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$\alpha_V\beta_3$ Integrin Crystal Structures and their Functional Implications

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Abstract

Many questions remain about the significance of structural features of integrin $\alpha_V\beta_3$ for its mechanism of activation. We have determined and re-refined, respectively, crystal structures of the $\alpha_V\beta_3$ ectodomain linked to C-terminal coiled-coils ($\alpha_V\beta_3$ -AB) or four transmembrane (TM) residues in each subunit ($\alpha_V\beta_3$ -1TM). The α_V and β_3 subunits with four and eight extracellular domains, respectively, are bent at knees between the integrin headpiece and lower legs, and the headpiece has the closed, low-affinity conformation. The structures differ in occupancy of three metal binding sites in the β_I domain. Occupancy appears related to the pH of crystallization, rather than to physiologic regulation of ligand binding at the central, metal-ion dependent adhesion site (MIDAS). No electron density was observed for TM residues and much of the α_V linker. $\alpha_V\beta_3$ -AB and $\alpha_V\beta_3$ -1TM demonstrate flexibility in the linker between their extracellular and TM domains, rather than previously proposed rigid linkage. A previously postulated interface between the α_V and β_3 subunits at their knees was also not supported, because it lacks high quality density, required rebuilding in $\alpha_V\beta_3$ -1TM, and differed markedly between $\alpha_V\beta_3$ -1TM and $\alpha_V\beta_3$ -AB. Together with variation in domain-domain orientation within their bent ectodomains between $\alpha_V\beta_3$ -AB and $\alpha_V\beta_3$ -1TM, the structures are compatible with the requirement of large structural changes, such as extension at the knees and headpiece opening, in conveying activation signals between the extracellular ligand-binding site and the cytoplasm.

Introduction

Integrins have noncovalently associated α and β subunits, each with an ectodomain (~ 1,000 residues in α and ~ 700 in β), a single transmembrane domain, and a typically short cytoplasmic domain. Integrin ectodomains have four domains in α and eight in β , of which one in each associate tightly to form a ligand-binding head (Fig. 1). The remaining domains connect the heads through upper legs, knees, and lower legs, to the plasma membrane. In the bent conformation, the knees are bent, and the headpiece (head and upper legs) folds over the lower legs, to form a 2,000 Å² interface (Fig. 1A). In two extended conformations, the legs are extended at the knees, and the headpiece may either be closed, as in the bent conformation, or open (Fig. 1B and C). Headpiece opening involves a large conformational change at the hybrid domain interface where the β_I domain is inserted, i.e. attached at both its N- and C-termini. Headpiece opening is conveyed by α -helix connecting rod-like movements across the β_I domain to its interface with the α -subunit where the ligand is

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Supporting information available. Supplementary figure S1 shows SDS-PAGE of $\alpha_V\beta_3$ crystals. This material is available free of charge via the Internet at <http://pubs.acs.org>.

bound, and increases affinity for ligand many orders of magnitude (Fig. 1C). Most integrins interact with the actin cytoskeleton, and transmit signals in both directions across the membrane to regulate binding to extracellular ligands, and traction through the cytoskeleton for cell migration and signaling (1).

The key question in the integrin field is how signals are transmitted from integrin cytoplasmic and transmembrane domains to the ligand-binding site in the ectodomain to regulate affinity for ligand. We and others have presented extensive evidence for two key types of conformational changes: 1) integrin extension to give the extended-closed state (Fig. 1B) and 2) integrin headpiece opening to give the extended-open state (Fig. 1C) (1). The extended-closed conformation has low affinity for ligand, and only the extended-open conformation has high affinity (2-9). Thus integrin activation by inside-out signals increases the proportion of integrins with the extended-open conformation. These conclusions are supported by a large number of electron microscopy (EM), small angle X-ray scattering (SAXS), and crystallographic studies as well as affinity measurements on cell surfaces. The studies include work on diverse integrins including $\alpha_V\beta_3$, $\alpha_{IIb}\beta_3$, $\alpha_5\beta_1$, $\alpha_V\beta_6$, $\alpha_L\beta_2$, and $\alpha_X\beta_2$ (1). To communicate allostery over unusually large distances through the flexible lower leg of the β -subunit in the extended conformation, it has been emphasized that large separations between the integrin transmembrane and cytoplasmic domains would be required, and evidence for such separation has been obtained by transmembrane domain cross-linking and fluorescence resonance energy transfer (FRET) experiments (10-12).

On the other hand, another model proposes that integrin activation requires neither extension nor headpiece opening (13, 14). The body of evidence supporting this alternative model is smaller, and is based on studies of integrin $\alpha_V\beta_3$ (13, 14). Crucial in the latest support for this hypothesis was a crystal structure of integrin $\alpha_V\beta_3$ (14). Excellent density was reported for the ectodomain, as well as the first three residues of both the α_V and β_3 TM residues. It was unexpected that the α_V and β_3 TM residues were far from one another in the crystal structure (25 Å), because structures for the closely related α_{IIb} and β_3 TM domains show the same residues in close contact (15-17). Nonetheless, a model was made by bending the ectodomain-TM linkers in both α_V and β_3 to bring the TM domains close, and it was suggested that some rigidity in the linker region is essential to maintain the integrin in its inactive conformation (14). Linker rigidity sets $\alpha_V\beta_3$ apart from integrins $\alpha_{IIb}\beta_3$ and $\alpha_X\beta_2$. Ectodomain-TM domain linkers were disordered in $\alpha_{IIb}\beta_3$ and $\alpha_X\beta_2$ crystal structures, suggesting flexibility (18, 19). Furthermore, disulfide cross-linking of the α_{IIb} and β_3 linkers on intact $\alpha_{IIb}\beta_3$ on cell surfaces demonstrated lack of a specific interface (15).

Several other features of $\alpha_V\beta_3$ crystal structures appear unusual. The β I MIDAS locates in between two other divalent metal-binding sites, the adjacent to MIDAS (ADMIDAS) and synergistic metal-binding site (SyMBS). $\alpha_V\beta_3$ was found to lack metal ions at the MIDAS and SyMBS, but to contain them when a ligand was soaked in, and metal ions were suggested to regulate ligand binding (14, 20). In contrast, $\alpha_{IIb}\beta_3$ with an identical β -subunit, binds metal ions at all three sites in absence of ligand (18, 21). $\alpha_V\beta_3$ has been reported to have clear density at its β -knee (14), in contrast to $\alpha_{IIb}\beta_3$ (18). A specific, stabilizing interface between the α_V and β_3 knees was reported that is unusual for containing many hydrogen bonds between highly solvent-accessible carbonyl and carboxyl groups (14). Such carboxyls are considered to be deprotonated at physiologic pH, and interactions between oxygen lone pair electrons are highly unfavorable. A specificity-determining loop (SDL) in the β I domain of the β_3 subunit near the ligand-binding interface with the α -subunit was built markedly differently in a 2004 $\alpha_{IIb}\beta_3$ crystal structure (4) than in the 2001 $\alpha_V\beta_3$ crystal structure (22). The conformation in $\alpha_V\beta_3$ was retained in the 2009 crystal structure (14), perhaps implying an influence of the α -subunit on the conformation of the nearby SDL in β_3 .

To obtain an independent view of integrin $\alpha_V\beta_3$, we have determined a structure of its ectodomain fused to coiled-coil peptides to mimic associating TM α -helices ($\alpha_V\beta_3$ -AB). Furthermore, to better address special issues raised by the $\alpha_V\beta_3$ -1TM structure, we carefully examined this structure and its underlying electron density. After doing so, we decided that it would benefit from re-refinement. Therefore, we report here two different $\alpha_V\beta_3$ crystal structures. Using these structures and their objective electron density, we report several novel structural features, and come to novel conclusions on the bent-closed structure of $\alpha_V\beta_3$ and its implications for activation.

Materials and Methods

Clasped $\alpha_V\beta_3$ -AB ectodomain structure

The α_V ectodomain sequence ending at M960 was fused to sequences in the order GSGGEN, AQCEKELQALEKENAQLEWELQALEKELAQ, corresponding to a 6-residue vector-derived sequence and the ACID-p1 sequence of peptide Velcro (23) containing a Cys at the “d” position of the heptad repeat (24), respectively. The β_3 ectodomain sequence ending at P691 was fused to sequences in the order ESM, LENLYFQ, GGKN, AQCKKKLQALKKKNAQLKWKQLQALKKKLAQ, TG, and HHHHHH, corresponding to a three-residue linker, a tobacco etch virus (TEV) protease site, a four-residue linker, the BASE-p1 sequence of peptide Velcro (23) containing a Cys at the “d” position of the heptad repeat (24), a two-residue linker, and a His6 tag, respectively (2). Protein was expressed in CHO Lec 3.2.8.1 cells (2) and purified from culture supernatant using Ni-NTA agarose (QIAGEN) followed by gel filtration (Superdex 200 HR) chromatography in 0.01 M Tris HCl pH 7.5, 0.14 M NaCl (TBS), and 1 mM Ca^{2+} , and was concentrated to about 5.5 mg/ml.

Crystals from hanging-drop vapor diffusion at room temperature were in 2 M ammonium sulfate, 80 mM sodium cacodylate pH 5.8, 2 mM CaCl_2 and 6 mM MgCl_2 . Coverslips with their hanging drops were transferred to wells containing saturated ammonium sulfate for dehydration over 16 h. Crystals were cryoprotected in 95% saturated ammonium sulfate and 5% glycerol before plunging in liquid nitrogen.

Diffraction data from GM/CA-CAT beamline 23-ID of Advanced Photon Source (APS) at Argonne National Laboratory were processed using XDS (25). The structure was solved using molecular replacement with PHASER with both 3IJE and 4G1M $\alpha_V\beta_3$ -1TM structures (described below). The rotation function and translation function Z scores, respectively, were 15.0 and 26.5 for the 3IJE model, and 16.2 and 38.1 for the 4G1M model. Initial structure refinement with PHENIX (26) used rigid bodies for individual domains, individual sites, TLS, and individual B factor refinement. Using the same parameters, we obtained R_{work} and R_{free} of 27.4% and 33.7% with 3IJE and 24.4% and 31.4% with 4G1M models, respectively. Like the better molecular replacement scores with the 4G1M model, these refinement results showed that the 4G1M $\alpha_V\beta_3$ -1TM model fit better the $\alpha_V\beta_3$ -AB dataset than the 3IJE $\alpha_V\beta_3$ -1TM model. The initial $\alpha_V\beta_3$ -AB model obtained using replacement with the 4G1M $\alpha_V\beta_3$ -1TM model was therefore used as the starting model for subsequent refinement.

After several rounds of manual model rebuilding with Coot (27) and refinement with PHENIX, simulated annealing was used in refinement instead of rigid body refinement. Simulated-annealing composite omit maps were also calculated and used to guide manual rebuilding to decrease model bias. During iterative rebuilding and refinement, an 8% twin fraction was observed in the dataset with the twin law H, K, -L. After applying the twin law, both R_{work} and R_{free} dropped around 1.5%. The ACID and BASE α -helical coiled-coil (leucine zipper) peptides, but not the linkers to the C-termini of the α_V - and β_3 -subunits, were present in electron density and were added to the model.

Refinement was first completed at 3.0 Å resolution. Using a recent cross-correlation method (28), we found that useful data extended to 2.85 Å. Density improved, and refinement was extended to this resolution.

Refinement of 3IJE

We calculated simulated-annealing (SA) composite omit maps using the $\alpha_V\beta_3$ -1TM (PDB code 3IJE) coordinates and structure factor amplitudes with both the programs CNS (29) and PHENIX (26). Neither program's SA omit maps showed 1 σ electron density for a substantial portion of the β_3 I-EGF1 domain, portions of the I-EGF2 and PSI, much of the α -subunit linker, or the TM segments of the α - and β -subunits.

Based on these results, we started with a model lacking the PSI, I-EGF1, and I-EGF2 domains, and systematically rebuilt the $\alpha_V\beta_3$ structure in 16 iterative steps of manual rebuilding with Coot (27), attention to outliers and clashes identified with MolProbity (30), and TLS and maximum-likelihood refinement with REFMAC (31). PyMol scripts were used to superimpose individual domains from high resolution $\alpha_{IIb}\beta_3$ structures to provide hints for rebuilding. At each step, we computed PHENIX simulated annealing composite omit maps (26). The PSI and most of the I-EGF1 and I-EGF2 domains appeared in the 2Fo-Fc map during refinement and were added back. R_{free} dropped from 28.5% in 3IJE to 26.9%. Another SA omit map calculated with CNS showed most of I-EGF1.

Many further cycles of rebuilding in Coot utilized PHENIX refinement with TLS, maximum likelihood, and simulated annealing (26). R_{free} dropped to 24.2%. Subsequent refinement cycles without simulated annealing resulted in a final R_{free} of 23.4%. In the last cycle, EGF-1 was carefully examined. Only short segments were missing in the 1 σ 2Fo-Fc map; however, the overall quality of the map remained lower than for other domains, suggesting flexibility. A low-resolution map computed from data between ~ 65 and 3.7 Å was used to check this less-ordered domain. This map clearly showed the N-acetyl glucosamine residue attached to β_3 Asn-452, which was added back. The C-termini of α_V and β_3 were each extended about two residues past the last residue in the 1 σ map, to the last peptide moiety in $\sim 0.5 \sigma$ density, beyond which there could be no confidence in the path of the polypeptide chain.

$\alpha_V\beta_3$ furin site mutation and function

Full-length human α_V and β_3 plasmids were subjected to site-directed mutagenesis and co-transfected into HEK293T cells as previously described (2). Cell surface expression measured with non-functional Cy3-conjugated β_3 mAb AP3 and binding of Alexa-488 conjugated fibrinogen and fibronectin were determined by fluorescent flow cytometry (8). Briefly, transfected cells were detached, re-suspended in 20 mM HEPES-buffered saline, pH 7.4 (HBS), supplemented with 5.5 mM glucose and 1% bovine serum albumin and incubated in room temperature for 30 min with human fibrinogen or fibronectin in the presence of either 5 mM EDTA, 5 mM Ca^{2+} , or 1 mM Mn^{2+} . Cells were then stained with Cy3-conjugated AP3 on ice for 30 minutes.

Transiently transfected cells were metabolically labeled with [^{35}S] cysteine/methionine and subjected to immunoprecipitation as described (11). Lysates in 20 mM Tris-buffered saline, pH 7.4 (TBS), supplemented with 1 mM Ca^{2+} , 1% Triton X-100, 0.1% Nonidet P-40 and 1:100 v/v protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO) were immunoprecipitated with 1 μg of $\alpha_V\beta_3$ mAb LM609 (EMD Millipore Corporation, Billerica, MA) and protein G-Sepharose at 4 °C for 1 h.

Results

$\alpha_V\beta_3$ -AB ectodomain structure

Previously described $\alpha_V\beta_3$ - Δ TM (22) and $\alpha_V\beta_3$ -1TM proteins (14) were each expressed in insect cells, differ in length from one another only by four TM residues in each subunit, and crystallize with 10 to 12% PEG as precipitant in nearly identical lattices. The spacegroup is $P3_221$, and the unit cell dimensions are $a = b = 130.0 \text{ \AA}$ ($\alpha_V\beta_3$ - Δ TM) or $130.26 \text{ \AA}$ ($\alpha_V\beta_3$ -1TM) and $c = 307.3 \text{ \AA}$ ($\alpha_V\beta_3$ - Δ TM) or $305.98 \text{ \AA}$ ($\alpha_V\beta_3$ -1TM). Such small differences in lattice dimensions are typical for different crystals of the same protein.

To obtain an independent view of $\alpha_V\beta_3$ structure, we expressed in mammalian CHO-Lec cells the complete $\alpha_V\beta_3$ ectodomain, connected through 6-residue (α_V) and 14-residue (β_3) linkers to the ACID and BASE leucine zipper Velcro peptides (2, 23) ($\alpha_V\beta_3$ -AB). Crystals formed with a different class of precipitant, 2 M ammonium sulfate. The spacegroup was still $P3_221$, and the a and b dimensions of $128.46 \text{ \AA}$ were similar to those of $130.26 \text{ \AA}$ for $\alpha_V\beta_3$ -1TM; however, at $352.49 \text{ \AA}$ the c dimension was markedly larger than $305.98 \text{ \AA}$ for the previous crystals (Table 1). The structure includes the ACID-BASE leucine zipper; however, it has little if any effect on $\alpha_V\beta_3$ because the long linkers between the α_V and β_3 subunits and the ACID and BASE peptides are disordered. The $2.85 \text{ \AA}$ $\alpha_V\beta_3$ -AB structure (Fig. 2A) is well refined, with R_{free} of 26.2%, excellent Ramachandran and geometry statistics, and high MolProbity (30) percentiles (Table 1).

$\alpha_V\beta_3$ -1TM ectodomain structure

The $2.9 \text{ \AA}$ $\alpha_V\beta_3$ -1TM structure was solved by molecular replacement (14) and is therefore subject to model bias. The standard method for obtaining a less model-biased map is to compute a simulated-annealing (SA) composite omit map (32). Independent SA composite omit maps were calculated with CNS and PHENIX using the deposited $\alpha_V\beta_3$ -1TM (PDB code 3IJE) data. The maps showed absent or poor electron density for much of the PSI, I-EGF1, and I-EGF2 domains, the linker between the ectodomain and TM domains, and TM domain residues. Furthermore, difference map peaks showed a substantial number of places where the 3IJE model and the electron density did not correlate (Fig. 3A, red and green mesh).

Therefore, we rebuilt the $\alpha_V\beta_3$ -1TM molecular model using many iterative steps of rebuilding with Coot and refinement that included use of REFMAC, PHENIX, and simulated annealing. We initially omitted regions with poor density. As other domains improved, 1σ density for PSI, I-EGF1, and I-EGF2 reappeared, and these domains were added back. We re-calculated SA composite omit maps after each refinement step, and used MolProbity to find clashes, bad rotamers, and Ramachandran outliers after each refinement. Density for C-terminal portions of α_V and β_3 did not reappear and they are thus absent in the final model.

The new and previous $\alpha_V\beta_3$ -1TM molecular models are referred to here by their PDB accession codes, 4G1M and 3IJE, respectively. If the PDB code is not specified, $\alpha_V\beta_3$ -1TM refers to the 4G1M structure. The 4G1M structure has many fewer regions of disagreement with electron density (Fig. 3B) than 3IJE (Fig. 3A). Furthermore, 4G1M is refined against data from $2.9 - 65 \text{ \AA}$, as opposed to $2.9 - 20 \text{ \AA}$ for 3IJE (Table 1). The greater amount of low-resolution data used in 4G1M allows better definition of less ordered regions, i.e. the PSI, I-EGF1 and I-EGF2 domains (Fig. 2B). Differences in the β_3 PSI, I-EGF1, and I-EGF2 domains (C α RMSD of $1.2 - 1.4 \text{ \AA}$, Table 2) occur throughout their length, whereas differences in the larger integrin domains result primarily from rebuilding of loops. The rebuilding process resulted in a drop of R_{free} from 28.5 to 23.4%, a decrease in

Ramachandran outliers from 3.1% to 0.1%, and a decrease in poor rotamers from 7.9% to 0.6%, as reported by MolProbity (30) (Table 1).

Overall structures

Both $\alpha_V\beta_3$ structures reveal an overall bent conformation (Fig. 2A and B). The α_V β -propeller and β_3 β I domains interact over a large interface to form the integrin head and its ligand binding site. Acute bends define the knees, at the junction between the upper and lower legs. The α_V leg is bent between the thigh and calf-1 domains, at the disulfide-bonded, Ca^{2+} binding genu loop, or α -knee. The bend in β occurs at the β -genu between I-EGF domains 1 and 2. Thus the thigh domain in α_V and the PSI, hybrid, and I-EGF1 domains in β_3 form the upper legs. The calf-1 and calf-2 domains in α_V and the I-EGF2, I-EGF3, I-EGF4 domains, β -ankle loop, and β -tail domains in β_3 form the lower legs (Fig. 1). Large interfaces are buried between the head and the lower legs, and between the α_V and β_3 legs, that stabilize the bent conformation (2). Since structures of $\alpha_V\beta_3$ (14, 20, 22, 33) and $\alpha_{IIb}\beta_3$ (4, 18, 21, 34, 35) have previously been described, we focus our attention on the most notable differences and the structural features that are relevant for models of integrin ligand-binding and activation.

Lack of difference between the SDL of $\alpha_V\beta_3$ and $\alpha_{IIb}\beta_3$

The specificity-determining loop (SDL) extends from the β I domain at its interface with the β -propeller domain. It locates just outside the binding footprint for small peptide ligands, and based on exchange between different integrin β -subunits, contributes to binding of macromolecular ligands (36). All previous $\alpha_V\beta_3$ structures (Fig. 4A) differ from all $\alpha_{IIb}\beta_3$ structures (Fig. 4D) over β_3 SDL residues 168-176 in structure to sequence register, and by the absence and presence, respectively, of two *cis*-peptides in the SDL at Ser-162/Pro-163 and Ser-168/Pro-169 (Fig. 4A and D). We wondered if these differences were induced by differences between the α_{IIb} and α_V β -propeller domains with which the SDL associates. However, inspection of the electron density for the previous $\alpha_V\beta_3$ -1TM structure (14) reveals clear signatures for both *cis*-Pro, and a sequence-to-structure shift (Fig. 4E). For example, negative density on the carbonyl group of Pro-169 when it is *trans* (Fig. 4E), is eliminated when it is *cis* (Fig. 4F), and positive density between the sidechains of Pro-169 and Pro-170 (Fig. 4E) is satisfied by the sidechain of Pro-169 when it is *cis* (Fig. 4F). Furthermore, positive densities near Glu-174, Asn-175, and Pro-176, and negative density at Leu-173 (Fig. 4E) are corrected when the sequence-to-structure register is shifted and the backbone is flipped at Pro-176/Cys-177 (Fig. 4F). Rebuilding to follow the electron density resulted in SDL backbones in $\alpha_V\beta_3$ -1TM (Fig. 4B) and $\alpha_V\beta_3$ -AB (Fig. 4C) similar to that in $\alpha_{IIb}\beta_3$ (Fig. 4D).

A metal ion at the SyMBS or MIDAS in unliganded $\alpha_V\beta_3$

The $\alpha_V\beta_3$ -AB structure has no metal at the MIDAS or ADMIDAS, but has density for Ca^{2+} at the SyMBS (Fig. 5A and D). The $\alpha_V\beta_3$ -1TM structure has only weak density at the SyMBS, modeled as a water in 4G1M (Fig. 5B). Positive density at the MIDAS in the 3IJE dataset (Fig. 5E) has been satisfied by placing a Ca^{2+} ion at the MIDAS in 4G1M (Fig. 5F). Thus $\alpha_V\beta_3$ -1TM has metal ions at both the MIDAS and ADMIDAS (Fig. 5B). The Ca^{2+} at the MIDAS has higher B factors than the surrounding coordinating residues, and therefore may have partial occupancy; furthermore, a water or Mg^{2+} ion (which is lacking in buffers but could have been bound to $\alpha_V\beta_3$) could also be built at the MIDAS. The most reasonable interpretation is Ca^{2+} or Mg^{2+} , both because a metal ion is normally bound here, and because of the close proximity of three negatively charged residues. The position of the metal ion differs from that in $\alpha_{IIb}\beta_3$, which appears related to the lack of metal ion binding to SyMBS in $\alpha_V\beta_3$ -1TM and the consequent shift in position of the dual SyMBS and MIDAS-coordinating residue Glu-220 (Fig. 5B and C). Remarkably, the occupancy of the

three β I metal ion binding sites is completely reversed between $\alpha_V\beta_3$ -AB and $\alpha_V\beta_3$ -1TM (Fig. 5A and B).

Overall flexibility in the ectodomain

Crystallographic B-factors reflect atomic displacements in the crystal lattice and are hence related to protein flexibility. In the $\alpha_V\beta_3$ -1TM backbone, B-factors are much higher than the overall average in I-EGF1 and in the N-terminal portion of I-EGF2, which connects to I-EGF1 and is near to the thigh domain (Fig. 6A). In the $\alpha_V\beta_3$ -AB backbone, B-factors in the β_3 subunit are high in a long PSI domain loop, in the last disulfide-bonded loop of I-EGF1, and in the first loop of I-EGF2, which has a gap in continuous density (Fig. 6C). In $\alpha_{IIb}\beta_3$, the first loop in I-EGF2 also has high B factors, and is partially missing in density (Fig. 6E). The highest B-factors in $\alpha_{IIb}\beta_3$ occur in the β -tail domain, which is poorly ordered (Fig. 6F).

The overall position of the genu moves markedly between the two $\alpha_V\beta_3$ structures (Fig. 2A and B), and the α -genu in $\alpha_V\beta_3$ -AB also has high B-factors (Fig. 6C). Genu movement is tied to changes in position of both the thigh and calf-1 domains between the two structures. The genu-proximal end of the calf-1 domain is 3.5 Å closer to I-EGF2 in $\alpha_V\beta_3$ -AB than in $\alpha_V\beta_3$ -1TM. Furthermore, the thigh domain rotates along its long axis. Rotation is not about the center of the domain, but is centered on β -strand-F, which faces inward toward the lower α - and β -legs. Far from the center of rotation, the long C-D loop in thigh moves 6 Å closer to I-EGF2 in $\alpha_V\beta_3$ -1TM than in $\alpha_V\beta_3$ -AB.

The other large movement between $\alpha_V\beta_3$ -1TM and $\alpha_V\beta_3$ -AB occurs in the PSI and I-EGF1 domains and in the PSI- and I-EGF1-proximal end of the hybrid domain. While there is little change in position of the β I-proximal end of the hybrid domain, the PSI-proximal end of the hybrid domain moves 2 to 2.5 Å. This libration of the hybrid domain is coupled to displacement of 2 to 3 Å of the PSI and I-EGF1 domains, in a direction that places PSI and I-EGF1 further away from α_V in $\alpha_V\beta_3$ -1TM than in $\alpha_V\beta_3$ -AB.

Flexibility in I-EGF1 and I-EGF2

Flexibility occurs within I-EGF1 and I-EGF2, as well as at their junctions with other domains. Ca-traces colored in rainbow by B factor show marked flexibility within the loops at the C-terminal end of I-EGF1 and at the N-terminal end of I-EGF2, near the junctions between these domains (Fig. 7). Just before the final Cys-521 of I-EGF2 (C8, Fig. 7E-G), Tyr-520 (Y in Fig. 7E-G) participates in hydrophobic interactions with core elements of the domain, and is shielded on one side by Lys-519 (K in Fig. 7E-G). Tyr-520 is highly conserved as an aromatic or hydrophobic residue in I-EGF2 of other integrin β -subunits. In contrast, I-EGF1 lacks elements that stabilize the conformation of its C7-C8 loop, and has Ser-469 and Gln-470 (S and Q in Fig. 7A-D, respectively) at the sequence positions equivalent to Lys-519 and Tyr-520 (Fig. 7E-G).

The flexibility of the C7-C8 loop in I-EGF1 is emphasized in $\alpha_{IIb}\beta_3$ headpiece structures in which the β_3 subunit terminates in I-EGF1. This loop is disordered in most such structures (4, 34). However, in recent closed headpiece structures with two headpieces per asymmetric unit (21, 35), the electron density is good enough to build most of the C7-C8 loop in one molecule and all of the C7-C8 loop in the other molecule (Fig. 7D). Remarkably, this loop adopts a markedly different orientation. Although there is room in the crystal lattice for the C7-C8 loop to adopt the same conformation as seen in the complete ectodomain, as shown by superposition (Fig. 7H), the C7-C8 loop instead moves to another position in the lattice, where the backbones of Pro-464 and Gly-465 participate in a network of water-mediated

hydrogen bonds to the β -propeller domain of a neighboring molecule in the crystal lattice of the 2.2 Å structure.

Lack of a stable interface between the I-EGF2 and thigh domains

Substantial negative and positive Fo-Fc difference density is present in the interface between the β_3 I-EGF2 and α_V thigh domains in the original $\alpha_V\beta_3$ -1TM model (Fig. 3A) but not in the map calculated using improved coordinates (Fig. 3B). This is a region of high B-factors, particularly in I-EGF2, but also in the extended CD loop of the α_V thigh (Fig. 6A and C). Near this interface in the I-EGF1 domain, between the two $\alpha_V\beta_3$ -1TM models the C α atom positions shift 2.3 Å for Leu-467 and 1.2 to 1.5 Å for Gly-463, Pro-464, and Gly-465. In the C1-C2 loop of the I-EGF2 domain, at the interface with the thigh domain, the C α atoms of Glu-475, Glu-476, Asp-477 (Fig. 8A and B), Tyr-478, and Arg-479 shift 1.8, 1.8, 1.5, 1.6, and 3.5 Å, respectively, between the two models. Although these residues are more accurately located in our model, as shown by presence of backbone density in simulated annealing composite omit maps calculated with 4G1M but not 3IJE structures (Methods), the differences in position emphasize that these are among the regions with the poorest electron density, and the most difficult to build.

The C1-C2 loop is markedly longer in I-EGF2 than in other integrin I-EGF domains (18). Several residues in this loop were too disordered to build in $\alpha_V\beta_3$ -AB or $\alpha_{IIb}\beta_3$ (Fig. 7E and G). Missing density here and elsewhere in the molecular model is clearly not due to proteolytic cleavage, as demonstrated by migration of the α_V and β_3 subunits at the expected molecular weight in SDS-PAGE (Supp. Fig. S1).

Across from I-EGF2, the electron density on the thigh side of the interface is better; nonetheless, substantial rebuilding was required to fit the density. Thus, in the interface the C α atoms of thigh residues Gln-504, Lys-505, Gly-506, and Glu-547 shift 1.4, 2.1, 1.4, and 3.1 Å, respectively, between the two models (see E547 in Fig. 8A and B). There are no hydrogen bonds or significant hydrophobic interactions between I-EGF2 and thigh in our model of $\alpha_V\beta_3$ -1TM (Fig. 8B), consistent with the substantial shift in orientation at the I-EGF2/thigh interface between $\alpha_V\beta_3$ -1TM and $\alpha_V\beta_3$ -AB (Fig. 8B and C).

Lack of a stable interface between the β -tail and β I domains

The interface between the β -tail CD (deadbolt) loop and the β I domain is extremely small in both the 4G1M $\alpha_V\beta_3$ -1TM and $\alpha_V\beta_3$ -AB structures, at 40 Å² and 50 Å², respectively. Furthermore, there are no significant van der Waals interactions or hydrogen bonds between these domains. Moreover, the β -tail CD loop has high B-factors, particularly in $\alpha_V\beta_3$ -1TM (Fig. 6B).

Interaction of an α_V glycopeptide moiety with β_3

Near the furin cleavage site in the calf-2 domain of $\alpha_V\beta_3$ -AB, we found a polypeptide-like electron density protruding from calf-2 toward β_3 , into a cavity between the I-EGF4 and β -tail domains. A second large, branched density corresponding to an N-linked carbohydrate extends from the first density. This enabled us to identify the polypeptide density as α_V residues 841-848, and to build two N-acetylglucosamine and three mannose residues N-linked to Asn-844 of the consensus N-glycosylation sequence (Fig. 9A). Around the furin cleavage site at R860, residues 839-840 and 849-866 are disordered.

The α_V 841-848 glycopeptide moiety inserts in a cavity between the β_3 headpiece and lower leg, and forms contacts with the β I, hybrid, I-EGF4, β -ankle, and β -tail domains (Fig. 9A). It is unlikely to be able to occupy the same position prior to furin cleavage. Therefore, we tested the effect on $\alpha_V\beta_3$ function of an α_V -R860A mutation. This mutation eliminated the

cleavage by furin of the α_V -subunit (Fig. 9B). However, it had little effect on binding of fibrinogen or fibronectin to $\alpha_V\beta_3$ transfectants measured by fluorescent flow cytometry (Fig. 9C and D). In contrast, introduction of an N-linked glycan wedge into the βI /hybrid domain interface to favor the open headpiece conformation markedly increased binding in both Ca^{2+} and Mn^{2+} (Fig. 9C and D).

Linkage between the extracellular and transmembrane portions of $\alpha_V\beta_3$

Improvement of the molecular model of $\alpha_V\beta_3$ -1TM included rebuilding of the C-terminal portion of the calf-2 domain. The backbones of L755 and G954 were rotated, and the backbone of W953 was flipped (Fig. 10A and B). These changes allowed these residues to fit better into density and enabled the strong hydrogen bond between the backbones of α_V L755 and G954 to fit into continuous 1σ 2Fo-Fc electron density (Fig. 10B). We traced generously, i.e. using 0.5σ 2Fo-Fc electron density, the linkers between the ectodomain and the membrane, which have high B factors (Fig. 6B). Our $\alpha_V\beta_3$ -1TM model thus extends to the A958-P959 peptide bond in α_V and the P691-D692 peptide bond in β_3 (Fig. 10B). We were unable to discern any trace of the α_V and β_3 polypeptide chains beyond these positions. Thus, we were unable to build residues α_V M960-I967 or β_3 I693-V695 that were included in the 3IJE model (Fig. 10A). In $\alpha_V\beta_3$ -AB, we were able to build up to α_V residue P959 and β_3 residue P691 (Fig. 10C).

The difficulty of tracing the $\alpha_V\beta_3$ -1TM α_V linker is emphasized by the markedly different position of the A958 sidechain in the two models (Fig. 10A and B). Lack of experimental support for the C-terminal portion of α_V in the 3IJE model is further shown by negative 3σ Fo-Fc difference electron density on the backbone of α_V residues P957, P959, M960, P961, V962, P963, W965, and V966, as well as on the sidechain of W965 (Fig. 10A). Scattered 1σ 2Fo-Fc electron density in this region of the 3IJE backbone (Fig. 10A) did not appear in electron density calculated using the 4G1M model (Fig. 10B), suggesting that this density was due to model bias. Absence of α_V residues 960-967 in our model eliminated negative Fo-Fc difference electron density, and positive electron density did not appear along the omitted backbone (Fig. 10B).

We were also unable to build β_3 TM residues I693-V695 (Fig. 10B). Continuous negative 3σ Fo-Fc electron density is present along the backbone of β_3 residues 691-695 in the 3IJE dataset (Fig. 10A), providing evidence against the path built for these residues. In this region of the electron density map for the 4G1M $\alpha_V\beta_3$ -1TM model, no negative or positive difference electron density appears (Fig. 10B).

Discussion

Differences among crystal structures

Two crystal structures of $\alpha_V\beta_3$ have revealed unanticipated features in this well-studied integrin. The SDL is functionally important for binding macromolecular ligands (36). Although previous structures suggested major conformational differences between this β_3 loop in $\alpha_V\beta_3$ and $\alpha_{IIb}\beta_3$, new $\alpha_V\beta_3$ structures reported here reveal no significant differences. The SDL loop in β_3 is thus little influenced by the associating α -subunit.

Three metal binding-sites are present at the ligand binding site of the integrin βI domain. Density appropriate for a metal ion is present at the MIDAS in $\alpha_V\beta_3$ -1TM, when phases are calculated using 3IJE or 4G1M coordinates. This contrasts with the previous assertion that no metal was present at this site in $\alpha_V\beta_3$ -1TM (14); however, our interpretation of a metal ion at the MIDAS is consistent with report of a density feature at this site in the $\alpha_V\beta_3$ - Δ TM structure (22). In $\alpha_V\beta_3$ -AB only the SyMBS site is occupied, whereas in $\alpha_V\beta_3$ -1TM only the MIDAS and ADMIDAS are occupied (Fig. 5). In contrast, all three metal ion binding

sites are occupied in unliganded $\alpha_{IIb}\beta_3$ crystal structures (18, 21). The SyMBS had previously been designated LIMBS, for ligand-induced or -associated metal binding site (20). However, our $\alpha_V\beta_3$ -AB structure clearly demonstrates that ligand binding is not required for occupancy of this site, supporting earlier observations with $\alpha_{IIb}\beta_3$ and the SyMBS designation (18, 21). Previous studies have shown that binding of Ca^{2+} to the SyMBS and Mg^{2+} to the MIDAS is synergistic (37); this synergism is explicable both by the close proximity of these two metal ion-binding sites, and bidentate coordination of the Glu-220 sidechain to both the SyMBS and ADMIDAS metal ions (Fig. 5C).

The striking differences between metal ion binding to identical β_3 subunits in $\alpha_V\beta_3$ and $\alpha_{IIb}\beta_3$ may be due to crystallization conditions. The use of both Mg^{2+} and Ca^{2+} in $\alpha_{IIb}\beta_3$ (18, 21) and $\alpha_V\beta_3$ -AB crystals, and only Ca^{2+} or only Mn^{2+} in $\alpha_V\beta_3$ - Δ TM and $\alpha_V\beta_3$ -1TM crystals (14, 20, 22, 33) may be one factor. The use of PEG and ammonium sulfate as precipitants in crystallization of $\alpha_V\beta_3$ -1TM and $\alpha_V\beta_3$ -AB, respectively, may also contribute to differences. The different pH ranges at which $\alpha_V\beta_3$ and $\alpha_{IIb}\beta_3$ crystallize may be the major factor. $\alpha_V\beta_3$ - Δ TM crystallized at pH 6.0 (22); $\alpha_V\beta_3$ -1TM was variously reported to crystallize at pH 5.5 in the publication or pH 4.8 in the deposited coordinates (14); and $\alpha_V\beta_3$ -AB crystallized at pH 5.8. Among $\alpha_{IIb}\beta_3$ structures in absence of ligand, the complete ectodomain crystallized at pH 7.0 (18) and the headpiece at pH 8.9 (21). Asp and Glu have pK values of 3.9 and 4.3, respectively. When these residues are close to other negatively charged residues, as they are at the SyMBS, MIDAS, and ADMIDAS, Coulomb's law dictates an increase in pK (38). This effect could easily result in substantial protonation of metal-coordinating residues in the pH range 4.8 to 6.0 used in crystallization of $\alpha_V\beta_3$, and hinder occupation of metal-binding sites. In contrast, the same residues would be more ionized at the physiologic pH of 7.4, and at pH 7.0 and 8.9 as used in $\alpha_{IIb}\beta_3$ crystallization. In contrast to the βI domain sites, the β -propeller β -hairpin Ca^{2+} -binding sites and genu are well occupied in $\alpha_V\beta_3$ crystals. This difference is easily explicable by the close spatial relationship of the SyMBS, MIDAS, and ADMIDAS to one another (38). Furthermore, the MIDAS and ADMIDAS have more water coordinations than any of the other integrin metal ions.

These observations suggest that the lack of simultaneous metal ion-binding to all three βI domain sites in $\alpha_V\beta_3$ is most simply explained as an artifact of crystallization conditions. An alternate explanation, that the sidechain of SyMBS residue Asp-217 orients differently in $\alpha_V\beta_3$ than in $\alpha_{IIb}\beta_3$ (14) encounters the issues that 1) either orientation fits the electron density at 2.9 Å and we have built the same orientation in $\alpha_V\beta_3$ -1TM and $\alpha_V\beta_3$ -AB as in $\alpha_{IIb}\beta_3$; 2) the orientation we have built is required to coordinate the SyMBS metal ion and satisfy all six octahedral coordination positions; 3) Fig. 2B of (20) shows Asp-217 of $\alpha_V\beta_3$ - Δ TM in the same orientation as we have built in $\alpha_V\beta_3$ -1TM; and 4) the Asp-217 orientation built in the 3IJE model is disfavored by overlap of the electron orbitals of O δ 1 of β_3 Asp-217 and O δ 1 of α_V of Asp-219, which are only 2.8 Å apart.

The concept that Ca^{2+} and Mg^{2+} bind synergistically at the SyMBS and MIDAS (18), and that at low pH conditions with $\alpha_V\beta_3$, metal binding and ligand binding are also synergistic, is more plausible than the concept that ligand binding is regulated by or induces metal ion binding (39). It would be difficult for the Asp of RGD to bind to the MIDAS in the absence of a metal ion and in the face of strong repulsion from negatively charged MIDAS residues. Furthermore, the SyMBS site is buried, and metal ion access to this site would be further limited after ligand binding.

The remarkable contact of a furin cleavage site-proximal α_V glycopeptide moiety with the β_3 subunit seen here might also be dependent on crystallization conditions. $(NH_4)_2SO_4$ was the $\alpha_V\beta_3$ -AB precipitant, whereas in the $\alpha_V\beta_3$ -1TM crystal structure with PEG precipitant,

no density for a similar glycopeptide moiety is discernable. These differences are in agreement with the highly polar interactions of the α_V glycopeptide moiety with β_3 , which are expected to be easily replaceable with water or PEG. We found no effect of furin cleavage on basal or Mn^{2+} -stimulated $\alpha_V\beta_3$ ligand binding, although effects might emerge in other assays. Mutational elimination of furin cleavage sites has little effect on ligand binding in $\alpha_{IIb}\beta_3$ and $\alpha_4\beta_1$ (40, 41) but is required for inside-out stimulation of adhesion by $\alpha_6\beta_1$ (42).

Implications for integrin activation

A deadbolt model proposed that removing β -tail domain constraints on the βI domain could induce integrin activation without extension (43). In the original proposal of this hypothesis, it was acknowledged that the β -tail domain CD or “deadbolt” loop had high temperature factors, buried a very small surface area on the βI domain, and was unlikely to contribute stabilizing energy in the conformation seen in the $\alpha_V\beta_3$ - ΔTM structure; therefore, it was suggested that minor rearrangements might result in a larger interface in intact $\alpha_V\beta_3$ on the cell surface (43). However, deletion of the β -tail domain CD loop has no activating effect whatsoever on $\alpha_V\beta_3$ and $\alpha_{IIb}\beta_3$ on cell surfaces (44). Moreover, despite the negligible contact between the β -tail CD loop and the βI domain in $\alpha_V\beta_3$ - ΔTM , there is even less in $\alpha_V\beta_3$ -1TM, none in $\alpha_V\beta_3$ -AB where the CD loop is disordered, and none in $\alpha_{IIb}\beta_3$ (18).

Another interface, between the α_V thigh domain and β_3 I-EGF2 domains, was recently proposed to be an energetic barrier, that when overcome, could enable $\alpha_V\beta_3$ activation in the absence of extension (14). However, electron density is poor at this interface. We found it necessary to substantially rebuild PSI, I-EGF1, I-EGF2, and loops in thigh adjacent to I-EGF2 in our $\alpha_V\beta_3$ -1TM model. Ca atom positions in loops relevant to a proposed interface are shifted substantially between the 3IJE and 4G1M $\alpha_V\beta_3$ -1TM models. Furthermore, the sidechain orientations shown in Fig. 4D in (14) are inconsistent with the associated 3IJE coordinate file, and suggest that different coordinate files were used for figure preparation and coordinate deposition. Thus, Fig. 4D in (14), but not the 3IJE coordinates (Fig. 8A) show hydrogen bonds between the α_V Glu-547 sidechain and β_3 Asp-477 backbone, and between the α_V Phe-548 backbone and β_3 Glu-476 sidechain. However, questionable hydrogen bonds between the sidechains of α_V Asp-550 and β_3 Glu-500, and between the backbone carbonyl of α_V Glu-547 and sidechain of β_3 Glu-476, remain in the 3IJE coordinate file (Fig. 8A). The 4G1M $\alpha_V\beta_3$ -1TM coordinate file lacks favorable, as well as unfavorable polar interactions in the same region (Fig. 8B). The lack of a stable interface here, and its irrelevance for regulation of integrin affinity state, is further supported by its marked reorientation in $\alpha_V\beta_3$ -AB (Fig. 8C), with no attendant reshaping of the βI domain ligand binding site. These results are consistent with extensive mutagenesis evidence that the loop between cysteines 1 and 2 of I-EGF2 acts as an entropic spring, and that its length, but not its sequence, is important in regulating the equilibrium between bent and extended integrin conformations (45).

In $\alpha_V\beta_3$ -1TM, we were unable to trace α_V linker residues Met-960 to Pro-963, α_V TM residues Val-964 to Ile-967, or β_3 TM residues Ile-693 to Val-695. Strong evidence against the placement of α_V residues 960-967 and β_3 residues 693-695 is shown by the presence of 3 σ negative difference electron density along this backbone and on the sidechain of α_V Trp-965 in 3IJE (Fig. 10A), and the lack of appearance of positive difference electron density in the corresponding regions of the 4G1M $\alpha_V\beta_3$ -1TM model (Fig. 10B). Flexibility in the α_V and β_3 linkers is further evidenced by differences in orientation between $\alpha_V\beta_3$ -1TM and $\alpha_V\beta_3$ -AB beginning with α_V residue Gln-956 and β_3 residue Lys-689 (Fig. 10B and C).

Flexibility of ectodomain linker residues α_V 956-963 and β_3 689-692 is to be expected from the lack of stabilizing interactions in the linker structures (Fig. 10B and C). The last residue well supported by structural interactions in α_V is G954, which has a backbone hydrogen bond to L755 (Fig. 10B and C). Similarly, the last such residue in β_3 is C687, which is disulfide-bonded to C663 (Fig. 10B and C).

Our conclusion that the α_V and β_3 linkers are flexible is in excellent agreement with a similar lack of density for the corresponding regions in $\alpha_{IIB}\beta_3$ and $\alpha_X\beta_2$ crystal structures (18, 19). Furthermore, the use of disulfide cross-linking restraints in the linker regions of intact $\alpha_{IIB}\beta_3$ on cell surfaces showed that the α_{IIB} and β_3 linkers are flexible, and even without any motions internal to the bent conformation of $\alpha_{IIB}\beta_3$, enable marked change in orientation of the ectodomain with respect to the membrane (15). However, there are limits to linker flexibility; coupling of conformational change between extracellular and TM domains is disrupted by insertions of 10 to 11 extra residues (15, 46). The finding that signals can be transmitted across membranes despite flexibility of extracellular linkers has also emerged in the epidermal growth factor receptor field, where spatial proximity between two monomers, rather than precise orientation, is important in transmembrane signaling by dimers (47).

We also reveal differences in positions of the structured domains in the two snapshots of the ectodomain in $\alpha_V\beta_3$ -1TM and $\alpha_V\beta_3$ -AB. A large number of leg domains, three in α and six in β , intervene between integrin ligand-binding domains and the ectodomain-TM domain linkers. As demonstrated in variation in orientation between these leg domains in $\alpha_V\beta_3$ -1TM and $\alpha_V\beta_3$ -AB (Table 2), changes in TM domain orientation could be dissipated by change in leg domain orientation. Any alternative theory of integrin activation within the bent conformation should propose a specific interdomain pathway for signal transmission, and a change in orientation between the α and β subunit TM domains sufficiently large in magnitude to prevent damping of transmission by ectodomain flexibility. The only specific pathway in the ectodomain described to date for transmitting conformational change to the ligand-binding site is through hybrid domain swing-out (4). Such a large conformational change, including a 70 Å increase in separation at the knees, is incompatible with maintenance of a compact, bent conformation.

Many mutational studies on α_V and β_3 support the importance of integrin extension, lower leg separation, and headpiece opening in integrin activation. Interfaces between the α_V and α_{IIB} calf-2 domain and β_3 I-EGF4, β -ankle, and β -tail domains are required for maintaining the low-affinity state; these interfaces prevent lower leg separation (48, 49). Interfaces between hybrid domain and β -tail domain (50), and between the α_V β -propeller domain and the upper β -leg (51) stabilize the low-affinity state by preventing integrin extension. Mutations that open up disulfide bonded loops in the I-EGF domains (52, 53) or introduce bulky residues (54) or N-glycosylation sites (55) into portions of the β -leg that are buried in the bent conformation activate $\alpha_V\beta_3$ and $\alpha_{IIB}\beta_3$; these mutations destabilize the bent conformation. Mutational introduction of disulfides to prevent integrin extension (2, 56), headpiece opening (2, 56), or lower leg separation (56) abolish activation of $\alpha_V\beta_3$. Stabilizing the open headpiece relative to the closed headpiece with glycan wedges or computationally predicted mutations stimulated $\alpha_V\beta_3$ and $\alpha_{IIB}\beta_3$ integrin activation and LIBS epitope exposure (8, 57). Conversely, stabilizing the closed headpiece with a disulfide bond in the βI domain or computationally predicted mutations inhibited activation (57, 58).

In summary, extensive mutagenesis and structural studies of $\alpha_V\beta_3$ and $\alpha_{IIB}\beta_3$ have demonstrated the importance of multiple interfaces between the integrin headpiece and lower legs, and between the integrin α - and β -legs, that are present in the bent conformation and restrain integrin activation. These interfaces involve large buried surface areas and well-

ordered sidechains, and thus contrast with the interfaces discussed above between the β -tail and β I domains and between the α - and β -knees. Integrin extension, lower leg separation, and headpiece opening have each been shown to be required for integrin activation. The structures described here provide a sound foundation for better understanding of the complex molecular mechanisms that regulate integrin activation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

MIDAS	Metal ion-dependent adhesion site
ADMIDAS	adjacent to MIDAS
SyMBS	synergistic metal binding site
TM	transmembrane
LIBS	ligand-induced binding site
EM	electron microscopy
FRET	fluorescence resonance energy transfer
SAXS	small angle X-ray scattering
PDB	Protein Data Bank
TEV	tobacco etch virus
APS	Advanced Photon Source
MR	molecular replacement
ML	maximum likelihood
NMR	nuclear magnetic resonance
SDL	specificity-determining loop

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Figure 1.

The three major integrin conformational states. A. Bent (with closed headpiece). B. Extended with closed headpiece. C. Extended with open headpiece. The dashed lower β-legs show that because of β-leg flexibility in the extended conformation, the closed and open headpieces are each compatible with TM domain close association and separation. However, a lateral force exerted by the cytoskeleton on the β-subunit can enforce TM domain separation and the open headpiece conformation (1).

Figure 2. Overall structures of two $\alpha_v\beta_3$ crystal forms and comparison to $\alpha_{IIb}\beta_3$. A-C. Cartoons of $\alpha_v\beta_3$ -AB (A), $\alpha_v\beta_3$ -1TM (4G1M structure, B), and $\alpha_{IIb}\beta_3$ (C). Ca^{2+} and Mg^{2+} ions are shown as silver spheres. Disulfides are shown as gold sticks, and glycans are displayed with gray carbons.

Figure 3.

Difference maps. Difference maps of 3IJE (A) and 4G1M (B) $\alpha_V\beta_3$ -1TM structures. Fo-Fc difference density is shown in green (+3 σ) and red (-3 σ). The head and upper legs of α_V and β_3 are shown in light blue and wheat, respectively, and the lower α_V and β_3 legs are shown in yellow and black, respectively. C-termini are labeled for α_V and β_3 .

Figure 4.

The conformation of the specificity-determining loop in the β I domain. The SDL and surrounding area (A-D) or a portion of the SDL loop (E-G) are shown for the 3IJE (A and E) or 4GIM (B and F) $\alpha_v\beta_3$ -1TM structures, $\alpha_v\beta_3$ -AB (C and G), and $\alpha_{IIB}\beta_3$ (D). β - and α -subunits are wheat and light blue, respectively. Mesh in E-G shows 2 $F_o - F_c$ density at 1 σ (grey) or $F_o - F_c$ difference density at 3 σ (green) and -3 σ (red).

Figure 5. The metal ion binding sites in the βI domain. A-C. Differences in metal binding to the βI domain among $\alpha_v\beta_3$ -AB (A), $\alpha_v\beta_3$ -1TM (B), and $\alpha_{IIB}\beta_3$ (C). D-F. Density at the metal-binding sites. D. The SyMBS in $\alpha_v\beta_3$ -AB. E and F. The MIDAS in 3IJE (E) and 4G1M (F) $\alpha_v\beta_3$ -1TM structures. Black mesh shows $2F_o-F_c$ density at 1σ , and green and red mesh show F_o-F_c difference density at 3σ and -3σ , respectively. Ca^{2+} and Mg^{2+} ions are shown as gold and silver spheres, respectively. Oxygens are red and waters are small red spheres. Dashed lines in A-C show metal coordination.

Figure 6.

Flexibility among $\alpha_v\beta_3$ crystal forms and $\alpha_{IIb}\beta_3$ assessed by B factor distributions. The orientations are similar to those in Fig. 2A-C, except in the lower panels the integrins are rotated about the axis vertical in the page. Integrins were superimposed as in Fig. 2, and separated horizontally on the page. C α -traces show B factors in rainbow, set using the lowest and highest C α B factors in each integrin as the blue and red ends of the rainbow, respectively. The B factors shown include contributions from TLS. The genu Ca^{2+} ions are shown as spheres and also colored by B factor. Disulfide bonds are silver. The names of the α_v , α_{IIb} , and β_3 subunits are shown next to their C-termini.

Figure 7.

The I-EGF domains at the β ₃-knee. A-G. I-EGF1 and I-EGF2 domains from structures determined here, the α IIb β ₃ ectodomain (18), or the closed α IIb β ₃ headpiece (PDB ID 3T3P) (35) were superimposed, and are shown in identical orientations aligned vertically and horizontally on the page. C α ribbons are rainbow colored from lowest (blue) to highest (red) C α B-factor in each domain. Disulfide bonds are color-coded and cysteines numbered according to the same scheme as shown in panel I. Selected sidechains (S469 and Q470 in I-EGF1 and the equivalent K519 and Y520 in I-EGF2) are shown. The C-terminus of I-EGF1 is indicated by showing the C α -C bond of C8 in silver. H. The markedly different orientation of the I-EGF1 domain C7-C8 loop in a closed headpiece crystal structure (blue)

is shown by superimposing the I-EGF1 domain of $\alpha_v\beta_3$ -AB (red). Surrounding molecules in the closed headpiece crystal lattice are shown in different colors. I. Sequence alignment of the β_3 -integrin I-EGF domains.

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Figure 8.

Lack of stable interfaces between I-EGF2 and thigh domains. Interfaces are shown in identical orientations after superposition of the ectodomains using the super command of PyMol. Thigh (green) and I-EGF2 (yellow) are shown with red oxygen and blue nitrogen atoms. All inter-subunit hydrogen bonds found as polar contacts by PyMol are shown with dashed lines. Only 2 of the 11 hydrogen bonds shown in Fig. 4D of Xiong *et al.* (14) appear in (A), either because highly solvated carboxyl-carboxyl and carboxyl-carbonyl interactions are considered repulsive at neutral pH or the distances are too far; the orientations shown in Fig. 4D of reference (14) differ markedly from the deposited 3IJE coordinates. All sidechain or backbone atoms shown in Fig. 4D of reference (14) are shown in stick.

Figure 9.

Interaction of an α_v glycopeptide moiety near the furin cleavage site with β_3 . A. Interaction of the glycopeptide with the β -subunit. The glycan N-linked to α_v -Asn-844 of the calf-2 domain (orange) inserts into the pocket formed by I-EGF4 domain (light blue), β -ankle (pink), β -tail domain (green), hybrid domain (yellow) and β I domain (red). Carbohydrate residues are displayed with gray carbons. α_v residues 838, 841, 848, and 867 are marked, which are adjacent to positions missing in density. B. Verification of furin cleavage site mutation by immunoprecipitation of [³⁵S]-labeled $\alpha_v\beta_3$ followed by reducing SDS 7.5% PAGE and fluorography. C and D. Ligand binding to cells with wild-type or mutant $\alpha_v\beta_3$ measured by fluorescent flow cytometry. Binding of fluorescently labeled fibrinogen or fibronectin to transfectants was tested in 5 mM EDTA, 5 mM Ca²⁺, or 1 mM Mn²⁺. The wedge mutant (8) was used as positive control. Results are expressed as mean fluorescent intensity of Alexa-488 conjugated ligand as a percentage of mean fluorescent intensity of Cy3-conjugated AP3 antibody to β_3 .

Figure 10.

The structure of the linker between the $\alpha_V\beta_3$ ecto and transmembrane domains. A. 3IJE $\alpha_V\beta_3$ -1TM. B. 4G1M $\alpha_V\beta_3$ -1TM. C. $\alpha_V\beta_3$ -AB. Shown are the C-terminal α_V and β_3 extensions together with the C-terminal portions of calf-2 and β -tail domains as a Ca -ribbon, together with sidechains and backbones of selected regions. Hydrogen bonds near the end of the calf-2 and β -tail domains are shown as red dashed lines. Electron density is shown as mesh around α_V residues 755 and 953-967 and β_3 residues 663 and 685-695. Mesh for 1σ 2Fo-Fc, -3σ Fo-Fc, and 3σ Fo-Fc electron density is shown in blue, red, and green, respectively. CCP4 format maps were opened and displayed with the isomesh command in PyMol using $\text{carve} = 2.4$ and $1.9\text{ \AA}$ for 2Fo-Fc and Fo-Fc maps, respectively. Mesh was calculated in A using 3IJE data and in B using 4G1M data. In each of A and B, mesh was separately calculated (carved) around the positions of atoms in both the 3IJE and 4G1M models, and the combined mesh is displayed. The view is similar to that in Fig. 4A of (14).

Table 1

Statistics of X-ray diffraction and structure refinement

Protein	$\alpha_V\beta_3$ -AB	$\alpha_V\beta_3$ -1TM	
Data collection statistics			
Space group	P3221	P3 ₂ 21	
$\alpha, \beta, \gamma, ^\circ$	90, 90, 120	90, 90, 120	
Unit cell (a = b, c), Å	128.46, 352.49	130.26, 305.98	
Resolution range (Å)	50.0-2.85(2.85-2.92)	65-2.9	
Completeness (%)	99.9 (100.0)	99.2 (98.5)	
Number of unique reflections	79,656 (5,806)	66,702 (6,515)	
Redundancy	7.4 (6.6)	2.9 (2.2)	
R _{sym} (%)	17.5 (457.6)	8.8 (100)	
I/ σ	10.2 (0.6)	10.5 (100) [‡]	
CC _{1/2} (%) ^e	99.7(13.6)	NA	
Wavelength (Å)	1.034	1.0332	
Refinement statistics			
	4G1E	3IJE	4G1M
Resolution range (Å)	50.0-2.85	20-2.9	65-2.9
R _{work} (%)	24.1	24.4	18.0
R _{free} (%)	26.1	28.7	23.4
Model statistics			
Bond RMSD (Å)	0.005	0.006	0.005
Angle RMSD (°)	0.6	1.086	0.735
Ramachandran plot ^d (Favored/allowed/outlier)	95.8/4.0/0.2	88.4/8.5/3.1	93.2/6.7/0.1
MolProbity percentile ^d (Clashscore/Geometry)	97/100	91/66	97/99
Residue range			
α_V	1-619, 621-838, 841-848, 867-959	1-838, 868-967	1-837, 868-959
β_3	1-33, 35-478, 483-691	1-695	1-692
Number of metals	6	6	7
Number of carbohydrates	48	34	37
Number of <i>cis</i> -Proline	7	0	7
Number of waters	104	0	108

[†]The number in parentheses cannot be correct, but cannot be recalculated by us, and is shown as originally reported.

^aThe numbers in parentheses refer to the highest resolution shell.

^b $R_{\text{merge}} = \sum_i \sum_h |I_i(h) - \langle I(h) \rangle| / \sum_i \sum_h I_i(h)$, where $I_i(h)$ and $\langle I(h) \rangle$ are the i th and mean measurement of the intensity of reflection h .

^c $R_{\text{factor}} = \sum_h |F_{\text{obs}}(h) - F_{\text{calc}}(h)| / \sum_h |F_{\text{obs}}(h)|$, where $F_{\text{obs}}(h)$ and $F_{\text{calc}}(h)$ are the observed and calculated structure factors, respectively. No I/σ cutoff was applied.

^dCalculated with the described server (30).

^e Pearson's correlation coefficient between average intensities of random half-datasets for unique reflection (28).

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Table 2RMSDs between 3IJE and 4G1M $\alpha_V\beta_3$ -1TM structures.^a

Domain	Residues	3IJE/4G1M non-H atoms (Å)	3IJE/4G1M C α atoms (Å)
PSI	1-56+434-435	1.9	1.4
Hybrid	57-108+353-433	1.4	0.5
β I	109-352	1.3	0.7
I-EGF1	436-472	1.9	1.3
I-EGF2	473-522	2.9	1.4
I-EGF3	523-559	1.2	0.6
I-EGF4	560-600	1.5	0.8
Ankle	601-605	0.4	0.2
β -tail	606-690	1.3	0.7
linker	691-692	1.5	2.1
β -propeller	1-438	0.8	0.3
Thigh	439-594	1.3	0.6
Calf-1	595-737	1.8	0.9
Calf-2	738-954	1.5	1.1
Linker	955-959	2.0	1.6
Overall		1.4	0.8

^aRMSD was calculated using rms_cur command of PyMol.