmatic.

Despite being a major class of integrin, $\alpha_5\beta_1$ has been proposed not to follow the canonical integrin open–close paradigm, which has been derived mostly from studies on $\alpha_{1b}\beta_1$ and $\alpha_\beta_1$ integrins (13–18). In this classical $\beta_1$-based model, integrins exhibit an acutely bent conformation on resting cells in which the heads of the ectodomains point toward the plasma membrane and the legs and transmembrane domains of $\alpha$ and $\beta$ subunits are in close contact with each other (5, 13, 17–20). In this “unprimed” state, integrins show low affinity for their ligands with their heads occluding access to the ligand-binding site by facing toward the plasma membrane (5, 15). In the presence of $\text{Mn}^{2+}$, which mimics intracellular signals in vitro, classical integrins become “primed” and show an increased affinity for their ligands (5, 17, 21). The upright conformation releases the ligand-binding integrin heads from the steric hindrance by the membrane and therefore renders them more potent to capture ligands. The binding of the RGD peptide to the groove at the tip of the integrin heads (14, 16, 22) translates conformational changes from the metal ion–dependent adhesion site (MIDAS), leading to the allosteric swing-out motion of the $\beta$ hybrid domain (15) and further to the opening of the legs. However, this mode of action does not apply to integrin $\alpha_5\beta_1$. Truncated integrin $\alpha_5\beta_1$ ectodomains do not undergo the large conformational change in the presence of the RGD peptide alone (21). Moreover, negative-stain electron microscopy (EM) observations hinted that the $\alpha_5\beta_1$ ectodomain may not bend completely in its resting state (23), which challenges the view that bending occludes the ligand-binding site. These observations raised the question whether integrin $\alpha_5\beta_1$ uses a unique mechanism with only weak coupling between its global conformational change and its ligand-binding affinity. Considering...
Here, we report the cryo-EM structures of native human integrin α5β1 purified from human placenta (i) in its open conformation in complex with FN and the stabilizing antibody TS2/16 and (ii) in its resting state employing an incomplete bent conformation. The complex of integrin α5β1 with FN7-10 shows three simultaneous interactions critical for the stabilization of integrin opening, namely, (i) at the synergy site of FN9, (ii) at the RGD loop of FN10, and (iii) at the adjacent to MIDAS (ADMIDAS) of the integrin β1 domain. The dual binding of FN9 and FN10 oriented FN10 to stabilize the large opening of α5β1. In particular, key interactions centered at R1445 and Y1446 of FN and D137 of ADMIDAS in β1 β1 domain provide an additional anchoring point that triggers the opening of α5β1, which was previously unknown. Through this interaction, D137 of the β1 β1 domain moves toward FN, resulting in a translocation of helix α1, which then allosterically induces the known conformational changes (5, 13–15, 17, 21) for integrin opening. In the resting state, integrin α5β1 uses a unique, incompletely bent conformation. The incomplete bending does not sterically hinder the access of FN to the integrin head. Side-by-side structural and biochemical comparison indicated that integrin α5β1 changes its affinity for FN while still in its half-bent conformation. The integrin opening is observed when FN is bound rather than FN binding occurring as a consequence of integrin opening. Our study, thus, reveals a mechanism of integrin opening by FN in which FN structural domain acts as a scaffold to facilitate and the opening of integrin.

**RESULTS**

**Ligand-bound native integrin α5β1 displays an extended conformation**

To gain mechanistic insights into the regulation of integrin α5β1 on a molecular level, full-length native integrin α5β1 (Fig. 1 and fig. S1A) was purified from human placenta (24) and embedded into membrane-scaffolding nanodiscs (NDs) (fig. S1, B and C) (25). To understand integrin opening and ligand engagement, we determined the structure of integrin α5β1 in complex with its ligand FN7-10 in the presence of MnCl2 (Fig. 1, A and C, and figs. S2 and S3) and the stabilizing antibody fragment TS2/16 Fv-clasp (26) by cryo-EM at an overall resolution of 3.5 Å, with the head resolved up to 3.1 Å (figs. S2 and S3). Although the C-terminal portion including the flexible lower legs, the transmembrane helices, and the cytoplasmic tail had a lower resolution, we obtained a clear view of the integrin headpiece, allowing us to understand the key events leading to the opening of integrin.

**Fig. 1. Cryo-EM analysis of FN-bound integrin α5β1, reveals an open conformation.** (A) Two views of the FN7-10 (orange)–bound α5β1 subunit (green, α subunit; blue, β subunit) cryo-EM structure, stabilized by TS2/16 (pink). The headpiece reveals an open conformation. (B) Schematic of integrin α5β1 domain architecture and nomenclature of different parts (headpiece, head, upper, and lower legs), including FN7-10 and TS2/16. TM, transmembrane; PM, plasma membrane; CT, cytoplasmic tail. (C) Molecular model based on the cryo-EM density, showing the same two views as (A). (D) Close-up and overlay of cryo-EM density with the modeled synergistic metal ion–binding site (SyMBS or LIMBS), MIDAS, and ADMIDAS within the β1 domain.
FN9-10 locks the integrin \(\alpha_5\beta_1\) opening by a gear-like mechanism

The \(\alpha_5\beta_1\)–FN7-10 complex revealed the involvement of the RGD motif (residues 1493 to 1495) in FN10 and the synergy site (residues 1373 to 1380, DRVPHSRN) in FN9, both facing toward the integrin heads simultaneously (Fig. 2, A to D). The RGD loop was previously shown to be flexible in an isolated FN10 domain by nuclear magnetic resonance (28). In our structure, however, in complex with the headpiece of \(\alpha_5\beta_1\), the loop was fixed in the pocket between \(\alpha\) and \(\beta\) integrin heads (Fig. 2, A to C), resembling structures of single RGD peptides bound to the integrin headpiece (fig. S4) (29). In addition, we observed that the succeeding PI497 in FN10 made a prominent van der Waals contact with the backbone of helix \(\alpha_1\), adjacent to S134, and packed against Y133 in the \(\beta_1\) \(\beta\) domain. S134 was also part of the MIDAS metal coordination sphere (Fig. 2B). Engagement of MIDAS was reported to induce the translocation of the \(\alpha_1\) and \(\alpha_2\) helices in the \(\beta\) domain, triggering a piston-like movement, which then leads to the swing-out motion of the hybrid domain and the separation of the \(\alpha\) and \(\beta\) legs (5, 13–15, 17, 21).

However, to date, none of the tested RGD motif–containing peptides alone were sufficient to induce the large opening of integrin \(\alpha_5\beta_1\) (29, 30). That indicates that our structure contains key to optimally induce and stabilize the opening of integrin. A closer look at the interaction surfaces between FN and integrin allowed us to identify these interactions (Fig. 2C). Adjacent to the RGD loop, we found that R1445 in FN10 formed a salt bridge with E320 in integrin \(\beta_1\) and that Y1446 in FN10 was in hydrogen bonding distance to D137 in integrin \(\beta_1\). D137 is also part of AMDIMAS, which coordinates the metal ion. The hydroxyl oxygen of Y1446 was located close to Mn\(^{2+}\) (at a distance of 3.8 Å). The close approach of R1445 and Y1446 toward the AMDIMAS coordinating residues (juxta-AMDIMAS) would move helix \(\alpha_1\) and its upstream portion closer to FN (Fig. 2E), facilitating a large tilt of the \(\beta\) head. Together, the observed arrangement of FN10 R1445 and Y1446 at ADMIDAS and P1497 at MIDAS is part of the critical pivoting point for the induction of the large conformational change of integrin in coordination with the concomitant insertion of the RGD motif into the interaction pocket at the integrin head in the groove between \(\alpha_5\) and \(\beta_1\).

The third contacting point was at the interface between the FN9 synergy site and the \(\alpha\) head (Fig. 2, A and D). This interaction was facilitated by a salt bridge (R1379–D154) and a proline-aromatic interaction between P1376 in FN9 and Y208 in \(\alpha_5\) (Fig. 2D). In particular, the R1379–D154 interaction agrees with previous biochemical identifications of D154 as a key residue for the interaction with the synergy site (30) and of R1379 in FN9 as a residue involved in the force-induced reinforcement of the integrin–FN binding, called catch-bond formation (31, 32). Our structure explains the molecular basis of the markedly reduced cell adhesion activity of the triple mutant R1374A/P1376A/R1379A (33), although in this conformation, we saw no apparent interaction of FN R1374 with the integrin surface.

To validate the importance of the interactions derived from our structure analysis, we engineered point mutants of FN7-10 juxta-AMDIMAS R1445A, Y1446A, and R1445A/Y1446A as well as of the synergy site R1379A and R1374A/R1379A. We tested the binding of \(\alpha_5\beta_1\) to the FN7-10 mutants by a solid-phase equilibrium binding assay (Fig. 2F, left). For this enzyme-linked immunosorbent assay (ELISA assay)–like assay, wells were coated with FN7-10, and the binding of \(\alpha_5\beta_1\) to FN7-10 was measured. Compared to FN7-10 wild type, a reduction of binding was observed with all engineered mutants. This is also in excellent agreement with a previous cell biological report that an R1445A mutant displayed reduced cellular adhesion (33). We further tested binding of recombinant \(\alpha_5\beta_1\) ectodomain mutants on FN7-10, including \(\alpha_5\beta_1\) (E320A), \(\alpha_5\beta_1\) (E320Q), and \(\alpha_5\beta_1\) (D137A) (Fig. 2F, middle). Similar to FN7-10 mutants, all integrin mutants also displayed decreased binding to FN7-10 in comparison to the wild-type ectodomain.

Overall, the FN molecule covered the top of the \(\alpha\)- and \(\beta\)-integrin heads (Fig. 2A). The structure of FN9-10 was rigid reaching to a local resolution of 3.1 Å, while the upstream F7-F8 was less well resolved (local resolution of 4 to 7 Å), indicating its flexibility (fig. S2C). The arrangement of FN9-10 showed an interdomain tilt angle between FN9 and FN10 of 28°, which is more tilted than 12° observed in the crystal structure of FN7-10 (30) (root mean square deviation (RMSD) of 1.59 Å for the FN9-10 portion). This tilt accommodated the docking of the RGD motif into the groove between the integrin heads. We also note that our integrin-bound FN7-9 and the FN7-9 alone in the crystal structure had a similar topological arrangement with an RMSD of 1.04 Å, while FN10 was tilted away. This explains a previous observation that fixing the interdomain tilt angle of FN9-10 to 28° increases the affinity for integrin (34). Our results suggest that the bending of the FN9-10 connection (35) functions as a critical factor to scaffold the open conformation of integrin.

TS2/16 recognizes integrin \(\alpha_5\beta_1\) without interfering with key residues for conformational change

TS2/16 is a stimulatory antibody that recognizes the head domain of the \(\beta_1\) subunit [reviewed in (36)]. It is reported to reduce the off-rate of integrin-ligand interactions, thus promoting the integrin-ligand association. In our cryo-EM three-dimensional (3D) reconstruction, TS2/16 contacted the side of the \(\beta\) domain with a buried surface
area of 114.9 Å² (Figs. 1A, pink, and 2G) and centered around the reported epitope of residues 207 to 218 in the β₁ head (37). Residue K208 of the β₁ domain made a prominent contact by reaching into a hydrophobic pocket on the surface of TS2/16 and formed a cation-π interaction with Y100 of TS2/16 complementarity-determining region H3 (CDR-H3) (Fig. 2H). In addition, D93 of TS2/16 CDR-L3 formed a salt bridge to R161 in the β₁ domain. The recognition surface in β₁ was distant from the sites where the movements occur during the large conformational change, which explains why TS2/16 also recognizes the closed conformation of integrin α5β₁ (38). In this regard, we also note that the opening of integrin by FN7-10 occurred less frequently (fig. S2D) in the absence of TS2/16 and showing that TS2/16 is not essential for the opening of integrin α5β₁ but it stabilizes the opening conformation.

**Glycan N275 of α5 reinforces the FN engagement to integrin α5β₁**

Integrin was purified from a natural source, allowing us to evaluate the physiological relevance of posttranslational modifications,
particular glycosylation (Fig. 3). The identified N-glycosylation sites (Fig. 3A) validated the previous structural visualizations of recombinantly prepared samples (29, 30) and agreed well with previous biophysical analyses [α, (39); β, (40)]. Of the 14 and 12 predicted potential N-glycosylation sites in α and β subunits, 6 and 4 were found in our cryo-EM density map, respectively (Fig. 3A). The glycan attached to N275 (in the α5 head) reached out to the interface between FN9 and FN10 (Fig. 3, B and C). The glycan was surrounded by a positively charged surface and sandwiched between residues R220 and Y226 of integrin α5, which contacted a mannoside and an N-acetylgalacatosamine moiety, respectively. S223 (α5) further restricted the flexibility of the glycan chain (Fig. 3B). To evaluate the relevance of N275 glycan for the binding to FN, we introduced a point mutation N275A into recombinant α3β1 integrin (Fig. 3D) (42, 43); however, the previously reported structure of α3β1-FN10 showed a different FN contact surface (42). We interpret that the α5β1-FN10 structure lacks FN9, and therefore, the recognition of the glycan by FN was likely not fully engaged. Our structure, thus, offers a direct structural explanation why glycosylation is essential for integrin’s interaction with FN and also demonstrates the relevance of the glycosylation in cells and in vivo (44, 45).

Integrin α3β1 in its resting state shows incomplete bending

To understand the transition of the change of the integrin α3β1 conformation, we obtained the cryo-EM structure of integrin α3β1 in its resting conformation (Fig. 4 and figs. S5 to S7) in the presence of Ca2+ and Mg2+, but without Mn2+. Unlike the known resting structures of β3-containing integrins such as α5β3 or α5β3 (13, 20) showing an acutely bent conformation, α3β1 exhibited only a half-bent conformation (Fig. 4, A to F) with an angle of ~90° at the knee between the lower legs and the headpiece. Compared with the ligand-bound open conformation of integrin α5β1, the lower legs of both α and β subunits in resting integrin were less flexible, enough to be clearly visible. That is likely due to the parallel closure of the α and β legs (Fig. 4G), while the contact points of the legs are lost in the open conformation as a result of the opening of the upper legs. That shows that ligand binding to the head domains and the subsequent swing-out of the hybrid domain destabilizes the leg-leg interaction (Fig. 5A).

The upper part of the ectodomain was the most stable portion of the whole structure, with a resolution reaching to 4.6 Å in the core of the integrin head (figs. S5B, C, and S6), partially resolving the side chains (fig. S5D). The integrin heads were tilted in the closed conformation, making the three FN binding sites uncoordinated. In particular, a superposition of both structures showed that the ADMIDAS area was moved away from the possible reach of FN10 Y1446 (Fig. 5B, 6.8 Å in distance) so that ADMIDAS would not be readily available for FN binding. Furthermore, we found that the N-glycan at N343 of the β1 domain presented a steric hindrance to the docking of FN10 when the head was closed (Fig. 5C), while it moved away from the FN binding site when integrin α3β1 used an open conformation (Fig. 5C). That motion suggests that N343 has a critical inhibitive function in ligand binding.

In contrast, the resolution of the lower legs was insufficient for model building. Therefore, rigid-body molecular fitting of individual domains was performed using available α3β1 structures and homology models of calf1 and calf2 for the α subunit and plexin-semaphorin-integrin (PSI), epidermal growth factor 1 (EGF1) to EGF4 and β-tail domains for the β subunit. The main contacting surface between the lower legs of α5 and β1 was the interface between the α-thigh/calf1 domain and β1-EGF2/3 (Fig. 4G), which were aligned in parallel similarly to the available structures of acutely bent integrins (13, 20). Subsequent multibody analysis, implemented in the processing software RELION3 (46, 47), of the lower legs and the headpiece revealed fluctuation at the knee with variation of the angle between the upper and lower legs ranging between 71° and 93° (Fig. 4H, fig. S7, and movie S1), suggesting that the integrin is not rigidly fixed in this resting conformation but bears a certain degree of flexibility. It should be noted, however, that our analysis did not indicate subpopulation of molecules in the acutely bent conformation observed for the β1 integrins. Unlike the acutely bent conformation, no interaction between the hybrid domain and the lower legs of the β subunit was evident in our structure. Nevertheless, the interaction of the hinge-like interface at the knees, between calf1 and EGF2/3, was robust enough to hold the half-bent conformation.

Mn2+ is necessary for integrin α3β1 ligand binding but does not induce a large conformational change

To closely follow the key steps leading from the resting to the fully open conformation of integrin α3β1, we investigated the conformations of integrin in the presence of buffer containing various concentrations of Mn2+. Cryo-EM analysis showed that integrin α3β1 used the half-bent resting conformation even at the extreme concentration of 100 mM Mn2+ (Fig. 6A). Together with previous reports showing that the integrin α3β1 headpiece does not change its conformation when it binds to RGD peptides (29, 30), it highlights that neither the addition of Mn2+ nor that of RGD-containing peptide is enough to induce a large conformational change of the β subunit. That is in stark contrast to α5β3, for which Mn2+ was sufficient to induce full opening (23).

These findings led us to hypothesize that the large conformational change of integrin α3β1 is only stabilized as a result of FN binding rather than being the cause of FN binding. To test this notion, we performed solid-phase equilibrium binding assays (Fig. 6B). The binding of α3β1 to FN was measured in the presence or absence of Mn2+. When Mn2+ was added, we observed a marked increase in the binding of α3β1 to FN compared with that under control conditions without Mn2+, indicating that the affinity of integrin α3β1 for FN was increased. These results demonstrate that the affinity of integrin α3β1 for FN was increased with integrin still mostly using a half-bent conformation.

Furthermore, to systematically survey the global conformations of integrin α3β1, we performed reconstitution assays using size exclusion chromatography (SEC), followed by surveillance of the conformations using negative-stain EM (Fig. 7). While vitrified integrin samples for cryo-EM are instrumental in structural analyses of robustly folded populations, negative-stain EM allows the visualization of flexible entities that do not align well because of the low signal-to-noise ratio of cryo-EM images and would, therefore, be discarded during image analysis. First, the effect of Mn2+ on ligand binding was examined. Integrin α3β1 was mixed with FN10-10 and TS2/16 in the presence and absence of Mn2+ and subjected to analytical SEC (Fig. 7, A and B). The elution profiles revealed no observable shift in integrin α3β1 with or without Mn2+, indicating
Fig. 3. N-glycosylation of integrin $\alpha_5\beta_1$, bridges interface to FN. (A) Table listing the N-glycosylation sites and the types of glycan trees as determined in our structure. (B) Electrostatic potential map of the FN-integrin interface and the N275-glycan. (C) Two views of the N275-glycan on $\alpha_5$, which is part of the extensive $\alpha_5\beta_1$-FN interface. The N275-glycan contacts with FN9. (D) Comparison of glycan position between integrin $\alpha_5\beta_1$ and integrin $\alpha_v\beta_3$ [PDB ID: 3ije (43)] and $\alpha_v\beta_3$ in complex with FN10 [PDB ID: 4mmz (42)]. The N275-glycan ($\alpha_5\beta_1$) is a conserved glycosylation site among FN-recognizing integrins such as $\alpha_v\beta_3$. (E) Solid-phase binding assay to measure the binding affinity of integrin $\alpha_5$($\text{N275A})\beta_1$ ectodomain mutant in comparison to wild-type ectodomain. Both wild-type and N275A ectodomain mutant were unclashed and binding to FN7-10 wild type evaluated in the presence of Mn$^{2+}$. Bars represent means $\pm$ SD of three measurements. Unspecific binding of integrin to bovine serum albumin (BSA)-coated control wells was subtracted from the total absorbance values. Normalized binding is expressed as the ratio of mutant absorbance relative to wild-type absorbance (ratio = 1 represents wild-type binding).
no large structural change, which was further confirmed by negative-stain observations [Fig. 7A (green line/middle SDS–polyacrylamide gel electrophoresis (PAGE)), and C]. Those results were in agreement with our cryo-EM observations (Fig. 6A) showing the half-bent conformation. On the other hand, α5β1 coeluted with FN7-10 in the presence of Mn2+, indicating their interaction (Fig. 7A, right side, blue lines, peak marked with asterisk), whereas no binding took place without Mn2+ (Fig. 7A, left side). Subsequent negative-stain EM observation and conformational assessment revealed an increase in the proportion of molecules in the open conformation (41%), compared with that under the same condition without Mn2+ (0%) or without FN7-10 but with Mn2+ (7%) (Fig. 7C), although the FN density was only partially visible because of dissociation during the negative-stain preparation. Together, our results demonstrated that the large extension and opening of integrin are not coupled to the affinity of integrin for FN; instead, it is stabilized as a result of FN binding.

**DISCUSSION**

Integrin is the master adhesion receptor, controlling numerous signaling pathways and determining cell fates, such as migration and polarization (2). There is a strong incentive to understand the molecular mechanism of the activation of integrins because of their biological and medical relevance. In this study, we successfully...
purified human integrin α5β1 from a natural source (human placenta) and reconstituted the complex with FN in a membrane scaffold to facilitate a comprehensive biochemical and structural analysis. Our strategy has two important advantages: (i) Our results were obtained with native proteins in a biologically relevant context, and (ii) we were able to perform a direct and straightforward analysis without adjusting the conditions or impairing complex integrity.

Our study provides the first structure of the open, elongated conformation of integrin α5β1 in complex with FN. We found that three binding sites of the FN ligand at FN9 and FN10 were simultaneously engaged (Fig. 2), which explains the conundrum why the binding of RGD peptides was not sufficient for integrin opening in previous studies of α5β1 (29, 30). In particular, we identified that R1445 and Y1446 in FN10 approach close to the ADMIDAS region,
which caused the translocation of ADMIDAS and downstream helix α₁, leading to the stabilization of the open conformation of the β₁βI domain (Figs. 1 and 5A). That movement further allosterically triggers the swing-out motion to extend and open the α₅β₁ legs (15).

Mechanisms of integrin opening
The toolbox prepared for this study, including physiologically relevant full-length integrin together with our cryo-EM pipeline, allowed us to investigate the molecular response during integrin α₅β₁ ligand engagement. Our analysis revealed that the behavior of α₅β₁ is unexpectedly distinct from that of the well-studied β₃-containing integrins, which show a tightly coupled conformational change upon addition of either ligand-mimetic peptides or specific ions. For the FN receptor α₅β₁, the extended, open conformation was observed only when both Mn²⁺ and FN were present (Fig. 7C). That means that FN binding does not require prior integrin opening, but instead, the open conformation of α₅β₁ becomes dominant as a result of the FN binding. This model of ligand-driven conformational changes of integrin is somewhat reminiscent of an alternative model described for β₃ integrins (48, 49). Each integrin structure revealed its own distinct mode of ligand recognition and opening of the headpiece. For α₅β₆-proTGF-β₁, an additional helix is formed immediately after the proTGF-β₁ RGD motif. The helix then forms hydrophobic interactions with integrin, which appears to be important to move the specificity-determining loop 1 (SDL1), the loop upstream of the helix α₁ (50). Furthermore, proTGF-β₁ recognizes integrin β₆SDL2 as a secondary interaction surface (50). In contrast, the SDL2 (residues 176 to 195) of integrin β₁ did not exhibit any interaction with FN (fig. S8). Integrin α₅β₆ does not open its legs even after ligand binding, and notably, its ADMIDAS does not

![Fig. 6. FN-binding of NDs containing α₅β₁ is enhanced by Mn²⁺.](image)

(A) Cryo-EM image of α₅β₁ with various concentrations of Mn²⁺. 2D class averages are shown, revealing no integrin opening. (B) Solid-phase binding assays to measure integrin binding to full-length FN (FN-FL) in the presence of activating (Mn²⁺) or resting (Ca²⁺/Mg²⁺) buffer conditions and varying integrin α₅β₁ concentration. Three individual experiments were conducted, and all conditions were measured and averaged (points represent means + SD). Control measurements of α₅β₁ on BSA are shown.
contain metal ion (51). While various mechanisms of ligand-integrin interactions exist, these observations implicate the relevance of ADMIDAS for integrin opening.

Implication of integrin-ligand coupling in cellular function

Our conformational analysis together with previous studies (23, 38) showed that α5β1 is only half-bent in its resting state and the closure of α5β1 is not tightly fixed but is rather a dynamic shift within the equilibrium of different states, showing 51% of extended-leg conformation in the presence of resting buffer without Mn2+ (Fig. 7C).

Even within the half-bent structure, our multibody analysis hints at flexibility between the legs and the heads (Fig. 4H and fig. S7). That concept, termed molecular breathing, has been previously discussed with regard to integrins (13, 17). The half-bent conformation of α5β1 is still physically accessible to FN, but we speculate that a breathing motion toward extension might increase the probability of docking of the three interaction sites to FN. Last, when FN binds, integrin α5β1 is globally extended with its legs open, which is facilitated by the molecular constraint of the beads-on-string arrangement of FN9-10.
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It is intriguing that the ligand recognition mechanisms of integrin isoforms appear to be fine-tuned for their specific ligands, presumably, depending on how robust an interaction has to be for their biological functions. The \( \alpha_5 \beta_1 \) integrin is mostly expressed in immune cells, and it is plausible that the closed-extended conformation is advantageous for searching and binding to its ligands and subsequent cellular activation (51, 52). \( \alpha_5 \beta_1 \) integrin is enriched on the surface of platelets, and there is a clear distinction between its closed-inaccessible and open-extended conformations, because the aggregation of platelets has to be tightly regulated to avoid false activation and detrimental effects on blood circulation. In contrast, \( \alpha_5 \beta_3 \) is ubiquitously expressed throughout various tissues and is generally involved in cellular responses that are slower than those of the immune system. Therefore, the loose closure of \( \alpha_5 \beta_1 \) might reflect that functional aspect and, thus, provides a less acute response to extracellular cues.

The differences among integrin isoforms may provide important regulatory features and may be related to the different functions of the isoforms within specific cell types.

**Integrin-mediated intracellular focal adhesion**

We demonstrated a stepwise mechanism of a large conformational change in \( \alpha_5 \beta_1 \) from a half-bent closed to an extended open complex (summarized in Fig. 8). However, in a cellular context, it is unknown how ECM components activate integrin through the recognition of integrin heads (outside-in) or how intracellular factors such as the integrin activators talin and kindlin activate integrin through the binding to the integrin tail (inside-out). It will be particularly important to elucidate which cellular factors trigger integrin \( \alpha_5 \beta_1 \) to assume the primed state observed in vitro by the presence of Mn\(^{2+} \) in which integrin can readily associate with FN. Moreover, once integrin \( \alpha_5 \beta_1 \) is engaged with FN, force is applied through the intracellular focal adhesion machinery bound to the integrin tail (3). It has been proposed that the FN synergy site is involved in the reinforcement of the interaction with integrin under applied force (32). Although a partial synergy site mutation (R1374A/R1379A) did not affect the viability of mice, it caused a bleeding disorder and accompanying cellular biological experiments indicated that the site is not essential for the formation of focal adhesion but is critical for focal adhesion maturation (33). Those findings support the hypothesis that the key function of the FN synergy site–integrin interaction is to provide a site for catch-bond formation when force is applied (31–33). In that context, we note that FN R1374, which has been reported as a key residue for the force-transduction function (32, 33), was not engaged in the interaction with integrin in our structure. That suggests the possibility that the interaction surface might alter to engage FN R1374 in the presence of applied force. In this study, we observed a comprehensive picture of the molecular interaction of FN and integrin \( \alpha_5 \beta_1 \) in the absence of force, which can be used as a basis for further analyses.

FN acts as a dimer in its full-length context and polymerizes into a fibrous form when integrated in the ECM network. The polymerization is induced by interaction with integrin \( \alpha_5 \beta_1 \) (1). In turn, we speculate that FN within the ECM might also act as a platform to induce integrin clustering, which contributes to stabilize the large intracellular multilayered focal adhesion machinery consisting of hundreds of proteins (4). Furthermore, the phenomenon of molecular breathing has been observed for the intracellular focal adhesion proteins talin (54) and vinculin (55) as well, which, together with integrin, may play an important role in the dynamic behaviors of focal adhesions. Molecular breathing might be advantageous in the cellular context of focal adhesion formation that requires associated molecules to promptly respond to diverse stimuli. It also allows a degree of flexibility in the recruitment and disassociation of various factors from focal adhesions, thus enabling fast assembly and disassembly during cell migration.

**MATERIALS AND METHODS**

**Integrin \( \alpha_5 \beta_1 \) purification**

Human endogenous integrin \( \alpha_5 \beta_1 \) was purified from placenta by adapting a previously published protocol (24). Deidentified human placentas were obtained from Ludwig Maximilian University (LMU) Klinikum Munich, Klinik und Poliklinik für Frauenheilkunde und Geburtshilfe, Campus Großhadern. Amnion and umbilical cords were first removed from the human placentas. The remaining placenta tissue was then cut into small pieces, mixed with tris-buffered saline (TBS) [50 mM tris and 150 mM NaCl (pH 7.5)], 1 mM MgCl\(_2\), 1 mM CaCl\(_2\), and protease inhibitor cocktail (Roche cOmplete) and homogenized. Debris...
was removed by centrifugation at 12,000g for 15 min at 4°C (Beckman centrifuge, J14 rotor). The supernatant was then sonicated (VS70T, BANDELIN) and centrifuged again at 10,000g for 10 min at 4°C (Beckman Avanti J-26 centrifuge, JA-14 rotor). Next, the membrane fraction was pelleted by ultracentrifugation (150,000g, 4°C; Beckman, Ti 45 rotor), and the pellet was washed five times in 50 ml of 50 mM tris (pH 7.5), 1 M NaCl, 1 mM MgCl2, 1 mM CaCl2, and protease inhibitor cocktail. The membrane fraction was then solubilized by incubation with detergent [1 % Triton X-100 in TBS (pH 7.5), 1 mM MgCl2, 1 mM CaCl2, and protease inhibitor cocktail] overnight at 4°C. After ultracentrifugation (150,000g, 4°C; Beckman, Ti70 rotor), the supernatant was incubated with 2 ml of KH72 monoclonal antibody–coupled Sepharose beads overnight at 4°C. The beads with bound protein were then washed five times with 10 ml of TBS (pH 7.5), 1 mM MgCl2, 1 mM CaCl2, and 0.1% Triton X-100 in a gravity flow column. Protein was eluted with 20 ml of 20 mM tris (pH 8.8) and 150 mM NaCl. Bound protein was eluted with low-pH immunoglobulin G elution buffer (Pierce, Thermo Fisher Scientific) and collected in 1-ml fractions, which were immediately neutralized by addition of tris (pH 8.8) to a final concentration of 150 mM. Fractions containing purified KH72, as determined by SDS-PAGE, were pooled and concentrated using an Amicon ultrafiltration device to a concentration of ~1 mg/ml. Coupling to N-hydroxysuccinimide (NHS)–Sepharose (GE Healthcare) was performed in a coupling buffer containing 200 mM NaHCO3 and 500 mM NaCl (pH 8.3). KH72 was dia lyzed against a coupling buffer for 4 hours at room temperature using 7000 molecular weight cutoff (MWCO) Slide-A-Lyzer dialysis tubes with one exchange of the buffer. Then, ~1 mg of KH72 was added to 250 µl of activated NHS-Sepharose and incubated overnight at 4°C. The next day, 150 mM tris (pH 8.8) was added to stop the reaction, and the KH72-bound Sepharose was stored in a solution of 50 mM tris and 150 mM NaCl (pH 7.5) containing 0.05% NaN3.

**Preparation of membrane scaffold protein**

Membrane scaffold protein (MSP) was purified using a previously published protocol (25) with the following details. MSPE3D1 or MSPEAH5 (57) with an N-terminal 6xHis tag was expressed in BL21 gold DE3 cells grown in Terrific broth medium containing kanamycin (50 µg/ml). The cells were grown until the optical density at 600 nm (OD600) reached 2 at 37°C. Then, expression was induced by incubation with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) overnight at 18°C. The cells were then harvested and lysed by sonication in phosphate-buffered saline (Sigma-Aldrich), 1% Triton X-100, and phenylmethylsulfonyl fluoride. After removal of debris by centrifugation at 20,000g for 30 min at 4°C (Beckman centrifuge, JA 25.50 rotor), the supernatant was filtered with a 0.22-µm-pore PES membrane filter (Corning), incubated on an Ni–nitrilotriacetic acid column (5 ml; Protino, Macherey-Nagel), and washed with 50 ml of each of the following buffers: first with 40 mM tris (pH 8), 300 mM NaCl, and 1% Triton X-100; then with 40 mM tris (pH 8), 300 mM NaCl, and 50 mM sodium cholate; and, last, with 40 mM tris (pH 8), 300 mM NaCl, and 5 mM imidazole. Protein was then eluted with 40 mM tris (pH 8), 300 mM NaCl, and 0.5 mM EDTA using a Superdex 200 column on an ÄKTA pure (GE Healthcare) machine. The protein peak was then pooled, concentrated using an Amicon ultrafiltration device (10,000 MWCO), and the buffer was exchanged through SEC to 20 mM tris (pH 7.4), 100 mM NaCl, and 0.5 mM EDTA using a Superdex 200 column on an ÄKTA pure (GE Healthcare) machine. The protein peak was then pooled, concentrated using an Amicon ultrafiltration device (10,000 MWCO) to a concentration of ~4 mg/ml, flash-frozen, and stored at ~80°C.

**Purification of FN7-10**

FN7-10 constructs were purified as described previously (12) with the following adaptations. FN7-10 was expressed in *Escherichia coli* BL21 gold (DE3) in ZY autoinduction medium containing ampicillin (100 µg/ml). Cells were grown until the OD600 reached ~1.5 to 2 at 37°C and then overnight at 18°C. Cells were pelleted and resuspended in 50 mM tris (pH 7.5), 200 mM NaCl, 1 mM EDTA, 1% Triton X-100, and protease inhibitor cocktail (Roche Complete) and stirred for 30 min at room temperature. Cells were then lysed by sonication (VS 70 T, BANDELIN), and the lysate was cleared by centrifugation at 76,000g for 30 min at 4°C (Beckman centrifuge, JA 25.50). The supernatant was subjected to precipitation by 40% (NH4)2SO4. After addition of solid (NH4)2SO4, the solution was
stirred for 30 min at 4°C and pelleted by centrifugation at 76,000g for 30 min at 4°C (Beckman centrifuge, JA 25.50). The pellet was resuspended in 20 mM tris (pH 7.5) and 50 mM NaCl, filtered using a 0.22-µm-pore PES membrane filter (Corning), and loaded onto a HiTrap Q column (5-ml column volume; Cytiva). Bound protein was washed with 10 column volumes of 20 mM tris (pH 7.5) and 50 mM NaCl and then eluted in a gradient from 50 to 500 mM NaCl. The peak containing FN7-10 (as determined by SDS-PAGE) was pooled, concentrated using an Amicon ultrafiltration device (10,000 MWCO), and subjected to SEC using a Superdex 200 column on an ÄKTA pure (GE Healthcare) machine in 50 mM tris (pH 7.5) and 150 mM NaCl. The protein peak was pooled, concentrated using an Amicon ultrafiltration device (10,000 MWCO) to a concentration of 10 to 20 mg/ml, flash-frozen, and stored at −80°C.

**TS2/16 Fv-clasp purification**

TS2/16 Fv-clasp protein was purified as described previously (26). VH-SARAH and VL-SARAH fragments were individually expressed in BL21 gold DE3 cells grown in LB medium. The cells were grown until the OD₆₀₀ reached ~0.6 at 37°C. Expression was then induced by incubation with 0.4 mM IPTG for 4 hours at 37°C. The TS2/16 Fv-clasp protein was expressed in inclusion bodies. After the cells were lysed by sonication, the inclusion bodies were isolated by centrifugation and solubilized in 50 mM tris, 150 mM NaCl, 6 M guanidine-HCl, and 375 µM β-mercaptoethanol (pH 8). VH-SARAH and VL-SARAH were then mixed at a 1:1 molar ratio in a total volume of 12 ml. To induce protein refolding, 50 mM tris, 4 M urea, 0.4 M L-arginine, 375 µM oxidized glutathione (GSSG), and 150 mM NaCl (pH 8) were added, bringing the total volume up to 300 ml (25× dilution), and the mixture was incubated for 4 hours at 4°C. Then, 300 ml of 50 mM tris, 0.4 M L-arginine, 375 µM GSSG, and 150 mM NaCl (pH 8) was added (2× dilution) and incubated for 16 hours at 4°C. After precipitation by 80% (NH₄)₂SO₄, the protein was dissolved and dialyzed in TBS. The protein was then subjected to SEC on a HiLoad 16/600 Superdex 200 prep grade column (GE Healthcare) in 20 mM tris and 150 mM NaCl (pH 8). The peak containing TS2/16 Fv-clasp, as determined by SDS-PAGE, was then pooled, concentrated, diluted in 20 mM tris and 50 mM NaCl (pH 7.5), and further subjected to MonoQ chromatography. Elution was carried out in a linear gradient from 100 to 500 mM NaCl. The major eluting peak contained fully folded TS2/16 Fv-clasp and was pooled, concentrated using an Amicon ultrafiltration device (10,000 MWCO), flash-frozen, and stored at −80°C.

**Assembly of integrin αβ into NDs**

NDs containing integrin αβ were assembled according to a previously published protocol (25) with the following adaptations. Isoform MSPE3D1 was used for all experiments, except that we used MSPAH5 to test the effects of different Mn²⁺ concentrations on the integrin conformation in cryo-EM (Fig. 6A). Lipid stock (from Folch fraction I, bovine brain, Sigma-Aldrich) was stored in chloroform:methanol: H₂O (20:9:1) at a concentration of 25 mg/ml at −20°C. Before use, the required amount of lipid was dried in a stream of nitrogen and further vacuum-dried for 2 hours. The resulting lipid film was solubilized in 20 mM Hepes (pH 7), 150 mM NaCl, and 1% n-dodecyl-D-maltopyranoside on a thermoshaker for 1 hour at 37°C, resulting in a final concentration of 25 mg/ml. MSPE3D1 or MSPAH5, αβ, and lipids were mixed in a ratio of ~29:1:3460 for MSPE3D1 or ~45:1:110 for MSPAH5 and then incubated for 1 hour at room temperature.

SM-2 Bio-Beads (Bio-Rad) were added to the mix to remove detergent overnight at 4°C. The mixture was then subjected to SEC (Superose 6 GL10/300 on an ÄKTA pure, GE Healthcare) in TBS (pH 7.5), 1 mM MgCl₂, and 1 mM CaCl₂. The peak containing ND with inserted αβ (ND-αβ; as determined by SDS-PAGE and negative-stain EM) was then pooled, concentrated (Amicon; 50,000 MWCO) at a maximum speed of 4,000 g until an absorption at 280 nm of ~1.2 to 1.6 mg/ml (~5 µM) was reached, flash-frozen, and stored at −80°C.

**Solid-phase integrin-FN binding assay**

Solid-phase binding assays were performed on the basis of a previously published protocol (30) with some adaptations. Ninety-six-well microtiter plates were coated with 50 µl per well of either full-length FN (0.01 mg/ml; Sigma-Aldrich) in TBS (pH 7.5) or FN7-10 in TBS and its variant or 5% (w/v) bovine serum albumin (BSA) (Sigma-Aldrich) in TBS (pH 7.5) and incubated overnight at 4°C. The wells were then blocked with 5% BSA (200 µl per well) in TBS (pH 7.5) for 1 hour at room temperature. Then, ND-αβ, or the recombinant αβ ectodomains were added in various concentrations in either TBS (pH 7.5), 1 mM MgCl₂, and 1 mM CaCl₂ or TBS (pH 7.5) and 1 mM MnCl₂. Unclamping of ectodomains was achieved by overnight incubation with TEV protease at room temperature, at a ratio of 1:30 (w/w) and confirmed via SDS-PAGE. For Fig. 6B, the concentration of ND-αβ was varied in the range between 0.1 and 100 nM for all other assays, a fixed concentration of 10 nM for both ND-αβ and ectodomains was used. The mixture (total volume, 50 µl per well) was then incubated for 3 hours at room temperature. The wells were then washed three times with 200 µl per well of the respective buffers (containing either MgCl₂/CaCl₂ or MnCl₂), and antibody (50 µl per well) (TS2/16-biotin, 0.005 mg/ml) in the respective buffers containing additional 0.1% BSA was added. After incubation for 2 hours at room temperature, the wells were washed three times with 200 µl per well of the respective buffers, and streptavidin-horseradish peroxidase (SA-HRP; 1.25 µg/ml; Life Technologies) in the respective buffers containing additional 0.1% BSA was added at 50 µl per well. After incubation for 1 hour at room temperature, the wells were washed four times with 200 µl per well of the respective buffers, and 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS)/H₂O₂ solution (100 µl per well; Sigma-Aldrich; ready to use) was added. Absorption at 420 nm was measured after the addition of ABTS/H₂O₂ using a plate reader (Tecan, Infinite M1000 PRO). Graphs were generated and assessed using GraphPad Prism software.

**Analytical SEC**

To assess the binding between ND-αβ and FN7-10 in the presence or absence of TS2/16 Fv-clasp, the reaction components were mixed, incubated, and injected into a Superose 6i column (6i, 3.2/300 on ÄKTA micro system, GE Healthcare), either in TBS (pH 7.5), 1 mM MgCl₂, and 1 mM CaCl₂ or in TBS (pH 7.5) and 1 mM MnCl₂. When the effect of manganese was tested, ND-αβ was used in the range between 0.1 and 100 nM; for all other assays, a fixed concentration of 10 nM for both ND-αβ and MNCl₂. Then, TS2/16 Fv-clasp was added to a final ND-αβ/FN7-10:TS2/16 Fv-clasp molar ratio of 1:2:2, and the mixture was incubated for 10 min at 4°C. If a component was left out for a control run, then it was replaced with a corresponding amount of buffer.
Electron microscopy

**Negative-stain EM**

For negative-stain EM, 5 μl of sample (protein concentration ranging from ~4 to 12 μg/ml) was incubated on glow-discharged carbon-coated copper grids (homemade carbon layer) for 1 min at room temperature and then washed twice in 100-μl drops of H2O. Excess H2O was removed by touching the rim of the grids with blotting paper. The grids were then stained with 5 μl of 2% uranyl acetate solution for 1 min at room temperature, and excess uranyl acetate solution was removed by touching the rim of the grids with blotting paper. Datasets were collected using a Titan Halo (FEI) equipped with a Falcon 2 detector in nanoprobe mode at an operating voltage of 300 kV. Micrographs were recorded with EPU software (Thermo Fisher Scientific) at ×58,000 magnification (1.847 Å per pixel) with varying defoci from −1.5 to −2.5 μm in 0.5-μm steps at an exposure of 4 s with 10 frames per micrograph and a total dose of 105 e/Å². Recorded, gain-normalized frames were aligned, motion-corrected, and dose-weighted using MotionCor2 software (58). Defocus values were assessed using either Gctf (59) or CTFFIND4 software (60). Using RELION3 (46), ~300 particles were manually picked and boxed out with a box size of 240 pixels. 2D alignment and classification were then performed. The resulting five initial 2D class averages were used as templates for automated picking with Gautomatch software (www2.mrc-lmb.cam.ac.uk/research/locally-developed-software/zhang-software/). For all Quantifications, 20,000 particles (with a box size of 340 pixels) were aligned and 2D classified in RELION3. After the first round of 2D classification, ~10,000 particles with clear signals were selected for the second round, which was used for quantification of the particles into the tent, intermediate, and extended conformational states (related to Fig. 7C). For the representative 2D classes displayed in fig. S1C, the whole dataset of resting ND-αβ1 (81,249 particles in total) were boxed out (with a box size of 240 pixels) and submitted to alignment and 2D classification in RELION3.

**Cryo-EM sample preparation**

FN-bound and resting αβ1 were used at concentrations of 0.15 and 0.3 mg/ml, respectively. For the samples testing the effect of different Mn²⁺ concentrations (Fig. 6A), a concentration of 0.2 mg/ml was used for ND-αβ1 (MSPAH5). Three microliters of ND-αβ1 supplemented with 0, 10, or 100 mM MnCl₂ (final concentrations) was incubated for 1 hour at 4°C. Then, the sample was placed on a glow-discharged (GloQube; 30 s, 20 mA) R 1.2/1.3 Cu 200-mesh grid (QUANTIFOIL), blotted for 3.5 s, and plunge-frozen immediately in liquid ethane-propane using a Vitrobot Mark IV (FEI) at 4°C and 100% humidity.

**Cryo-EM data collection for screening and initial reconstruction**

The assessment of the large conformational change by cryo-EM, as well as the initial screening and reconstruction necessary for the final structural analysis, was performed as follows. A screening dataset for FN-bound αβ1 was collected using EPU software (Thermo Fisher Scientific) and a Talos Arctica (Thermo Fisher Scientific) equipped with a Falcon 3 direct detector in nanoprobe mode with the following parameters: operating voltage of 200 kV, ×92,000 magnification (1.61 Å per pixel), varying defoci from −2 to −3 μm, 0.5-μm steps, exposure of 3 s, 40 frames per micrograph, and total dose of 67 e/Å². The recorded, gain-normalized frames were aligned, motion-corrected, and dose-weighted using MotionCor2. Defocus values were assessed by Gctf. Using RELION3, 754 particles were manually picked and boxed out (with a box size of 300 pixels). Then 2D alignment and classification were performed. Of four generated 2D averaged classes, the three classes with distinct patterns were used as a template for automated picking with Gautomatch. Of 105,351 particles that were initially picked, four of 2D classes containing 10,949 particles (with a box size of 340 pixels) were selected. Those four classes were also used as a template for automated picking with Gautomatch of the dataset used for the high-resolution structural analysis of FN-bound αβ1 described below. The 10,949 particles of the four classes were further used to reconstruct an initial model using the initial model option in RELION3. After a 3D classification run using the initial model as a reference, of eight generated classes, the reconstruction with the most prominent features (16,765 particles) was selected as an initial reference for 3D reconstruction of the dataset of FN-bound integrin αβ1 for high-resolution analysis, as described below.

For resting integrin αβ1, 2600 micrographs were recorded using SerialEM software (61) and a K2 Summit direct detector with a quantum energy filter (20- and 40-eV slit width) (Gatan) with the following parameters: counting mode, ×130,000 magnification (1.06 Å per pixel), varying defoci from −1.5 to −3.5 μm, 0.2-μm steps, and total dose of 51 e/Å² (6-s exposure, 30 frames, and frame dose of 1.7 e/Å²). The micrograph frames were gain-normalized, motion-corrected, aligned, and dose-weighted using MotionCor2. Defocus and image resolution values were assessed using Gctf. Then, 502 particles were manually picked and boxed out with a box size of 220 pixels, and then 2D alignment and classification were performed using RELION3. Six selected 2D class averages were used as a template for automated picking with Gautomatch. A total of 103,534 automatically picked particles were boxed out with a box size of 300 pixels, binned to 150 pixels, and used to generate an initial 3D map using the ab initio reconstruction option in cryoSPARC (62). After the model was imported from cryoSPARC into RELION3 and further sorting by 3D classification, 23,332 remaining particles were refined. The refined 3D map was used as a reference for the main dataset of resting integrin αβ1 used for high-resolution analysis as described below.

For testing the effect of various Mn²⁺ concentrations on integrin αβ1 conformation, three datasets were obtained using a Talos Arctica and a Glacios (Thermo Fisher Scientific). The datasets for αβ1 at final concentrations of 10 and 100 mM MnCl₂ were collected using EPU software and a Talos Arctica with a Falcon 3 direct detector with the following parameters: nanoprobe mode, operating voltage of 200 kV, ×92,000 magnification (1.61 Å per pixel), varying defoci from −2 to −3 μm, 0.5-μm steps, exposure of 3 s, 40 frames per micrograph, and total dose of 67 e/Å². The recorded, gain-normalized frames were aligned, motion-corrected, and dose-weighted using MotionCor2. Defocus values were assessed using CTFFIND4. A total of 965 and 88 micrographs were acquired for 10 and 100 mM MnCl₂, respectively. Automated picking with Gautomatch was performed, and 202,156 and 18,822 particles were picked for 10 and 100 mM MnCl₂, respectively. Those particles were boxed out with a box size of 200 pixels; 2D alignment and classification were performed using RELION3. Representative images and classes are displayed in Fig. 6A. The dataset for the control sample with 0 mM MnCl₂ was collected using SerialEM software and a Glacios electron microscope equipped with a K2 Summit direct detector with the following parameters: nanoprobe mode, operating voltage of 200 kV, ×22,000 magnification (1.885 Å per pixel), varying defoci from −1.5 to −3 μm, 0.5-μm steps, exposure of 15 s, 50 frames per micrograph,
and total dose of 58.5 e/Å². The recorded frames were gain-normalized, aligned, motion-corrected, and dose-weighted using MotionCor2 software. Defocus values were assessed using CTFIND4 software. A total of 1935 micrographs were acquired with automated picking by Gautomatch. The particles were boxed out with a box size of 174 pixels. Then, 2D alignment and classification were performed in RELION3. Representative images and classes are displayed in Fig. 6A.

After particle alignment and 2D classification, only densities for the ectodomains of αβ1 were visible under both conditions. Despite extensive efforts to optimize the cryo-EM grid preparation, we were not able to visualize the ND density. However, SEC, corresponding negative-staining, peptide sequencing, and mass spectrometry showed full coverage of the sequence. Therefore, we concluded that the lack of membrane density was due to the flexible movement of the leg portions and also the specific characteristics of the cryo-EM sample preparation, such as the interaction of the membrane scaffolding with the air-water interface. That interaction in particular is known to cause local instability on embedded proteins during cryo-EM sample preparation, which might cause the integrin to compromise its rigidity at the lower leg portion. Therefore, the remaining transmembrane helices and cytoplasmic domains would be too flexible to appear as shown in the 2D and 3D classes, a notion supported by the progressive decrease in local resolution at the lower leg and the further downstream portion of the 3D reconstruction.

Cryo-EM data collection for high-resolution structural analysis

Datasets for high-resolution image analyses of FN-bound (Fig. 1) and resting (Fig. 4) integrin αβ1 were collected using SerialEM software and a Titan Krios (Thermo Fisher Scientific) equipped with a K3 Summit direct detector and a quantum energy filter (20–eV slit width) (Gatan) in nanoprobe mode at an operating voltage of 300 kV. For the resting integrin αβ1 dataset (Fig. 4), 2818 micrographs were recorded at ×105,000 magnification (0.85 Å per pixel) in counting mode with varying defoci from −0.5 to −3.2 μm in 0.3-μm steps at a total dose of either 65.8 e/Å² (2-s exposure, 20 frames, and frame dose of 3.13 e/Å²) or 82.3 e/Å² (2.5-s exposure, 50 frames, and frame dose of 1.65 e/Å²). The frames of the combined micrographs were gain-normalized, motion-corrected, aligned, and dose-weighted using MotionCor2. Defocus and image resolution values were assessed using either Gctf or CTFIND4. A total of 1424 particles were manually picked and boxed out with a box size of 300 pixels. Then, 2D alignment and classification were performed using RELION3. Six of the 2D class averages with distinct features were used as a template for automated picking with Gautomatch. A total of 496,356 automatically picked particles were boxed out with a box size of 300 pixels, downscaled to 150 pixels, aligned, and 2D-classified in RELION3. After initial sorting, 255,514 particles remained and were further sorted by 3D classification using a 30-Å low pass–filtered map obtained from the previous dataset created on the same sample grid (see the paragraph below for details on how 3D reference was initially obtained). From the six initially generated 3D classes, the two with the clearest signal were selected, which resulted in 680,610 particles. Those particles were rescaled to their original size (360 pixels) and further refined in the RELION3 processing pipeline, resulting in the final density map of the whole molecule with 3.5-Å total resolution, as determined by the FSC = 0.143 criterion in the RELION3 postprocessing option. Using Chimera (63) and the implemented Segger plugin (64, 65), the map was divided into two parts: the stable core, comprising the β-propeller, βII and hybrid domains, and the FN 9-10 and TS2/16 Fv-clasp; and the flexible, protruding densities, combining FN7-8 and the remaining integrin leg densities (thigh domain and PSI) (see Fig. 1 and fig. S3). Both maps were imported into RELION3, low pass–filtered to 15 Å, and used to generate masks. Within RELION3, the signal of the flexible, protruding parts was partially subtracted, and the remaining density was subjected to focused 3D refinement, using the whole molecule as a reference and applying the mask of the “core.” That procedure improved the resolution of the core part to 3.1 Å, as determined by the FSC = 0.143 criterion in the RELION3 postprocessing option.

Model building

To prepare an initial model of the FN-bound and TS2/16-bound integrin αβ1, the available crystal structures 4wk0 [ααβ1 headpiece (29)], 3v13 [αβ1 headpiece with upper legs (30)], 5xxc [TS2/16 Fv-clasp (26)], and 1nfl [FN7-10 (12)] were fit as rigid bodies into the EM density maps using Chimera (63). The model was further refined by manual building and real-space refinement in Coot (67), using maps of the core density of integrin αβ1, FN9-10, and TS2/16 Fv-clasp at a resolution of 3.1 Å and that of the whole molecule at a resolution of 3.5 Å as references. B factors were then used to generate masks, which were subsequently used for the multibody refinement option in RELION3 (47). The multibody refinement displayed movement between the two parts. Next, partial signal subtraction of either the headpiece or the lower leg signal was performed within RELION3, taking their different orientations as determined by multibody refinement into account. With focused 3D refinement after subtraction, the headpiece resolution was improved to 4.6 Å, as determined by the FSC = 0.143 criterion using the RELION3 postprocessing option. The resolution of the lower leg part was more flexible and determined to be 8 Å according to the local resolution analysis.

For the FN7-10–bound and TS2/16 Fv-clasp–bound αβ1 dataset (Fig. 1), 9431 micrographs were recorded at ×64,000 magnification (1.38 Å per pixel) in counting mode with varying defoci from −0.8 to −2.6 μm in 0.3-μm steps at a total dose of 59.7 e/Å² (7.39-s exposure, 37 frames, and frame dose of 1.61 e/Å²). Initial image processing steps were automatically performed on a graphics processing unit workstation running the modular Focus software (66). Those steps included gain normalization, motion correction, and dose weighting by MotionCor2; contrast transfer function estimation by Gctf; and particle picking by Gautomatch, using a template obtained from the screening dataset of the same sample (see above). A total of 4,218,951 particles were boxed out with a box size of 360 pixels, initially downscaled to 90 pixels, aligned, and 2D classified in RELION3. After initial sorting, 1,492,856 particles remained and were further sorted by 3D classification using a 30-Å low pass–filtered map obtained from a previous dataset created on the same sample grid (see the paragraph below for details on how 3D reference was initially obtained). From the six initially generated 3D classes, the two with the clearest signal were selected, which resulted in 680,610 particles. Those particles were rescaled to their original size (360 pixels) and further refined in the RELION3 processing pipeline, resulting in the final density map of the whole molecule with 3.5-Å total resolution, as determined by the FSC = 0.143 criterion in the RELION3 postprocessing option. Using Chimera (63) and the implemented Segger plugin (64, 65), the map was divided into two parts: the stable core, comprising the β-propeller, βII and hybrid domains, and the FN 9-10 and TS2/16 Fv-clasp; and the flexible, protruding densities, combining FN7-8 and the remaining integrin leg densities (thigh domain and PSI) (see Fig. 1 and fig. S3). Both maps were imported into RELION3, low pass–filtered to 15 Å, and used to generate masks. Within RELION3, the signal of the flexible, protruding parts was partially subtracted, and the remaining density was subjected to focused 3D refinement, using the whole molecule as a reference and applying the mask of the “core.” That procedure improved the resolution of the core part to 3.1 Å, as determined by the FSC = 0.143 criterion in the RELION3 postprocessing option.
added, and the structure was validated using PHENIX (68). The final structure fitted well to the cryo-EM density, with a correlation coefficient around the model (CCmask) of 0.75 (table S1). Note that the B factors of the periphery of the model (FN7 and integrin legs) are high because of the flexibilities of the corresponding portions. For the resting integrin αβ3, the crystal structures 4wjk (29) and 3vi3 (30) showing the headpiece and headpiece with upper legs, respectively, were used for initial fitting in Chimera. The missing parts of the lower legs (calf1 and calf2, PSI, EGF1 to EGF4, and β-tail domains) were prepared by homology modeling based on the crystal structure 3fc5 [ectodomain of integrin αβ3(1.3)] using MODELLER (69). The initial model was manually adjusted, and domains were linked using Coot. B factors were added, and the structure was validated using PHENIX. The final structure fit to the cryo-EM density, with a correlation coefficient around the model (CCmask) of 0.66 (table S1). Note that the B factors of the periphery of the model (integrin lower legs) are high because of the flexibilities of the corresponding portions.

SUPPLEMENTARY MATERIALS
Supplementary material for this article is available at http://advances.sciencemag.org/cgi/content/full/7/19/eabe9716/DC1

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Structural insights into integrin $\alpha_5\beta_1$ opening by fibronectin ligand

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