Crystal structure of the complete integrin αVβ3 ectodomain plus an α/β transmembrane fragment

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We determined the crystal structure of 1TM-αVβ3, which represents the complete unconstrained ectodomain plus short C-terminal transmembrane stretches of the αV and β3 subunits. 1TM-αVβ3 is more compact and less active in solution when compared with ΔTM-αVβ3, which lacks the short C-terminal stretches. The structure reveals a bent conformation and defines the α–β interface between IE2 (EGF-like 2) and the thigh domains. Modifying this interface by site-directed mutagenesis leads to robust integrin activation. Fluorescent lifetime imaging microscopy of inactive full-length αVβ3 on live cells yields a donor–membrane acceptor distance, which is consistent with the bent conformation and does not change in the activated integrin. These data are the first direct demonstration of conformational coupling of the integrin leg and head domains, identify the IE2–thigh interface as a critical steric barrier in integrin activation, and suggest that inside-out activation in intact cells may involve conformational changes other than the postulated switch to a genu-linear state.

Introduction

Integrins are α/β heterodimeric type I membrane receptors that mediate divergent cation–dependent interactions with components of the extracellular environment (cells and soluble and matrix proteins) leading to changes in cell shape, movement, growth, differentiation, and survival (Hynes, 2002). Determination of the crystal structure of the ectodomain of αVβ3 (ΔTM-αVβ3) in the absence and presence of a prototypical RGD ligand revealed the modular nature of integrins and clarified the basis of its divalent cation–mediated interaction with extracellular ligands (Xiong et al., 2001, 2002). The 12 extracellular integrin domains are assembled into a head segment mounted on top of two leg segments. The integrin head comprises a seven-bladed β-propeller domain from αV and a vWFA (βA or I-like) domain from β3 and contains the RGD-binding site. The αV leg is composed of an upper Ig-like thigh domain and a lower calf module consisting of two large β-sandwich domains, Calif-1 and -2. The β3 leg consists of an upper leg segment comprising a plexin-semaphorin-integrin (PSI), an Ig-like hybrid, and integrin IE1 (EGF-like 1) domain, followed by a lower leg segment made up of three IE domains (IE2–4) and a novel β-tail domain (βTD). ΔTM-αVβ3 is bent in half at two knee-like joints, the α- and β-genus, the former between the thigh and Calif-2 domains and the latter predicted between IE1 and -2 (Xiong et al., 2001), such that the head and upper leg segments of the heterodimer contact the lower leg segments of the same molecule.

Binding of the F3 subdomain of the talin head to the cytoplasmic tail of the β subunit breaks an α/β salt bridge (that normally stabilizes the inactive state; Wegener et al., 2007), triggering a conformational wave that travels through the αβ transmembrane (TM) and lower leg domains to switch the conformationally sensitive βA domain to a high affinity state, which is a process
called inside-out activation (Hynes, 2002). Direct evidence for the conformational coupling of the lower leg and head domains is indirect. And how inside-out activation converts the ectodomain to the high affinity state is still ill defined. Modeling the lower integrin legs of the ectodomain perpendicular to the plasma membrane placed the ligand-binding head near the lipid bilayer, seemingly blocking access to macromolecular ligands (for review see Arnaout et al., 2005). A switchblade model (Takagi et al., 2002) proposed that disruption of the α/β cytoplasmic–TM domain interfaces converts the bent ectodomain to a genu-linear conformation, providing ligand access and allowing βA to switch to high affinity. An alternate model, the deadbolt, proposed that lifting the constraints exerted on βA by the lower leg BTD can induce activation without linearity (Xiong et al., 2003). Complete structural information on the remaining α/β residues in the lower leg segments and the conformationally sensitive IE1/IE2 region are missing or incomplete in current structures of the integrin ectodomains (Xiong et al., 2001; Zhu et al., 2008), and knowledge of the distance separating the ligand-binding integrin head from the plasma membrane in the inactive and active states is crucial to elucidate the conformational transitions and energetics of affinity switching in integrins.

In this communication, we engineered a new construct, 1TM-αVβ3, encoding the complete sequence of the αVβ3 ectodomain plus the first four TM residues of each subunit. 1TM-αVβ3 was water soluble at neutral pH and was monomeric, which allowed characterization of its biophysical and functional properties and a determination of its crystal structure. 1TM-αVβ3 was hydrodynamically more compact and less active than ΔTM-αVβ3 in solution. Its crystal structure revealed a bent conformation and defined the complete structure of the ectodomain, including that of the IE1/IE2 region and TM extensions, permitting us to build a structure model comprising the bent ectodomain plus the complete TM domains (αVβ3-CTM). Functional experiments showed that modifying the defined IE2–thigh interface leads to constitutive activation. Fluorescent lifetime imaging microscopy (FLIM) using ligated Fab fragment of the anti-αV propeller mAb 17E6 (Mitjans et al., 1995) as fluorescence donor and the plasma membrane dye FM4-64 FX as acceptor yielded donor–membrane acceptor separation distances consistent with the αVβ3-CTM structure model, which did not vary between the inactive and active states of the full-length integrin in live cells. These data demonstrate a conformational link between the lower leg and head domains, define the crystal structure of a near native complete ectodomain, identify a critical steric barrier to activation at the IE2–thigh interface, and suggest that the genu-linear state is not an obligate feature of inside-out activation.

Results

Expression and biophysical characterization of 1TM-αVβ3

Published structures of ΔTM-αVβ3 (Xiong et al., 2001) and ΔTM-αIbβ3 (Zhu et al., 2008) lacked the last seven extracellular residues from the α subunit and P691-D692 from the β3 subunit (Fig. 1A). We expressed in insect cells an αVβ3 ectodomain, 1TM-αVβ3, encoding all the missing extracellular residues in addition to a four-residue TM extension of each subunit. 1TM-αVβ3 was secreted into the culture supernatant of virus-infected High-Five insect cells (Fig. 1B) and was purified by affinity chromatography (Fig. 1C).

The isocratic elution profiles of purified 1TM-αVβ3 and ΔTM-αVβ3 were compared on molecular sieve chromatography columns in neutral buffer containing the physiological divalent cations 1 mM Ca²⁺ + 1 mM Mg²⁺ (Ca²⁺/Mg²⁺) or 1 mM of the activating cation Mn²⁺. The apparent hydrodynamic radius (Stokes
radius ($R_s$) of 1TM-αVβ3 was calculated by reference to the elution position of standard proteins (Fig. 2). $R_s$ of 1TM-αVβ3 changed from 55 ± 0.41 Å (mean ± SD; n = 4) in Ca$^{2+}$/Mg$^{2+}$ to 58 ± 0.3 Å in Mn$^{2+}$. The $R_s$ values for ΔTM-αVβ3 in Ca$^{2+}$/Mg$^{2+}$ and Mn$^{2+}$ were 57 ± 1.1 Å and 60 ± 1.1 Å, respectively. Thus, the mean $R_s$ value for 1TM-αVβ3 in Mn$^{2+}$ differed little from that of ΔTM-αVβ3 in Ca$^{2+}$/Mg$^{2+}$. The small amounts of oligomers in the ΔTM-αVβ3 preparations in the presence of Mn$^{2+}$ were largely absent in 1TM-αVβ3, and the peak width at half height, which is a measure of molecular heterogeneity, was narrower for 1TM than for ΔTM. These data suggest that the short C-terminal α/β extensions introduced in 1TM result in a more compact and homogenous molecule in solution.

### Binding of soluble 1TM-αVβ3 to physiological ligands and to the activation-sensitive mAb AP5

We performed dose–response curves to quantify the binding of increasing concentrations of 1TM-αVβ3 to immobilized FN7–10 in Mn$^{2+}$ and in Ca$^{2+}$/Mg$^{2+}$ (Fig. 3, A and B). Half-maximal binding was achieved at 0.1 µg/ml and 6.0 µg/ml, respectively (n = 2). For soluble ΔTM-αVβ3, half-maximal binding was achieved at 0.15 µg/ml and 1.6 µg/ml. No binding took place to uncoated wells or fibronectin (FN)-coated wells in the presence of cilengitide (unpublished data). Soluble 1TM-αVβ3 formed a stable complex with either FN7–10 or full-length FN in Ca$^{2+}$/Mg$^{2+}$ buffer (Fig. 3 D, inset; Table S1; and not depicted). However, soluble 1TM-αVβ3 formed a stable complex in 2 mM of CaCl$_2$ (or Ca$^{2+}$/Mg$^{2+}$) buffer with the Fab fragment of AP5 (Fig. 3 E), a ligand-inducible binding site mAb which binds αVβ3 in its active or RGD-bound states but not in its inactive state (Honda et al., 1995; Faccio et al., 2002).

#### Crystal structure of 1TM-αVβ3

A new native dataset derived from one 1TM-αVβ3 crystal let us determine the structure of the 1TM-αVβ3, including the TM extensions, at 2.9-Å resolution (Fig. 4). The structure was superposable on the ΔTM-αVβ3 structure (Xiong et al., 2001, 2004) except for those regions newly expressed, the C-terminal exofacial, and the TM residues (Fig. 4 A) and now resolved the IE1 and -2 domains (Fig. 4, B–D). The nearly parallel exofacial extensions I955-P959 (in αV) and P688-G690 (in β3) remain close through A958 and G690 (Ca–Ca = 6.2 Å) and then diverge at an extended Pro-rich loop structure in αV (P959APMPVP963) before the first putative α TM residue V964. Structure search of this Pro-rich sequence yielded such loops in two contexts (Protein Data Bank numbers 2w55 and 2q0s), suggesting that this extended structure is not a crystal artifact but also exists in the native integrin. The first TM residues V964 (in αV) and I963 (in β3) occupy similar positions in the structure, but the TM extensions do not assume the α-helical turns found in the nuclear magnetic resonance (NMR) structure of the α/β TM domains (Lau et al., 2009) and do not interact as the result of unfavorable crystal contacts.

The three tandem integrin domains IE2–4 are related by an approximately twofold screw axis symmetry and form an extended module (Fig. 4 C). IE1 is roughly antiparallel to IE2, as a result of the bend in the β-genu (Fig. 4 C). Each IE domain has three disulphides (a–c) having the same connectivity $\alpha_2c_2\beta_2$, $\beta_2\alpha_2$, $c_2\alpha_2\alpha_2$ as that found in EGF domains, and $\alpha_2\beta_2\alpha_2$ as that found in other EGF domains (Fig. 4 E; Wouters et al., 2005). IE1 lacks disulphide a ($\alpha_2\alpha_2c_2$), thus potentially avoiding a clash with the adjacent PSI domain. A characteristic forth N-terminal intradomain disulphide d ($d_3d_3$) occupies an analogous site to the Ca coordination site found in the Ca-binding subset of EGF domains (Wouters et al., 2005). IE1–4 each contain
three β strands (A–C), with the first two antiparallel strands (A and B) forming a major β sheet found in classical EGF domains. Strand C is hydrogen bonded to strand D, which is contributed in part by the consecutive domain, with strands C and D forming a minor β sheet-like conformation. A short strand D caps the IE4 domain, which is stabilized by the C601-C604 disulphide linker that precedes the start of the βTD. In IE1–4, strand D has a characteristic β bulge at a conserved E/N residue (E472, E522, N559 and E599, respectively) to allow accommodation of disulphide c; its cross-strand partner in strand C is an invariant Gly (G468, G518, G555, G595, respectively), which is found typically in class II EGF domains (Wouters et al., 2005) and some laminin-type epidermal growth factor–like domains (Stetefeld et al., 1996).

IE2 has two characteristically long loops, c (between Cc and Cc′) and d (between Ck and Ck′; Fig. 4E), with the latter housing the β-genu (Fig. 4B). The β-genu is clearly visible in the electron density map despite lack of crystal contacts from symmetry-related molecules, which is stabilized by hydrogen bonds to the major sheet of IE2. The two long loops and strand A of IE2 face the bottom of the thigh domain, making mainly electrostatic contacts with its CC′ and EF loops (Fig. 4D). The minor strand of IE2 faces the N-terminal segment of PSI and forms ionic and van der Waals contacts involving mainly T7-R8 of the PSI domain.

Extending the 1TM structure into the α/β TM fragment, which overlaps with structured residues in the NMR structure of αIIbβ3 TM domains (Lau et al., 2009), allowed us to build a structure model of the inactive ectodomain plus the complete TM domains (Fig. 4, F–H). In this model, the ligand-binding site is accessible to macromolecular ligands, and the extracellular membrane proximal segment is structured. By comparison, in the recent structure model of the αIIbβ3 binding increasing concentrations of ΔTM- or 1TMαVβ3 to wells coated with FN7–10 in the presence of 1 mM MnCl₂ [A] or 1 mM CaCl₂ + 1 mM MgCl₂ [B]. The data shown are from a representative experiment, one of two conducted. Each point was taken at the end of the assay, and the amount of integrin present was measured by quantitative ELISA (see Materials and methods for details). No binding took place to uncoated wells run in parallel (not depicted). (C) Molecular sieve chromatogram showing the stable binding of 1TMαVβ3 to FN7–10 in solution containing 0.2 mM MnCl₂. Peak elution volumes for the 1TMαVβ3–FN complex and FN7–10 are 10.82 ml and 14.75 ml, respectively. (inset) Coomassie-stained SDS-PAGE under non-reducing conditions. Lane 1, molecular mass markers (in kilodaltons); lane 2, 1TMαVβ3; lane 3, FN7–10; lane 4, blank; lane 5, FN7–10 from the faster peak; lane 6, blank; lane 7, 1TMαVβ3–FN7–10 complex in the slower peak. An ~1:1 integrin/FN molar ratio was calculated from the scanned gel (see Materials and methods), which is in agreement with previous results (Adair et al., 2005). (D) Molecular sieve chromatography of 1TMαVβ3 with intact plasma FN in the presence of 0.2 mM MnCl₂ or 1 mM CaCl₂ + 1 mM MgCl₂. In MnCl₂-containing buffers, 1TM (Kav of 0.229, 11.58 ml) forms a complex with intact FN, which elutes at a Kav of 0.66 (9.0 ml). FN alone elutes as a discrete peak at a Kav of 0.127 (10.0 ml). In CaCl₂/MgCl₂ buffers, 1TM runs as a more compact molecule, and coelution with FN reveals no indication of complex formation (inset). (E) Molecular sieve chromatogram showing complex formation of 1TMαVβ3 with the Fab fragment of mAb AP5 in TBS containing 2 mM CaCl₂. The dashed line shows the peak elution volume of purified 1TMαVβ3 alone run on the same column and in the same buffer. (inset) Coomassie-stained 12% SDS-PAGE under non-reducing conditions. Lane 1, molecular mass markers (in kilodaltons); lane 2, blank; lanes 3 and 5, purified 1TMαVβ3 and AP5 Fab, respectively, before mixing; lane 4, 1TMαVβ3–AP5 Fab complex from the slower peak. A 1:1 integrin/AP5 Fab molar ratio was calculated from the scanned gel (see Materials and methods). mAU, milli-absorbance unit.

Figure 3. Binding of 1TMαVβ3 to physiological ligands and to the Fab fragment of the activation-sensitive mAb AP5. (A and B) Receptor-binding assay to immobilized ligand. Dose–response curves showing
ectodomain plus TM domains. This region is not structurally defined and assumed to be very flexible in the inactive integrin model. However, inserting a flexible linker into the extracellular membrane proximal Pro-rich sequence of the β2 integrin α subunit CD11b leads to a constitutively active integrin (Kamata et al., 2005), suggesting that some rigidity in this region is essential to maintain the integrin in its inactive conformation.

**Functional effects on activity of full-length αVβ3 after mutation of features structurally defined in the 1TM structure**

We assessed the effects on cellular αVβ3 caused by modifying the newly defined IE2–thigh interface through a Δβ-genu deletion (Δβ-genu), altering the βTD–hybrid–IE3 interfaces by breaking two salt bridges (R404A + R633A), and changing the βA–βTD–hybrid interfaces by deleting the βTD CD loop plus

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**Figure 4. New features of 1TM-αVβ3 crystal structure and hypothetical model of αVβ3 plus the complete TM domains (αVβ3-cTM).** (A) Density map (in blue; contoured at 1.0 σ) of the exofacial and TM extensions. The superposed main chains of 1TM-αVβ3 and ΔTM-αVβ3 (only the lower parts of Calf-2 and βTD shown from each; Xiong et al., 2004) are in light and dark gray, respectively, except for the new exofacial and TM extensions of 1TM-αVβ3, which are shown in red, and the last residues in the ΔTM-αVβ3 structure (G956 and G690), which are shown in green. (B) Density map (in blue; contoured at 1.0 σ) and main chain tracing of the α-genu (in red) and main chain tracing of the β-genu (in red) in IE2. The orange sphere in this panel and in C represents the metal ion at the α-genu. (C) Ribbon diagram showing electrostatic interactions at the IE2–thigh interface. Residues (shown in ball and stick representation) forming a salt bridge, main chain, or side chain H-bonds are labeled. Thigh and IE2 are labeled in gray and blue, respectively. Oxygen, nitrogen, and carbon atoms are in red, blue and green, respectively. Hydrogen bonds and salt bridges (distance cutoff, 3.5 Å) are represented with red dotted lines. (D) Structure alignment and Cys pairing of IE domains. The sequence housing the α-genu in IE2 is in blue. The secondary structure elements (strands are underlined, and a helix is represented by a cylinder) are shown. The atomic coordinates are deposited in the Protein Data Bank (3IJE). (F–H) Structure model of αVβ3 ectodomain plus the complete TM domains (αVβ3-cTM). The model is built by releasing the C termini in the 1TM-αVβ3 structure from their respective crystal contacts, such that extracellular P691 and P963 initiate the respective β3 and αV TM helices, which is consistent with the known propensity of prolines to strongly stabilize α-helical conformations (Senes et al., 2004). (F and G) The resulting movements included a 2.9-Å inward movement of P691 of β3 (F) and a rotation of αV’s Pro-rich loop at G956, such that P963 initiates the αV TM helix (modeled after αIIb’s TM NMR structure; G; Lau et al., 2009). The structure model was energy minimized with Modeller (Fiser and Sali, 2003), and the αVβ3 TM side chains were optimized and repacked using Rosetta (Rohl et al., 2004). The ribbon diagrams in F and G were generated using PyMOL (DeLano Scientific LLC). (H) A ribbon diagram, which was generated using Chimera, of the αVβ3-cTM model showing the orientation of the ectodomain relative to the TM domains. The model predicts that the TM domains are at an ~30° angle relative to the long axis of Calf-2, with ADMIDAS (adjacent to MIDAS) metal ion (green sphere) at an ~45 Å distance from the plane parallel to the hypothetical membrane drawn at the Cα of β3’s Pro691. The α-genu and propeller metal ions are in orange.
an R633A substitution (ΔCD + R633A). The activating G989FF/GAA mutation (Zhu et al., 2007) served as a positive control. None of the mutations impaired expression or heterodimer formation compared with wild type (WT), as judged by reactivity with the β3-specific AP3 mAb and the heterodimer-specific LM609 mAb (unpublished data). Mn$^{2+}$ increased binding of the WT receptor to soluble Alexa Fluor 488–labeled FN9–10 from 7% in Ca$^{2+}$/Mg$^{2+}$ buffer to 48% of the AP3-positive cell population in Mn$^{2+}$. The Δβ-genu mutation induced constitutive activation that was significantly more robust than the G989FF/GAA mutant (Fig. 5), with a more modest activation induced by the R404A + R633A mutation. The activating effect of the ΔCD + R633A mutation was small but significant (P < 0.005; Fig. 5).

**FLIM analysis of αVβ3 in live cells**

We used FLIM to assess the orientation of the αVβ3 ectodomain relative to the plasma membrane in K562 cells stably expressing WT αVβ3. For lifetime calculations, FLIM collects only photons emitted from the donor fluorophores, thus avoiding the problem of mis-excitation of the acceptor and the analogous problem of spectral break through bleeding of the donor signal into the acceptor spectral window, which are common concerns in spectral fluorescence resonance energy transfer (FRET) applications (Chigaev et al., 2001; Kim et al., 2003; Coutinho et al., 2007). Importantly, FLIM allows picosecond measurements and is independent of concentration of the fluorophores.

As fluorescence energy transfer between a donor–acceptor pair described by the Förster equation depends both on the distance and the relative orientation of donor and acceptor (Jares-Erijman and Jovin, 2003; Giepmans et al., 2006), it is critical that a structurally defined probe be used as donor. Therefore, we used the Fab fragment of mAb 17E6 whose low resolution epitope mapping (Mould et al., 2000) corresponds to its crystal structure bound to the β-propeller of ATM-αVβ3, which we have determined (17E6 binds at the top of the propeller contacting the DA loop between blades 2 and 3 and the CD loop in blade 3; unpublished data). Binding of unlabeled 17E6 Fab to the cell surface Mn$^{2+}$-activated WT αVβ3 did not affect binding of fluorescently labeled soluble monomeric FN10, either as WT or as a high affinity form (Fig. 6 A). The RGD ligand cilengitide also triggered an increase in hydrodynamic radius of the 17E6–1TM-αVβ3 complex (Fig. 6 B), indicating that 17E6 Fab did not freeze either membrane-bound or soluble αVβ3 in an inactive state. The plasma membrane was labeled with FM4-64 FX (FM) as acceptor. This dye preferentially inserts into the outer leaflet of the membrane of live cells in 1–5 min at 4°C, fluoresces brightly, and can be rapidly fixed with ice-cold paraformaldehyde, eliminating diffusion on the time scale of FLIM measurements. The standard equation to calculate FRET efficiency applies to populations of fluorophores in such circumstances and is frequently used in biological systems to report mean donor–acceptor distances.

Alexa Fluor 488 fluorescence lifetime was first measured in inactive WT αVβ3–expressing K562 cells stained with Alexa Fluor 488–Fab in 1 mM of CaCl$_2$-containing buffer. A lifetime of 2,306 ± 52 ps (mean ± SD) was determined in the absence of acceptor (Fig. 6 C and D). When the Alexa Fluor 488–Fab-labeled K562 cells were further labeled with the FM membrane dye, Alexa Fluor 488 lifetime decreased to 2,056 ± 118 ps (P < 0.0001), corresponding to a FRET efficiency of 10.8% and a mean donor–acceptor separation distance (r) of 88 Å. The respective values for the Mn$^{2+}$-activated αVβ3 were 2,322 ± 43 ps (P < 0.0001) after addition of the FM dye, corresponding to a FRET efficiency of 10.1% and a mean donor–acceptor separation distance (r) of 89 Å. This distance is consistent with the hypothetical structure model depicted in Fig. 4 H, which predicts an ~90-Å distance from the centroid of the integrin-bound 17E6 Fab to the plane of the membrane parallel drawn at the Cα of β3’s P691 residue. Mean donor–acceptor separation distances of 78 ± 9 Å (mean ± SD) and 83 ± 3 Å were obtained with unliganded αVβ3 and with high affinity FN10-bound αVβ3, respectively, each in 1 mM of Mn$^{2+}$-containing buffer. FLIM measurements conducted on transiently transfected HEK 293T cells expressing WT, G989FF/GAA, or Δβ-genu αVβ3 in 1 mM of Mn$^{2+}$-containing buffer
yielded mean donor–acceptor separation distances of 96 Å, 94 Å, and 90 Å, respectively. Thus, the donor–acceptor separation distances are comparable in the inactive, Mn²⁺-activated, constitutively active, or FN10-bound states of the full-length αVβ3 expressed on the surface of live cells. It is appropriate to mention here that the calculated length of the genu-linear αVβ3 molecule is >200 Å (Xiong et al., 2001).

**Discussion**

Our major findings in this study are that (a) the C-terminal extensions of the αVβ3 ectodomain by the remaining exofacial residues and four αβ TM residues gave a water soluble and conformationally stable integrin that is predominantly inactive in Ca²⁺/Mg²⁺ buffer in solution, (b) crystal structure of the near native 1TM-αVβ3 in Ca²⁺ defined the first complete structure of an integrin ectodomain plus an αβ TM fragment and allowed us to build a structure model of the ectodomain plus the complete TM domains, (c) functional analyses using the 1TM-αVβ3 crystal structure identified a critical role for the newly defined I2–thigh interface in integrin activation, and (d) FLIM of WT αVβ3 in live cells revealed that the apparent distance between the integrin head and the plasma membrane changes little in inactive, Mn²⁺-activated, constitutively active, or FN10-bound full-length integrin.

We found that 1TM-αVβ3 is recognized in solution by the activation-sensitive mAb AP5 in 2 mM Ca²⁺ but did not form a stable complex in solution with FN7–10 or full-length FN, unless the integrin is activated by Mn²⁺. Quantitative binding assays showed that $K_{d(app)}$ values of 1TM-αVβ3 and ΔTM-αVβ3 binding to immobilized FN7–10 in Mn²⁺ are comparable (0.15 and 0.1 µg/ml). However, binding of 1TM-αVβ3 and ΔTM-αVβ3 in Ca²⁺/Mg²⁺ buffer yielded $K_{d(app)}$ values that were ~60-fold and ~10-fold lower, respectively, than in Mn²⁺ (Fig. 3, A and B), reflecting the proportion of the active species in each preparation. Thus, 1TM, which differs only in the added αβ lower leg, is less active and

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**Figure 6.** FLIM analysis of αVβ3 in live cells. (A) Histogram (mean ± SEM; n = 2) showing binding of subsaturating Alexa Fluor 488–labeled WT (open bars) and high affinity (h; shaded bars) FN10 to full-length WT αVβ3 stably expressed on K562 in the absence (−) or presence (+) of saturating amounts of unlabeled 17E6 Fab in 1 mM Mn²⁺ (see Materials and methods). (B) Isocratic molecular sieve elution profiles in Mn²⁺-containing TBS buffer. 1TM-αVβ3 (black) and its complexes with cilengitide (red), 17E6 Fab (blue), and 17E6 Fab complex followed by the addition of cilengitide to 10 µM (green) were resolved. The elution profile of 17E6 Fab alone is also shown (violet). Cilengitide runs in the column volume. Cilengitide triggers an increase of the apparent Stokes radius of the 1TM-αVβ3–17E6 Fab complex. mAU, milli–absorbance unit. (C) Histogram (mean ± SD) showing lifetimes (in picoseconds) of Alexa Fluor 488 fluorescence determined by FLIM in inactive (Ca²⁺) and active (Mn²⁺) full-length αVβ3. *P < 0.0001 versus donor only. (D) Representative Alexa Fluor 488 fluorescence intensity of the unliganded WT integrin. The pseudocolored FLIM images represent donor fluorescence lifetimes on a pixel by pixel basis, where shorter lifetimes are located toward the red area of the spectrum and longer lifetimes toward the blue area. Bar, 8 µm.
hydrodynamically more compact than \( \Delta T \), reflecting a conformational coupling between the lower leg and head domains that regulates integrin activation. Our present findings are also consistent with the integrin existing in equilibrium between two major quaternary states, inactive (T state) and active (R state; Perutz, 1989), with the T state predominating in soluble 1TM-\( \alpha V \beta 3 \) in Ca\(^{2+}\)/Mg\(^{2+}\) buffer. mAb AP5 binds preferentially in Ca\(^{2+}\) or Ca\(^{2+}\)/Mg\(^{2+}\) buffer to the minor active species (estimated at \( \sim 2\% \) and \( \sim 10\% \) in 1TM- and \( \Delta T \)-\( \alpha V \beta 3 \) preparations, respectively), driving the quaternary equilibrium toward the active quaternary state (s) and stabilizing it there (Fig. 3 E). The same shift can be accomplished by FN7–10 or by native FN but requires Mn\(^{2+}\) not Ca\(^{2+}\), presumably because of the higher affinity of Mn\(^{2+}\) to the metal ion–dependent adhesion site (MIDAS) that mediates \( \alpha V \beta 3 \) interaction with ligands. Using the measured \( K_{\text{diss}} \) Values as approximations of binding affinities, the mean difference in energy between the two major quaternary states of 1TM- and \( \Delta T \)-\( \alpha V \beta 3 \) is estimated at \( \sim 2.4 \) kcal/mol and 1.4 kcal/mol, respectively. It is expected that this value will be even higher for the full-length integrin.

The crystal structure of \( \Delta T \)-\( \alpha I I b \beta 3 \) at 2.55-Å resolution reported (Zhu et al., 2008) while this work was under review ends at the equivalent residues to \( \Delta T \)-\( \alpha V \beta 3 \) and thus lacks those lower leg residues and TM fragments defined in the current 1TM-\( \alpha V \beta 3 \) structure and shown to regulate affinity. Superposition of the \( \Delta T \)-\( \alpha I I b \beta 3 \) structure onto that of 1TM-\( \alpha V \beta 3 \) using Matchmaker in the Chimera software suite (Pettersen et al., 2004) revealed several interesting differences between these two structures. First, a significant inward rotation of Calf-1 and -2 at the \( \alpha \)-genu in \( \Delta T \)-\( \alpha I I b \beta 3 \) resulting from crystal contacts with symmetry-related molecules was associated with translational movements in the IE2-4 domains. Second, the \( \beta \)-genu is missing in the \( \Delta T \)-\( \alpha I I b \beta 3 \) structure, where it is presumed disordered (Zhu et al., 2008). Yet, significant changes appear in the main chain flanking the \( \beta \)-genu in \( \Delta T \)-\( \alpha I I b \beta 3 \) compared with 1TM-\( \alpha V \beta 3 \), suggesting proteolytic cleavage within this site in \( \Delta T \)-\( \alpha I I b \beta 3 \) as another likely explanation. Third, the \( \beta TD \) of \( \Delta T \)-\( \alpha I I b \beta 3 \) veers toward IE4 and away from the hybrid/\( \beta A \) domains, breaking the R633-coordinated salt bridge, which stabilizes the inactive state of full-length \( \alpha I I b \beta 3 \) (Matsumoto et al., 2008). The engineered disulfide linking \( \beta TD \) to Calf-2 in \( \Delta T \)-\( \alpha I I b \beta 3 \) may impose these quaternary changes in the \( \beta TD \) environment.

Fourth, the \( \beta A \) domain of unliganded \( \Delta T \)-\( \alpha I I b \beta 3 \) contained the MIDAS and the stimulatory ligand-associated metal-binding site (LIMBS) metal ions (Mg\(^{2+}\) and Ca\(^{2+}\), respectively), but no metal ions were present in the 1TM-\( \alpha V \beta 3 \) 2.9-Å structure formed in the presence of Ca\(^{2+}\) (Fig. S1) or in our published 3.1-Å unliganded \( \Delta T \)-\( \alpha V \beta 3 \) structure (Xiong et al., 2001), even when Mn\(^{2+}\) was diffused into the crystals. Lower resolution (Zhu et al., 2008) is an unlikely explanation for this difference, as both metal ions are overt in the 3.2-Å structure of the \( \Delta T \)-\( \alpha V \beta 3 \)-cRGD complex (Xiong et al., 2002). Furthermore, in native \( \beta A \) isolated from the \( \beta \) subunit, LIMBS did not bind Ca\(^{2+}\) (Pesho et al., 2006), so the absence of a LIMBS Ca\(^{2+}\) in unliganded \( \beta A \) is independently supported. Whether clamping the legs covalently by an artificial disulfide permits metal occupancy at LIMBS through allosteric mechanisms remains an open question. A likely interpretation is that the difference reflects an influence of the associated \( \alpha \) subunit on the metal ion coordination in \( \beta A \). In the unliganded \( \Delta T \)-\( \alpha I I b \beta 3 \) structure, the LIMBS residue D\(^{107}\) points toward LIMBS (and not away from it as in 1TM-\( \alpha V \beta 3 \)) to avoid a clash with a hydrophobic residue (Phe191, which is a Trp in all other \( \alpha \) subunits; Fig. S1). This orientation provides five coordination sites for a LIMBS Ca\(^{2+}\), with the sixth coordination site completed by OE1 of E220, which is thus pulled out of the MIDAS pocket, allowing coordination of an Mg\(^{2+}\) at the MIDAS.

As 1TM-\( \alpha V \beta 3 \) is the fourth integrin crystal structure resolved in a bent conformation under conditions that activate ligand binding in biochemical and cell biological assays, we once again addressed the contentious issue of the conformation of the integrin \( \alpha V \beta 3 \) at the cell surface. We positioned a FLIM donor on the integrin head and an acceptor in the outer face of the plasma membrane. FLIM measurements revealed no significant change in mean separation distance of the integrin head relative to the plasma membrane outer face in Mn\(^{2+}\)-activated WT \( \alpha V \beta 3 \) compared with the inactive integrin (Fig. 6, C and D). A previous FRET study measured the distance between an FITC-labeled ligand-mimetic peptide as donor and a plasma membrane dye as acceptor (Chigaev et al., 2003). There, the change in mean distance of closest approach was \( \sim 50 \) Å between resting and Mn\(^{2+}\)-activated \( \alpha V \beta 1 \). However, as stated by the authors, the membrane dye used, R18, has a tendency to flip-flop between the outer and inner leaflets of the plasma membrane, introducing uncertainty in the measurements. The FLIM methodology we have used minimizes this problem and supports a more recent cryoelectron tomography study of liposome-embedded and Mn\(^{2+}\)-activated \( \alpha I I b \beta 3 \) (Ye et al., 2008). Interestingly, high affinity soluble monomeric FN10 bound to the WT \( \alpha V \beta 3 \) in Mn\(^{2+}\) or the constitutive activation of \( \alpha V \beta 3 \) by G989FF/GAA or \( \Delta \beta \)-genu mutants also failed to trigger an increase in the mean donor–membrane acceptor separation distance compared with the inactive WT integrin. The simplest explanation for these data is that a switch of the integrin from the inactive to the active state or its binding to soluble FN10 in live cells can occur with little or no genu extension, which is consistent with a recent modeling study (Rocco et al., 2008).

The functional experiments presented in this study identify a previously unappreciated but critical role for the IE2–thigh interface in stabilizing the inactive state (Fig. 7). This is reflected by the robust constitutive activation introduced in the surface-expressed receptor upon deletion of the \( \beta \)-genu, which contributes to this interface. Our results also show that stability of the IE2–thigh interface appears to be conformationally linked to the lower leg extensions introduced in 1TM-\( \alpha V \beta 3 \). Other mutations in the lower leg domain that interrupt two salt bridges linking the \( \beta TD \) and IE3 to the top and bottom of the hybrid domain, respectively (Fig. 7), revealed that these make a modest contribution to stability of the inactive state: in \( \alpha V \beta 3 \), the steric barrier is mainly mediated by the IE3–\( \beta TD \) hybrid contact, whereas the \( \beta TD \)-hybrid contact appears to predominate in \( \alpha I I b \beta 3 \) (Matsumoto et al., 2008). Our functional experiments also show that \( \beta TD \) contacts with both \( \beta A \) and hybrid domains contribute to stability of the inactive conformation, although this contribution is minor in \( \alpha V \beta 3 \) and absent in \( \alpha I I b \beta 3 \) when the \( \beta TD \)-\( \beta A \) contact alone is removed by deleting the CD loop in \( \beta TD \) (Zhu et al., 2007).
The shape of the quaternary R (active) state of the integrin ectodomain remains to be defined structurally. But it includes the high affinity state of βA and a proposed genu-linear conformation, which, it is argued, is required for switching βA to the active state (Takagi et al., 2002). In the homologous αA domain, the high affinity state is characterized by an inward movement of the N-terminal α1 helix and a two-turn downward movement of the C-terminal α7 helix, which is permitted by a flexible long linker distally (Lee et al., 1995). The former movement was observed in the crystal structure of the bent cRGD-bound ΔTM-αVβ3 (Xiong et al., 2002) but in the absence of the axial movement of α7 helix, which is constrained distally in the bent crystal structure by the hybrid domain. The activating effect of releasing constraints on the hybrid in the R633 + R404 mutant is consistent with previous work performed on integrin ectodomains with modified, truncated, or entirely amputated legs (Mould et al., 2003; Xiao et al., 2004), which found a correlation between opening the βA-hybrid hinge and the high affinity state of βA. An ∼70° hinge opening in the legless ectodomain structure (Xiao et al., 2004), which is associated with a one-turn downward movement of the C-terminal α7 helix, led to the conclusion that this feature characterizes the high affinity state of βA. However, a recent study (Chigaev et al., 2009) found that the hybrid domain movement and the high affinity state are regulated separately and independently of each other. And an EM study of the intact ΔTM-αVβ3 ectodomain complexed with FN7–extra domain B–10 detected only a small 11 ± 4° opening of the βA–hybrid hinge angle (Adair et al., 2005), which is sufficient to break the IE2–thigh interface, suggesting that a rather modest hinge opening may be all that is needed to switch βA to high affinity. Consistently, a molecular dynamics study of the legless αIIbβ3 ectodomain detected a spontaneous βA–hybrid hinge opening of ∼20° when a single N303-K417 bond linking βA to hybrid is broken (Puklin-Faucher et al., 2006), but over the course of this increase, a lateral rather than the axial shift of the βA domain C-terminal α7 helix was observed. The same study found that subsequent pulling on bound FN10 caused the βA–hybrid domain hinge to further increase to 70° in the legless ectodomain, suggesting that the wider hinge opening requires force applied on a ligand-bound integrin. Whether such an extreme movement requires preconversion of the ectodomain to a genu-linear structure or can occur in a genu-bent conformation requires further study. Our data suggest that overcoming the critical IE2–thigh steric barrier through the activating β-genu deletion does not require a genu-linear conversion. It may also be relevant that binding of soluble monomeric high affinity FN10 did not induce genu linearity in the full-length αVβ3, suggesting that the high affinity state and genu linearity are not conformationally linked events, an interpretation which is consistent with experimental observations in WT and modified integrins (Takagi et al., 2003; Coutinho et al., 2007; Gupta et al., 2007). It has been proposed that genu linearity requires force applied by the cytoskeleton to the cytoplasmic tails of the unliganded integrin (Zhu et al., 2008). However, our FLIM data on constitutively active αVβ3 suggest that this remains as bent as the inactive molecule. It is possible that the transition to the genu-linear state requires that force be exerted on both ends of an integrin, as when it is bound simultaneously to an immobilized ECM ligand and to the cytoskeleton in mechanically stressed tissues (for review see Arnaout et al., 2005).
Molecular sieve chromatography of purified 1TM-αVβ3

All molecular sieve chromatography of purified αVβ3 was performed as previously described (Adair et al., 2005) by mixing 1TM-αVβ3 pacUW31 vector was used to confirm the presence of the WT subunit into HEK 293T cells using Lipofectamine 2000 serum, 50 IU/ml penicillin and streptomycin, and 0.5–1.0 mg/ml G418.

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Data collection, refinement, and model statistics

| Statistics                                      | Value                      |
|------------------------------------------------|----------------------------|
| **Data collection statistics**                 |                            |
| Space group                                    | P2221                      |
| Unit cell dimensions (Å)                       | a = b = 130.256, c = 305.982 |
| Resolution range (Å)                           | 50–2.9                     |
| Completeness (%)                               | 99.2 [98.5]                |
| Number of unique reflections                   | 66,702 (6,515)             |
| Redundancy                                     | 2.9 [2.2]                  |
| R_space [%]                                    | 8.8 (100)                  |
| I/σ                                            | 10.5 (100)                 |
| Wavelength (Å)                                 | 1.0332                     |
| **Refinement statistics**                      |                            |
| Resolution range (Å)                           | 20–2.9                     |
| R_factor (work set) [%]                        | 24.4                       |
| R_factor (free set) [%]                        | 28.7                       |
| Mean B factors (Å²)                            | 39.1                       |
| Atoms in the model                             | 13,027                     |
| Number of GlcNAc                               | 14                         |
| Number of Ca²⁺                                 | 6                          |
| **Model statistics**                           |                            |
| (RMSD from ideality)                          |                            |
| Bond lengths (Å)                               | 0.006                      |
| Bond angles (°)                                | 1.086                      |

RMSD, root mean square deviation. Values in parentheses are for the highest resolution shell [0.1 Å].

Online supplemental material

**Fig. S1** shows a ribbon diagram of the MIDAS face in the 1TMαVβ3 and 1TMαllbβ3 structures. Table S1 shows the elution profiles after molecular sieve chromatography of 1TMαVβ3 or full-length FN alone and in complex. Online supplemental material is available at [http://www.jcb.org/cgi/content/full/jcb.200905085/DC1](http://www.jcb.org/cgi/content/full/jcb.200905085/DC1).

Fluorescent labeling of 17E6 Fab and FN

The Fab fragment of the αV-specific mAb 17E6 (Mitjans et al., 1995) was prepared by papain digestion followed by anion exchange and size-exclusion chromatography (Andrew, 2002), and its purity was confirmed by SDS-PAGE followed by Coomassie staining. Fab 17E6 and high affinity αVβ3-specific FN10 were labeled with Alexa Fluor 488 N-hydroxysuccinimidyl ester dye using the Alexa Fluor 488 protein labeling kit (Invitrogen) according to the manufacturer’s instructions. The final antibody and FN concentrations and the dye to protein molar ratios (F/P) were determined spectrophotometrically, giving F/P molar ratios of ~3–7 (for Fab) and ~1 (for FN9–10 and FN10; FN9 has no lysines). Binding of the fluorophore-labeled FN to αVβ3-expressing cells was evaluated by flow cytometry, and dose–response curves were established to determine the optimal (saturating or subsaturating) concentration of ligand used in subsequent experiments.

Time-correlated single photon counting–FLIM acquisition and analysis

Wells of nonfluorescent Labtek II four-chamber microscope slides (Thermo Fisher Scientific) were coated with poly-Lys (Sigma-Aldrich) overnight at 4°C. 25,000–50,000 αVβ3-expressing K562 or HEK 293T cells were transfected in serum-free iscove’s modified dulbecco’s medium to each well and incubated for 30 min at 37°C in a total volume of 200 µl. Nonspecific sites were blocked by incubation with 10% serum-rich medium for 10 min at RT and then washed twice to remove nonadherent cells. Adherent live cells were stained with 20 µg/ml 17E6 labeled with the fluorescence donor Alexa Fluor 488 (Alexa Fluor 488–Fab) in 25 mM Tris, pH 7.4, and 145 mM NaCl (TBS) containing 1 mM CaCl₂, 1 mM CoCl₂, and 1 mM MgCl₂ or 1 mM MnCl₂ for 20 min at 37°C. After two washes, some cells were labeled with 6–12 µM FM4-64 FX (FM) in TBS containing 1 mM CaCl₂, 1 mM CoCl₂ and 1 mM MgCl₂, or 1 mM MnCl₂ for 5 min on ice, washed once, immediately fixed with ice-cold 4% paraformaldehyde, washed, and mounted with GVA mount (Invitrogen) under a coverslip. The GVA-mounted slides were kept in the dark and used the next day for FLIM acquisition (Bacsakai et al., 2003). In some experiments, adherent K562 expressing WT αVβ3 were pre-incubated with saturating amounts (10 µg/ml) of unlabeled high affinity FN10 followed by the addition of Alexa Fluor 488–Fab and then processed as in the previous section.

FLIM measurements were made on a two-photon microscope (Radiance 2000, Bio-Rad Laboratories) with a femtosecond-pulsed Ti:Sapphire Laser (Mai Tai; Spectra-Physics) at 800-nm excitation. Photons were detected by microchannel plate photomultiplier tube (MCP R3809; Hamamatsu Photonics) with time-correlated single photon counting (SPC830; Becker & Hickl) to measure fluorescent decay profiles. Decay curves were best fit into monoexponential curves using SPCImage software (version 2.6.1.2711; Becker & Hickl). Lifetimes for multiple cells (n = 9–14) for each experimental condition were compared and evaluated for statistically significant differences from control cells (labeled only with donor fluorophore) by analysis of variance with Fisher’s post-hoc correction. Percent lifetime decrease, or FRET efficiency (E), was calculated as the difference between the excited state of the donor in absence of acceptor (τ₀) and in presence of acceptor fluorophore (τE) according to the equation E = 1 – τE/τ₀. The distance between donor (Fab) and acceptor fluorophores, r, was calculated using the following equation (Duncan et al., 2004): r = R₀/Fₚ₀/τ₀ − 1/1-θ, where R₀ is the Förster radius, the distance at which energy transfer is 50%. We assumed random orientation for the fluorophore (orientation factor, θ = 2/3) in the Fab molecule. We approximated R₀ as 62 Å based on spectral similarities to the published Alexa Fluor 488–Alexa Fluor 568 pair (Invitrogen).

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