Structural basis for pure antagonism of integrin $\alpha_v\beta_3$ by a high-affinity form of fibronectin

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Integrins are important therapeutic targets. However, current RGD-based anti-integrin drugs are also partial agonists, inducing conformational changes that trigger potentially fatal immune reactions and paradoxical cell adhesion. Here we describe the first crystal structure of $\alpha_v\beta_3$ bound to a physiologic ligand, the tenth type III RGD domain of wild-type fibronectin (wtFN10), or to a high-affinity mutant (hFN10) shown here to act as a pure antagonist. Comparison of these structures revealed a central $\pi-\pi$ interaction between Trp1496 in the RGD-containing loop of hFN10 and Tyr122 of the $\beta_3$ subunit that blocked conformational changes triggered by wtFN10 and trapped hFN10-bound $\alpha_v\beta_3$ in an inactive conformation. Removing the Trp1496 or Tyr122 side chains or reorienting Trp1496 away from Tyr122 converted hFN10 into a partial agonist. These findings offer new insights into the mechanism of integrin activation and a basis for the design of RGD-based pure antagonists.

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a physiologically relevant ligand: the FN10 domain. We show that in contrast to wtFN10, a high-affinity form of this ligand (hFN10) acts as a pure antagonist for αvβ3. To clarify the structural basis for this activity, we also determined the crystal structure of hFN10–αvβ3. Comparisons of the structures revealed a novel mechanism underlying pure antagonism by an RGD-containing ligand that suggests a path to the generation of RGD-based drugs that can act as pure antagonists.

RESULTS

Binding of hFN10 to resting and activated cellular αvβ3

We compared the integrin binding properties of wtFN10 to those of a high-affinity form, hFN10, that was selected for specific αvβ3 binding from a FN10 phage display library, in which five residues N- and C-terminal to the RGD motif were randomized. In hFN10, the sequence 1492PRGDWEG1499 replaces 1492GRGDSPAS1499 of wtFN10. Interestingly, RGDW is also the core sequence in the disintegrin barbourin (on which the drug eptifibatide was based), excepting an arginine-to-lysine substitution, which enhances the specificity of barbourin for platelet αIIbβ3 (ref. 14).

The binding of fluoresceinated fluid-phase wtFN10 to stably expressed wild-type αvβ3 in K562 cells (K562-αvβ3) was a low background in physiological concentrations of Ca2+ and Mg2+ (Ca2+/Mg2+, 1 mM each) (Fig. 1a), as we expected, because soluble physiologic ligands do not bind to the inactive integrin in physiologic Ca2+-Mg2+ containing buffers. Binding of soluble wtFN10 was increased ten-fold in the presence of the integrin activator Mn2+, a mimic of inside-out activation (Fig. 1a), or was increased six-fold by an N339S mutation in the β3 domain, which constitutively activates αvβ3 both in vitro and in vivo (Fig. 1b).

By contrast, strong binding of soluble fluoresceinated hFN10 to K562-αvβ3 occurred in Ca2+-Mg2+ (six-fold compared to that of wtFN10), and was increased a further ~1.5-fold by Mn2+ (Fig. 1a). Binding of hFN10 was similar both on wild-type αvβ3 and αvβ3 N339S, each of which was transiently expressed on HEK293T cells (Fig. 1b).

Effects of hFN10 binding to αvβ3

The integrin activator Mn2+ induces expression of the epitopes of LIBS monoclonal antibodies (mAbs) AP5 (ref. 5), LIBS-1 and LIBS-2 (ref. 6), each of which recognizes distinct epitopes in the β3 subunit. The mAb AP5 recognizes an N-terminal sequence in the PSI (plexin-semaphorin-integrin) domain5, the mAb LIBS-1 recognizes a different epitope from AP5 and LIBS-6 binds the C-terminal membrane proximal βTD domain. LIBS expression is further increased after binding of physiologic ligand5.

As we expected, Mn2+-driven binding of wtFN10 to K562-αvβ3 increased the expression of the LIBS for mAbs AP5, LIBS-1 and LIBS-6 above that induced by Mn2+ alone (Fig. 1c). In contrast, binding of hFN10 not only failed to induce LIBS expression but also substantially decreased LIBS expression induced by Mn2+ alone (Fig. 1c). We saw this effect whether αvβ3 was expressed artificially (on K562 cells) or constitutively (on melanoma M21 cells). And whereas binding of wtFN10 to the constitutively active αvβ3 N339S integrin increased AP5 epitope expression by approximately two-fold, binding of hFN10 did not (Fig. 1d).

Figure 1 Binding properties of hFN10 and wtFN10 to αvβ3 (a–d) Binding of fluoresceinated wtFN10 and hFN10 (a, b) or LIBS mAbs alone or in the presence of wtFN10 and hFN10 (c, d) to αvβ3 cells. In e, mAb binding was assessed using K562-αvβ3 and M21 cells. FMI, mean fluorescence intensity. The histograms in a–d represent the mean ± s.d. of n = 3 independent experiments. (e) Hydrodynamic analyses of unliganded αvβ3 and αvβ3–FN10 complexes in the presence of Ca2+, Ca2+/Mg2+ or Mn2+. Stokes radii (in nm) are shown in parentheses. AU, absorbance unit; OD280, optical density at 280 nm. (f) Mn2+-induced spreading of K562-αvβ3 on wtFN10, hFN10 or full-length FN (for wtFN10 and hFN10, the data are shown as the mean ± s.d. of n = 3 independent experiments; for full-length FN, results from two independent experiments are shown). Spreading under all conditions was eliminated by the mAb LM609 against αvβ3 (not shown). (g, h) Representative phase contrast images of K562-αvβ3 spreading on wtFN10 (g) and hFN10 (h). Scale bars, 20 μm.
It is known that Mn\(^{2+}\) or the binding of soluble RGD-based ligands induces conformational changes in the \(\alpha_5\beta_3\) ectodomain, as detected by changes in the hydrodynamic radius of the ectodomain.\(^{4,26,27}\) We therefore examined the effects of binding of hFN10 to the \(\alpha_5\beta_3\) ectodomain in solution by comparing the hydrodynamic radii of \(\alpha_5\beta_3\)-hFN10 and \(\alpha_5\beta_3\)-wtFN10 complexes by molecular sieve chromatography.\(^{3,4}\) We performed chromatography on preformed \(\alpha_5\beta_3\)-hFN10 and \(\alpha_5\beta_3\)-wtFN10 complexes (Supplementary Fig. 1a) on a molecular sieve column equilibrated in the relevant metal ion–containing buffer, and we derived Stokes' radii as described previously.\(^{4}\) The complexes displayed markedly different profiles (Fig. 1e and Supplementary Fig. 2b): wtFN10 increased the Stokes' radius of \(\alpha_5\beta_3\) in Mn\(^{2+}\) (6.6 nm) compared to the integrin size in Mn\(^{2+}\) alone (\(R_s = 6.3\) nm), as we expected. However, hFN10 had little effect on the \(R_s\) of \(\alpha_5\beta_3\) in Mn\(^{2+}\) (6.3 nm) or in Ca\(^{2+}\)-Mg\(^{2+}\) (6.0 nm compared to 5.9 nm in the absence of hFN10).

Cell spreading is a reporter of ligand-induced outside-in signaling.\(^{28}\) To determine the effect of hFN10 on spreading, we compared the spreading of \(\alpha_5\beta_3\)-expressing cells on surfaces coated with native full-length FN (positive control) (Fig. 1f), wtFN10 (Fig. 1f,g) or hFN10 (Fig. 1f,h). After 2 h, approximately 90% of attached cells spread on native FN, and 60% spread on wtFN10. In contrast, less than 20% of attached cells spread on hFN10. Cell attachment under all conditions was eliminated when assays were carried out in the presence of the function-blocking LM609 mAb against \(\alpha_5\beta_3\) (data not shown).

Crystal structures of \(\alpha_5\beta_3\)-wtFN10 and \(\alpha_5\beta_3\)-hFN10 complexes

To clarify the structural basis for the inhibitory effects of bound hFN10 on conformational changes and function of \(\alpha_5\beta_3\), we soaked ligands hFN10 or wtFN10 into crystals of the \(\alpha_5\beta_3\) ectodomain\(^4\) in 2 mM MnCl\(_2\) and determined the crystal structures of the resulting \(\alpha_5\beta_3\)-hFN10 and \(\alpha_5\beta_3\)-wtFN10 complexes (Fig. 2a,b, Supplementary Fig. 2 and Table 1). hFN10- or wtFN10-bound \(\alpha_5\beta_3\) remained gemini, with each ligand bound at the integrin head, as we expected. However, orientation of FN10 relative to the \(\beta\)A domain differed markedly between the two complexes, with a ~60° rotation around the RGD loop (Fig. 2c). \(F_o - F_c\) omit maps (generated after omitting the FN10 ligand) revealed clear positive densities (Supplementary Fig. 2c,d), reflecting stable engagement of the integrin head by ligand. The omit maps showed clear density for the complete hFN10 domain but for only ~60% of wtFN10 (that portion facing the integrin), with the wtFN10 portion farthest away from the integrin showing minimal density, consistent with its low affinity and the likely flexibility of this region in the crystal.

The RGD motif of each ligand bound the \(\alpha_5\beta_3\) head in an identical manner (Fig. 3a,b), and as shown previously for the RGD-containing pentapeptide cilengitide\(^{1,3}\), RGD inserted into the crevice between the propeller and \(\beta\)A domains and contacted both. The \(\alpha_5\beta_3\)-wtFN10 interface was modestly larger than the \(\alpha_5\beta_3\)-cilengitide interface, mainly because of contacts wtFN10 made with the glycans at the propeller residue Asn266, which included hydrogen bonds with mannos 2271 (MAN2271) (Fig. 3a). An N266Q

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Table 1 Data collection and refinement statistics

|             | \(\alpha_5\beta_3\)-hFN10 | \(\alpha_5\beta_3\)-wtFN10 | \(\alpha_5\beta_3\)-hFN10/8 |
|-------------|--------------------------|--------------------------|--------------------------|
| **Data collection** |                           |                           |                           |
| Space group  | \(P3_121\)               | \(P3_121\)               | \(P3_121\)               |
| Cell dimensions | \(a, b, c\) (Å)         | 129.8, 129.8, 307.6      | 129.7, 129.7, 305.8      | 130.0, 130.0, 308.2       |
| \(\alpha, \beta, \gamma\) (°) | 90, 90, 120             | 90, 90, 120              | 90, 90, 120              |
| Resolution (Å) | 50–3.1 (3.21–3.1)        | 75.49–3.32 (3.5–3.32)    | 50–3.17 (3.28–3.17)      |
| \(\text{R}_{\text{work}}^{\alpha} / \text{R}_{\text{free}}^{\alpha}\) | 27.1 (2.3)               | 13.9 (3.3)               | 12.6 (2.2)               |
| Completeness (%) | 99.9 (100)               | 88.0 (88.0)              | 99.7 (99.7)              |
| Redundancy | 6.1 (6.1)               | 6.2 (6.4)               | 5.3 (5.1)               |
| **Refinement** |                           |                           |                           |
| Resolution (Å) | 49.3–3.1                 | 42.5–3.32                | 49.4–3.18                |
| Number of reflections | 55,243                 | 39,536                   | 51,260                   |
| \(R_{\text{work}} / R_{\text{free}}\) | 20.5 / 25.5             | 21.1 / 25.8              | 20.5 / 23.9              |
| Number of atoms | 13,501                   | 13,626                   | 13,000                   |
| Protein | 12,794                   | 12,922                   | 12,922                   |
| Ligand / ion |                             |                           |                           |
| FN10 | 690                       | 694                       | 65                       |
| Mn\(^{2+}\) | 8                        | 8                        | 8                        |
| Water | 9                         | 2                         | 5                        |
| **B factors** |                           |                           |                           |
| All atoms (Å\(^2\)) | 116.7                    | 102.8                    | 75.8                     |
| Protein | 114.2                    | 98.5                      | 75.7                     |
| Ligand / ion |                             |                           |                           |
| FN10 | 163.2                     | 181.8                     | 83.7                     |
| Mn\(^{2+}\) | 135.9                     | 102.9                     | 80.5                     |
| Water | 95.2                      | 67.7                      | 54.8                     |
| **r.m.s. deviations** |                           |                           |                           |
| Bond lengths (Å) | 0.004                    | 0.003                     | 0.005                    |
| Bond angles (°) | 0.89                     | 0.9                       | 0.98                     |

\(^{\text{a}}\)Values in parenthesis are for the highest-resolution shell. One crystal was used for each data set.
substitution in cellular αβ3 did not impair heterodimer formation (as judged by binding of the heterodimer-specific mAb LM609; data not shown) but did reduce the adhesion of HEK293T cells expressing the constitutively active mutant integrin αvβ3266Qβ339S to immobilized full-length FN by 56% compared to adhesion mediated by αvβ3266Qβ339S in Ca2+-Mg2+-buffer (P = 0.003, n = 3 independent experiments) (Supplementary Fig. 3a).

One structural feature that coincided with the higher affinity of hFN10 compared to wtFN10 was the more extensive αvβ3–hFN10 interface, which was largely due to the additional and distinct contacts hFN10 made mainly with the MIDAS face and with the specificity-determining loop of the βA domain (Fig. 3b). These contacts contributed to the different orientation of hFN10 on αvβ3 and the coordination patterns of the Mn2+ ion at MIDAS and ADMIDAS (Fig. 3b). In contrast to the αvβ3–wtFN10 interface, hFN10 made no contacts with the propeller glycans at Asn266. At the center of the hFN10–βA contacts was a π–π edge-to-face interaction of the mutant Trp1496 in hFN10 with Tyr122 in the αβ3 helix of βA (Fig. 3b). Tyr122 also hydrogen bonded to Glu1462 of hFN10, which formed a salt bridge with Lys125 of βA. The hydroxyl oxygen of hFN10 Tyr1446 also coordinated the Mn2+ at ADMIDAS through a water molecule. The outcome of these interactions was that the tertiary changes induced by the physiologic ligand wtFN10 (i.e., inward movement of the N-terminal α helix toward MIDAS and reorganization of the C-terminal F strand and α7 loop) were absent in the hFN10-bound βA domain (Fig. 3c).

Indeed, the structure of hFN10-bound βA was superimposable on that of the unliganded βA domain (Fig. 3c). That is to say, the hFN10-bound βA domain assumed the same conformation as the unliganded βA domain. The hFN10-bound αvβ3 structure also displayed other features of an inactive state4 that were absent from the αvβ3–wtFN10 structure. These included electrostatic interactions between the two subunits at the β-subunit ‘knee’ (β-genus) (Supplementary Fig. 3b) and between βA and βTD within the β-subunit (Supplementary Fig. 3c). Together these findings suggest that when bound to hFN10, αvβ3 at or close to an inactive ground state that would not transduce outside-in signals, despite ligand occupancy.

The Trp1496–Tyr122 π–π interaction was crucial for blocking conformational changes induced by the binding of ligand, as shown in mutational studies. Removing the Trp1496 side chain through Trp1496 substitution to serine (hFN10W/S) converted hFN10 into a partial agonist that could induce binding of the LIBS mAb AP5 to M21 cells in the absence (control) or presence of unlabeled wtFN10, hFN10 or hFN10W/S. Right, binding of fluoresceinated AP5 to αvβ3+ or αvβ3Y122A+ HEK293T cells in presence of unlabeled wtFN10 or hFN10. Histograms represent the mean ± s.d. of n = 3 independent experiments.

Reorienting Trp1496 converts hFN10 into a partial agonist

The core RGDWN sequence in hFN10 matches that in barbourin, a partial agonist of αvβ3. Yet superposition of the RGD-containing loops in hFN10, barbourin (PDB 1IQ7) and the drug epifibatide (PDB 2VDN14) revealed drastically different orientations of the tryptophan side chain in hFN10 compared to those in barbourin and epifibatide. In contrast to the Trp1496 side chain in hFN10, this tryptophan side chain points inwards toward the center of the RGD loop in the barbourin and epifibatide structures, away from

Figure 3 αβ3–FN10 interfaces, conformational changes and structure validation. (a,b) Ribbon diagrams showing key electrostatic and hydrogen bond interactions and metal-ion coordinations in the structures of αβ3–wtFN10 (a) and αβ3–hFN10 (b). The chain colors are as in Figure 2. The inset in b shows an enlarged view of the αα-weighed 2Fo – Fc map contoured at 1.0σ of the Trp1496 and Tyr122 side chains in the αβ3–hFN10 complex. Inward movement (blue arrow) of Tyr122 (light green) in wtFN10-bound βA would clash with the Trp1496 side chain. (c) Left, the βA domain from αβ3–hFN10 (pink) superimposed on that of αβ3–wtFN10 (light green) and on the βA domain (dark green) from unliganded αβ3 (PDB ID 3JIE) (right). The blue arrow in the left structure in c indicates the direction of wtFN10-induced inward movement of the α1 helix (and ADMIDAS ion) toward MIDAS. Spheres representing the three metal ions bear the color of the respective βA. The major tertiary change observed in the F-α7 loop of wtFN10-bound βA (c, left) was not translated into a one-turn displacement of α7, possibly because of crystal contacts when the complete ectodomain is used in crystallization. Except for ligand occupancy and the resulting changes in the specificity-determining loop (SDL), the structures of the unliganded and hFN10-bound βA domains are identical (c, right) (LIMBS and MIDAS are not occupied by metal in unliganded βA). (d) Left, binding of fluoresceinated AP5 to M21 cells in the absence (control) or presence of unlabeled wtFN10, hFN10 or hFN10W/S. Right, binding of fluoresceinated AP5 to αvβ3 or αvβ3Y122A+ HEK293T cells in presence of unlabeled wtFN10 or hFN10. Histograms represent the mean ± s.d. of n = 3 independent experiments.
the critical Tyr122 side chain of the βA domain (Fig. 4a). Binding of either barbourin or eptifibatide induces expression of AP5 LIBS14. We attempted to modify the orientation of the Trp1496 side chain in hFN10 to resemble that in barbourin or eptifibatide. We mutated three loop residues (underlined) flanking the RGWDWN sequence in hFN10 (TPRGDWNE) to match the barbourin sequence (IAKGDWND) and purified this domain (hFN10/B). The crystal structure of the αvβ3–hFN10/B complex (Supplementary Fig. 4) showed that the main chain and side chain structures of the RGD-loop residues IARGDW were clear in the complex, but the rest of the hFN10/B domain was not visible, likely because the orientation of the Trp1496 side chain in hFN10 so that it no longer faces the Tyr122 side chain of βA (Fig. 4c). This reorientation was associated with the unhindered inward movement of Tyr122 in the α1 helix, as in the eptifibatide-bound integrin14. The binding of hFN10/B to cellular αvβ3 consistently induced expression of the LIBS for mAb AP5, similar to that induced by wtFN10 (Fig. 4d and Supplementary Fig. 5).

DISCUSSION

In this study we report the crystal structures of integrin αvβ3 in complex with an RGD-bearing domain of the physiologically relevant macromolecular ligand fibronectin (wtFN10) and with a high-affinity form of this domain of fibronectin (hFN10) carrying substitutions adjacent to the RGD sequence. Our major finding is that hFN10 unexpectedly acts as a pure antagonist of αvβ3 and lacks the partial agonism of native ligands that is also observed in other RGD-like ligands.

When it bound to cellular αvβ3, hFN10, unlike wtFN10, did not induce activation-specific conformational LIBS mAb epitopes in the integrin N- and C-terminal domains, and it reduced LIBS expression induced by both the activating cation Mn2+ and the constitutive (mutational) activation of αvβ3. In addition, hFN10 bound to but did not substantially alter the hydrodynamic behavior of the soluble αvβ3 ectodomain, unlike wtFN10 or cyclic RGD-based peptides. Cell spreading on immobilized hFN10 was also significantly reduced compared with wtFN10 (P = 0.002) and was substantially reduced compared with native FN. Cell spreading reports outside-in signaling by ligand–occupied integrins28.

The αvβ3–hFN10 structure defines the interface an integrin makes with a macromolecular physiologic ligand. Interestingly, this interface was surprisingly modest even relative to the αvβ3–ciligideitide interface13 and was distinguished by contacts with the glycan at Asn266 of the α-subunit propeller domain. These contacts significantly (P = 0.003) contributed to the adhesion function of cellular αvβ3 (Supplementary Fig. 3a). The glycan at Asn266 is conserved in FcγR1–FN interface26 of the fibronectin receptor αvβ1 (ref. 30), and mutation of the equivalent residue in αv (N275Q) impaired αvβ3–mediated cell adhesion31, suggesting that the glycan contact may also be in the αvβ3–FN interface. This interface is also expected to be more robust than the αvβ3–FN interface because of an interaction of FN-type III domain 9 with the α-subunit propeller, an interaction that is not used by αvβ3 (ref. 21). This may explain the greater susceptibility of the smaller αvβ3–FN interface to force-induced binding or unbinding events, which may make it more suitable than the more extensive αvβ3–FN interface for mediating dynamic outside-in signal transduction32. Thus, the wtFN10–αvβ3 structure provides a molecular explanation for the distinct cellular responses seen when different integrins bind to the same ligand domain.

The structure of the αvβ3–hFN10 complex identified a basis for the unexpected action of FN10 as a pure antagonist despite its prototypical RGD motif. Structural and mutational studies support a critical role for the novel Trp1496–Tyr122 π–π interaction in ‘freezing’ the integrin in an inactive conformation. First, removing the Trp1496 side chain from hFN10 resulted in a domain that acted as wtFN10, as reported by LIBS expression. LIBS were also induced by hFN10 binding to cellular αvβ3, lacking the Tyr122 side chain. Second, changing the orientation of the Trp1496 side chain in hFN10 so that it no longer faces Tyr122 (as seen in the structure of the αvβ3–hFN10/B complex) also led to induction of LIBS when cellular αvβ3 bound hFN10/B. These data strongly argue that blocking the inward movement of the α1 helix towards MIDAS is sufficient to halt the associated tertiary changes in the βA domain that lead to outside-in signaling. Thus, altering the side chain orientation of Trp1496 by design or selection of its local environment can substantially affect the tertiary or quaternary changes induced by binding of RGD-based ligands. This effect could perhaps be replicated. For example, this may be accomplished in a cyclized form of the RGD-containing loop of hFN10, by changing the orientation of the tryptophan side chain in eptifibatide by replacing d-phenylalanine with d-tryptophan in a modified form of cilengitide. The critical βA Tyr122 is also conserved in αvβ3 (ref. 30) and β3 integrins33, which, like αvβ3, are drug targets34. Our results thus clearly suggest a path to structure-based drug design of...
a new generation of ligand-mimetic integrin inhibitors that can act as pure antagonists.

METHODS
Methods and any associated references are available in the online version of the paper.

Accession codes. The coordinates and structure factors of αVβ3–hFN10, αVβ3–wFN10 and αVβ3–hFN10/B have been deposited in the PDB under accession codes 4MMZ, 4MMX and 4MMY, respectively.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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AUTHOR CONTRIBUTIONS
M.A.A. conceived and designed experiments, J.F.V.A., J.-P.X. and S.L.G. made and purified proteins, J.F.V.A. and J.-P.X. performed the crystallographic studies, J.L.A., X.R., J.F.V.A., M.A.A. and B.D.A. performed the biophysical, biochemical and cell-based assays, M.A.A., J.F.V.A., J.-P.X. and J.L.A. interpreted data. M.A.A. wrote the manuscript with the assistance of S.L.G. and J.-P.X.

COMPETING FINANCIAL INTERESTS
The authors declare no competing financial interests.

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Plasmids, mutagenesis, protein expression and purification. The human αVβ3 ectodomain was expressed in insect cells and purified as described38. The activating N339S mutation in the β3 subunit was generated as described27. Expression plasmids encoding wild-type human N-terminally His-tagged FN10 (S1417–T1509) were generated by PCR from a plasmid containing human FN7–10 kindly provided by H.P. Erickson (Duke University Medical Center, Durham, NC)39. The plasmid encoding His-tagged high-affinity FN10 (hFN10)25 was PCR generated by replacing the cDNA encoding the loop sequence 1492GRGDSAPASS1499 in wtFN10 with 1492PGRWNE1499. RG-loop substitution W1496S and TPRGDWNE to IARGDWND (substituted residues underlined) in hFN10 to produce hFN10 W/S and hFN10/B, respectively, were generated using PCR-based mutagenesis with the QuikChange kit (Agilent Technologies), cloned into the bacterial expression plasmid pET11a and verified by DNA sequencing. The double mutation N266Q (in the β3αV propeller) plus N339S (in the β3αV propeller) plus Y122A mutation in the human FN10 W/S was generated by PCR in pcDNA3 expression plasmids and confirmed by DNA sequencing. FN10 forms were expressed in bacteria and purified by affinity chromatography on nickel columns followed by gel filtration. Thrombin-cleaved FN10 was further purified by gel filtration. Protein purity was confirmed by SDS-PAGE.

Cell lines, cell culture and transfection. The human erythroblastic leukemia cell line K562 stably expressing recombinant αVβ3 (K562-αVβ3)4 and the human melanoma cell line H22 (M21), which constitutively expresses αVβ3, have been described previously38. Cells were maintained in Iscove’s modified Dulbecco’s medium plus 5% FBS and 100 μl ml−1 penicillin, streptomycin (100 μg/ml), and 2 mM L-glutamine. The cell lines were incubated for 10 min at 37 °C with anti–LIBS-1– or anti–LIBS-6–labeled M21 cells for an additional 30 min at 4 °C. Cells were then washed, resuspended, fixed in 4% paraformaldehyde and analyzed by flow cytometry. LIBS epitope expression was measured and expressed as MFI (in the case of K562-αVβ3 or M21 cells) and normalized (in the case of HEK293T cells) as described above.

Hydrodynamic shift assays. The αVβ3 ectodomain was incubated alone with FN10 (at a 2:1 FN10:αVβ3 ratio) in 145 mM NaCl and 25 mM Tris–HCl, pH 7.4 (TBS), containing Ca2+, Mg2+ or Mn2+ (20 °C, 1.5 h). Aliquots were then taken and chromatographed at room temperature on a pre-calibrated Superdex S-200 GL column equilibrated in buffer having the same metal-ion composition used during the incubation, and Stokes radii were derived as described previously4. The elution profiles resolved by molecular sieve chromatography were monitored in-line by UV adsorption at 280 nm. Unliganded with or without FN10–treated αVβ3 species were resolved as single discrete symmetrical peaks. Excess FN10 served as an internal standard. The identity of the resolved peaks was confirmed formally by SDS-PAGE.

Cell adhesion assays. wtFN10, hFN10 or full-length FN (each at a 100 μg/ml in PBS) was adsorbed to demarcated areas in Maxisorp NunOmni Tray plates (Sigma–Aldrich, St. Louis, MO) overnight at 25 °C. The various FN-coated surfaces were washed with PBS and blocked with BSA (5% w/v) in PBS. Cells were incubated for 1 h at 37 °C. Cells were then washed, resuspended, fixed in 4% paraformaldehyde and analyzed by flow cytometry. LIBS epitope expression was measured and expressed as MFI (in the case of K562 αVβ3 or M21 cells) and normalized (in the case of HEK293T cells) as described above.

Crystalllography, structure determination and refinement. The αVβ3 ectodomain was crystallized at 4 °C by vapor diffusion using the hanging drop method as previously described4. hFN10, wtFN10 or hFN10/B (at 1.5 mM) was soaked into αVβ3 crystals in the crystallization well solution containing 2 mM MnCl2 for 2–3 weeks. Crystals were harvested in 12% PEG 3500 (polyethylene glycol, plus MgCl2 or MnCl2 (10 min, 37 °C), and incubated first with Alexa488-labeled wtFN10 or hFN10 (each at 3–10 μg/ml) in the dark and then with Alexa647-conjugated AP3 (10 μg/ml) for an additional 30 min on ice. Cells were washed in the respective metal ion–containing buffer, resuspended, fixed in 4% paraformaldehyde and analyzed using FACSCalibur or BD-LSRII flow cytometers (BD Biosciences). Binding of soluble FN10 to αVβ3 crystals was expressed as the mean fluorescence intensity (MFI) determined using FlowJo software. Binding of soluble FN10 to HEK293T cells was normalized by dividing its MFI by the MFI of Alexa647-conjugated AP3 to the same cells and multiplying by 100. The mean and s.d. from independent experiments were calculated and compared using Student’s t-test.

LIBS epitope expression. K562-αVβ3 cells, transiently transfected HEK293T cells or αVβ3+ M21 cells (0.5 × 106 in 100 μl WB) were incubated in the absence or presence of unlabeled soluble FN10 (5 μg/ml) in Ca2+–Mg2+ or in Mn2+ (30 min, at 25 °C). Alexa647-labeled AP5 Fab (10 μg/ml), unlabeled anti–LIBS-1 (each at 10 μg/ml) or LIBS-6 ascites (to 1:50 dilution) were added, and the cells were incubated for an additional 30 min before washing. Alexa647-labeled AP3 (10 μg/ml) was used with HEK293T cells in a separate set of tubes for normalization. APC-labeled goat anti-mouse Fc-specific antibody (10 μg/ml) was added to anti–LIBS-1 or anti–LIBS-6–labeled M21 cells for an additional 30 min at 4 °C. Cells were then washed, resuspended, fixed in 4% paraformaldehyde and analyzed by flow cytometry. LIBS epitope expression was measured and expressed as MFI (in the case of K562-αVβ3 or M21 cells) and normalized (in the case of HEK293T cells) as described above.

Cell culture and transfection. The human erythroblastic leukemia cell line K562 stably expressing recombinant αVβ3 (K562-αVβ3) and the human melanoma cell line H22 (M21), which constitutively expresses αVβ3, have been described previously38. Cells were maintained in Iscove’s modified Dulbecco’s medium plus G418 (0.5–1.0 mg/ml) (K562-αVβ3) or RPMI 1640 (M21) supplemented with 10% fetal calf serum (FCS), 2 mM L-glutamine, penicillin and streptomycin. HEK293T (ATCC) cells cultured in DMEM supplemented with 10% FCS, 2 mM L-glutamine, 1 mM sodium pyruvate, penicillin and streptomycin were transiently co-transfected with pcDNA3 plasmids encoding full-length wild-type αVβ3, αVβ3 Y122A, αVβ3 N339S or αVβ3 N266Q/N339S using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer’s protocol.

Fluorescent labeling of FN10 and mAbs. FN10 and the mAbs AP5 (Fab) and AP3 (IgG) were labeled with N-hydroxysuccinimidy esters of Fluor 488 (Alexa488) and Alexa647 (Invitrogen), respectively, according to the manufacturer’s instructions. Excess dye was removed using CentriSpin size-exclusion microcentrifuge columns (Pancreion Separations, Adelphia, NJ). The final FN10, AP5 and AP3 concentrations and dye-to-protein molar ratios (F/P) were determined spectrophotometrically, giving F/P molar ratios of 1.2 (for FN10) and 3 (for AP5 and AP3).

Ligand binding and flow cytometry. In cellular and biochemical assays in which calcium, magnesium or manganese ions were used, they were each used at final concentrations of 1 mM. Cells stably (K562) or transiently (HEK293T) expressing wild-type or mutant forms of αVβ3 were harvested by incubating in 10 mM EDTA in PBS (5 min, 25 °C), followed by washing three times in HEPES-buffered saline (20 mM HEPES and 150 mM NaCl, pH 7.4) containing BSA (0.1% w/v; washing buffer, WB). 1 × 106 cells were suspended in 100 μl WB containing CaCl2

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molecular weight 3500) in 100 mM sodium acetate, pH 4.5, 800 mM NaCl plus 2 mM Mn²⁺ and FN10 (at 1.5 mM), cryoprotected by the addition of glycerol in 2% increments up to a 24% final concentration and then flash frozen in liquid nitrogen. Diffraction data from cryocooled crystals were collected on the ID19 beamline fitted with a CCD detector at the APS Facility (Chicago, IL). Data were indexed, integrated and scaled with the HKL2000 program for αVβ3–hFN10 and αVβ3–hFN10/B and with iMosflm for αVβ3–wtFN10 using the same Rfree set imported from the PDB ID 3IJE structure factors. Phases were determined by molecular replacement using PHASER, with the structures of the unliganded αVβ3 ectodomain (PDB ID 3IJE) and the FN10 domain (PDB ID 1FNF) used as search models. The resulting models were refined with the 1.8.4 version of Phenix using simulated annealing, TLS, positional and individual temperature-factor refinement and default restraints. Several cycles of refinement and model building using Coot were applied to refine the complex structures of αVβ3–hFN10, αVβ3–wtFN10 and αVβ3–hFN10/B (Table 1), with automatic optimization of X-ray and stereochemistry and additional Ramachandran restraints in the last cycles. The Ramachandran statistics were as follows: αVβ3–hFN10 structure, 89% in the most favored regions, 10.18% in additional allowed regions and 0.56% outliers; αVβ3–wtFN10 structure, 89% in the most favored regions, 10.18% in additional allowed regions and 0.56% outliers; αVβ3–hFN10/B structure, 91% in the most favored regions, 0.56% in additional allowed regions and 0.56% outliers. σA-weighted Fo – Fc omit maps were generated by removing the FN10 ligand from the final complex models using phenix.maps. All structural illustrations were prepared with the Chimera software.