



# Dynamics of integrin $\alpha 5\beta 1$ , fibronectin, and their complex reveal sites of interaction and conformational change

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Yang Su<sup>1,2,‡</sup>, Roxana E. Iacob<sup>3,‡</sup>, Jing Li<sup>1,2</sup>, John R. Engen<sup>3</sup> , and Timothy A. Springer<sup>1,2,\*</sup> 

From the <sup>1</sup>Program in Cellular and Molecular Medicine, Boston Children's Hospital, Boston, Massachusetts, USA; <sup>2</sup>Departments of Biological Chemistry and Molecular Pharmacology and of Pediatrics, Harvard Medical School, Boston, Massachusetts, USA; <sup>3</sup>Department of Chemistry & Chemical Biology, Northeastern University, Boston, Massachusetts, USA

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**Integrin  $\alpha 5\beta 1$  mediates cell adhesion to the extracellular matrix by binding fibronectin (Fn). Selectivity for Fn by  $\alpha 5\beta 1$  is achieved through recognition of an RGD motif in the 10th type III Fn domain (Fn10) and the synergy site in the ninth type III Fn domain (Fn9). However, details of the interaction dynamics are unknown. Here, we compared synergy-site and Fn-truncation mutations for their  $\alpha 5\beta 1$ -binding affinities and stabilities. We also interrogated binding of the  $\alpha 5\beta 1$  ectodomain headpiece fragment to Fn using hydrogen-deuterium exchange (HDX) mass spectrometry to probe binding sites and sites of integrin conformational change. Our results suggest the synergistic effect of Fn9 requires both specific residues and a folded domain. We found some residues considered important for synergy are required for stability. Additionally, we show decreases in fibronectin HDX are localized to a synergy peptide containing contacting residues in two  $\beta$ -strands, an intervening loop in Fn9, and the RGD-containing loop in Fn10, indicative of binding sites. We also identified binding sites in the  $\alpha 5$ -subunit  $\beta$ -propeller domain for the Fn9 synergy site and in the  $\beta 1$ -subunit  $\beta 1$  domain for Fn10 based on decreases in  $\alpha 5\beta 1$  HDX. Interestingly, the dominant effect of Fn binding was an increase in  $\alpha 5\beta 1$  deuterium exchange distributed over multiple sites that undergo changes in conformation or solvent accessibility and appear to be sites where energy is stored in the higher-energy, open-integrin conformation. Together, our results highlight regions important for  $\alpha 5\beta 1$  binding to Fn and dynamics associated with this interaction.**

Integrins are  $\alpha\beta$ -heterodimeric cell-surface receptors that sense cellular environments and mediate cell adhesion and migration. Their  $\alpha$  and  $\beta$  subunits are comprised of large extracellular domains (ectodomains) that link to single-pass transmembrane domains and cytoplasmic tails. The two subunits associate noncovalently to form a ligand-binding head distal to the cell membrane (Fig. 1A). By binding glycoprotein ligands in the extracellular matrix (ECM) or on the surface of other cells and connecting to the actin cytoskeleton inside the cell, integrins transmit signals, including tensile force, between the extracellular and intracellular environments. The

prototypic  $\alpha 5\beta 1$  integrin mediates cell adhesion to fibronectin (Fn) in the ECM; it also directs Fn fibril formation and assembly into the ECM (1, 2). The interaction between  $\alpha 5\beta 1$  and Fn is important for vertebrate development (3) including vascular development and angiogenesis (4) and cancer progression (5).

Like most integrins, activation of  $\alpha 5\beta 1$  for high-affinity ligand binding is accompanied by large conformational changes in its ectodomain.  $\alpha 5\beta 1$  equilibrates between three overall conformations: bent-closed (BC), extended-closed (EC), and extended-open (EO) (Fig. 1, A and B) (6–8). In the resting state on the cell surface,  $\alpha 5\beta 1$  is predominantly (>99%) in the BC conformation where the ectodomain bends at the knees and the lower legs and transmembrane domains in each subunit associate with one another. Extension at the knees separates the headpiece from the lower legs in each subunit (6, 8–12). Headpiece opening reshapes the  $\beta 1$  domain around the ligand-binding site, which is linked to swing out of the  $\beta$ -subunit hybrid domain away from the  $\alpha 5$ -subunit thigh domain (6, 12–15) (Fig. 1, C and D). Both BC and EC conformations are low affinity; transition to the EO conformation increases  $\alpha 5\beta 1$  affinity for ligand by  $\sim 5000$ -fold (8). The divalent ion  $Mn^{2+}$  can activate high-affinity ligand binding *in vitro* by facilitating these conformational changes as well as increasing the intrinsic affinity of each conformation for the ligand (16). In the presence of  $Mn^{2+}$ , the  $\alpha 5\beta 1$  headpiece was closed when unliganded and became open when an RGD peptide or Fn was bound (Fig. 1, A–D) (6, 12, 17).

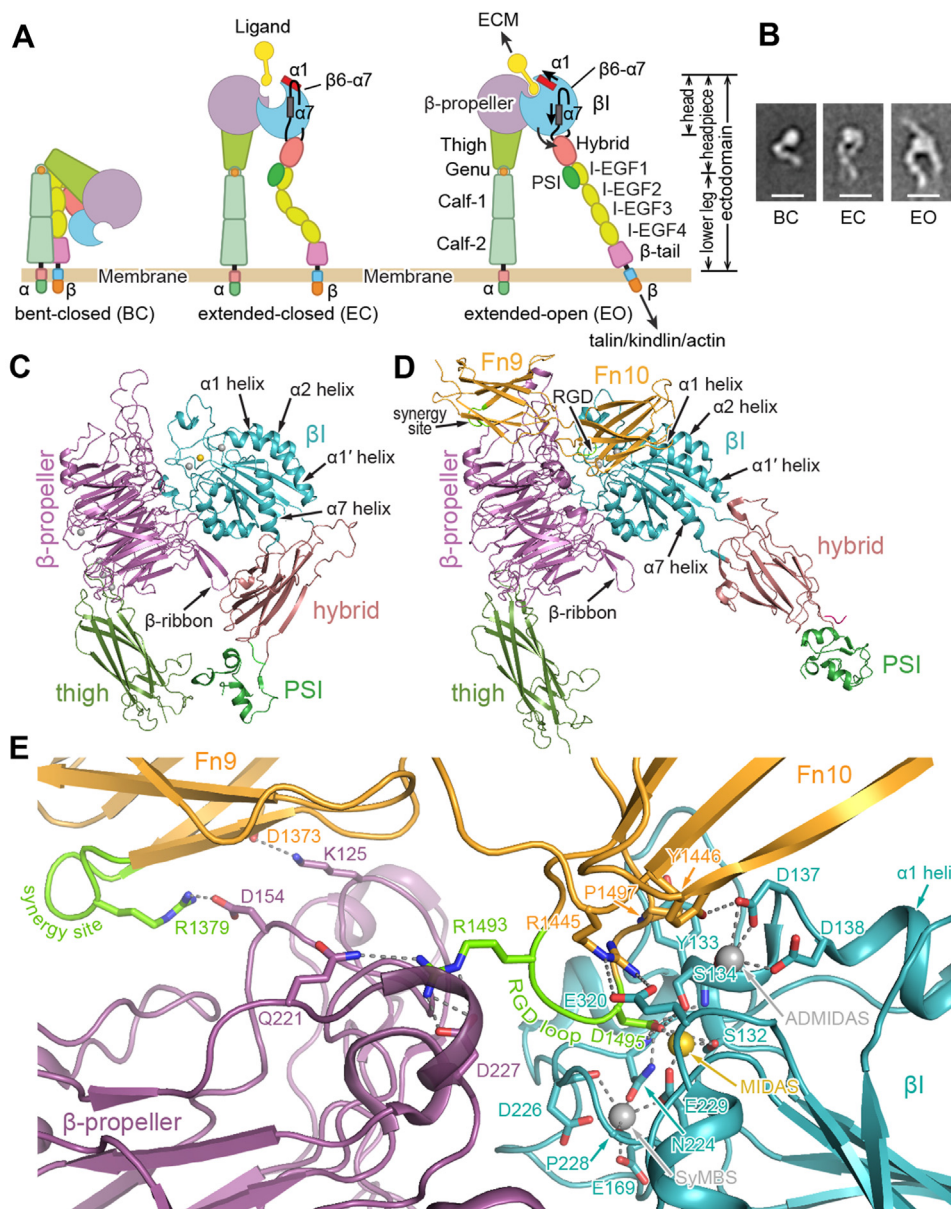
$\alpha 5\beta 1$  belongs to a subfamily of integrins that recognize an Arg-Gly-Asp (RGD) sequence in their ligands. While many integrin ligands contain the RGD motif,  $\alpha 5\beta 1$  binding to Fn is unusual in requiring both the RGD sequence located in the 10th type-III Fn domain (Fn10) and the synergy site located in the adjacent Fn9 domain (18–22). A recent cryo-EM structure of the  $\alpha 5\beta 1$ -Fn complex clarified this dual recognition mode by showing that the RGD-bearing loop in Fn10 is inserted in a cleft between the  $\alpha 5$ -subunit  $\beta$ -propeller domain and the  $\beta 1$ -subunit  $\beta 1$  domain, while the synergy site in Fn9 contacts the  $\alpha 5$ -subunit  $\beta$ -propeller domain (12) (Fig. 1E), as earlier predicted (13). However, much more remains to be learned about this important interaction.

Here, we have complemented static pictures from crystal and cryo-EM structures that reveal how RGD peptides and Fn

<sup>‡</sup> These authors contributed equally to this work.

\* For correspondence: Timothy A. Springer, [springer@crystal.harvard.edu](mailto:springer@crystal.harvard.edu).

## Dynamics of $\alpha 5\beta 1$ , fibronectin, and their complex



**Figure 1.  $\alpha 5\beta 1$  and fibronectin structures.** *A*, three overall conformations of  $\alpha 5\beta 1$ . *B*, representative negative stain EM class averages for each conformation of  $\alpha 5\beta 1$ ; the EO conformation is bound to Fn7–10. Scale bars are 10 nm. From (6). *C* and *D*, structures of  $\alpha 5\beta 1$  headpiece alone (PDB 3VI3) (*C*) and in complex with Fn7–10 (PDB 7NWL) (*D*). *E*, close-up view of the  $\alpha 5\beta 1$ /Fn7–10 interface.

bind to integrin  $\alpha 5\beta 1$  with studies of how their interaction affects the backbone dynamics not only of regions that interact but also of regions that undergo conformational changes. We explore the  $\alpha 5\beta 1$  headpiece, Fn, and their interactions by using hydrogen-deuterium exchange (HDX) coupled with mass spectrometry. The integrin headpiece fragment (Fig. 1A right) contains the ligand-binding site, has the same intrinsic affinity for Fn as the complete ectodomain and intact integrin on the cell surface, and undergoes the same closed-to-open conformational change as the complete ectodomain and intact integrin on the cell surface (8, 12–14, 17). HDX measures exchange of protein backbone amide hydrogen atoms with deuterium. The exchange is sensitive to hydrogen bonding,

solvent accessibility, and backbone movement and thus reports local backbone conformation and dynamics (23). HDX measurements provide unique insights into the dynamics of proteins and protein–protein interactions and are complementary to structural methods such as crystallography and EM that provide snapshots of low-energy positions of proteins within their energy landscapes. To understand how motions in fibronectin integrin-binding fragments (ligand) and fibronectin-binding integrin headpiece fragments (receptor) change upon complex formation, we have studied here HDX in the ligand, the receptor, and their complex. To complement this work, we have also studied how ligand-binding affinities vary in mutants and Fn domain truncations.

## Results

### Dynamics, stability, and affinity for $\alpha 5\beta 1$ of Fn fragments and mutants

We describe, in turn, deuterium uptake dynamics in fibronectin fragments, then in the integrin  $\alpha 5\beta 1$  headpiece fragment, and then in integrin  $\alpha 5\beta 1$  headpiece complexes with fibronectin fragments and RGD peptide (Table S1). We first measured HDX for Fn9–10 (30 peptides constituting 97.3% sequence coverage) and Fn7–10 (32 peptides constituting 69.8% sequence coverage) in the absence of  $\alpha 5\beta 1$  integrin. The individual Fn domains showed distinct deuterium uptake profiles (Figs. 2, A–D and S1). Fn7 and Fn10 incorporated the highest amount of deuterium; in most regions, more than 50 to 60% of available amide hydrogen atoms were exchanged with deuterium after 4 h. Importantly, the integrin-binding RGD loop in Fn10 had high exchange (Fig. 2E), showing that it is highly dynamic in agreement with NMR studies on Fn10 and Fn9–10 fragments (24, 25). Fn9 incorporated less deuterium than Fn10 in the Fn9–10 fragment, even though as isolated domains Fn9 was less stable than Fn10 by differential scanning calorimetry and chemical denaturation studies (26, 27). Most Fn9 peptides showed less than 50% exchange after 4 h. The synergy site in Fn9 was notably one of the least exchanging regions and exchanged only  $\sim 35\%$  after 4 h (Fig. 2, A–D and F).

The Fn9 domain was more dynamic in Fn9–10 than in Fn7–10. In particular, the Fn9 B-C loop, which contacts the Fn8 domain, incorporated more deuterium in Fn9–10 than in Fn7–10, particularly at early time points (Fig. 2G). Correlating with this difference in HDX, Fn8 stabilized Fn9 and Fn10 in thermal unfolding experiments measured by intrinsic tryptophan fluorescence (Fig. 3A).

The Fn7, Fn8, Fn9, and Fn10 domains each contain a single Trp residue that is buried in the hydrophobic core. In general, tryptophan fluorescent emission shifts from  $\sim 330$  nm to  $\sim 350$  nm as a result of increased solvent exposure during unfolding. Instead of stepwise transitions for each individual Fn domain, a single transition was observed for Fn9–10, Fn8–10, and Fn7–10 that suggested cooperative thermal unfolding for the less stable Fn8 and Fn9 domains (26, 27). The  $T_m$ s of Fn8–10 and Fn7–10 (Fig. 3A) were comparable and were 8 °C higher than that of Fn9–10, showing that Fn9 and Fn10 are stabilized by Fn8 and that Fn7 did not provide further stabilization. In agreement with previous reports on isolated Fn domains, the  $T_m$  of Fn10 was high (26, 27); however, its “folded” and “unfolded” states showed substantially higher F350-to-F330 fluorescence ratios than the corresponding states of the tandem domains in Fn9–10, Fn8–10, and Fn7–10 (Fig. 3B). These differences suggest that the folded and unfolded states of Fn10 may each be more disordered than those in the fragments with tandem domains.

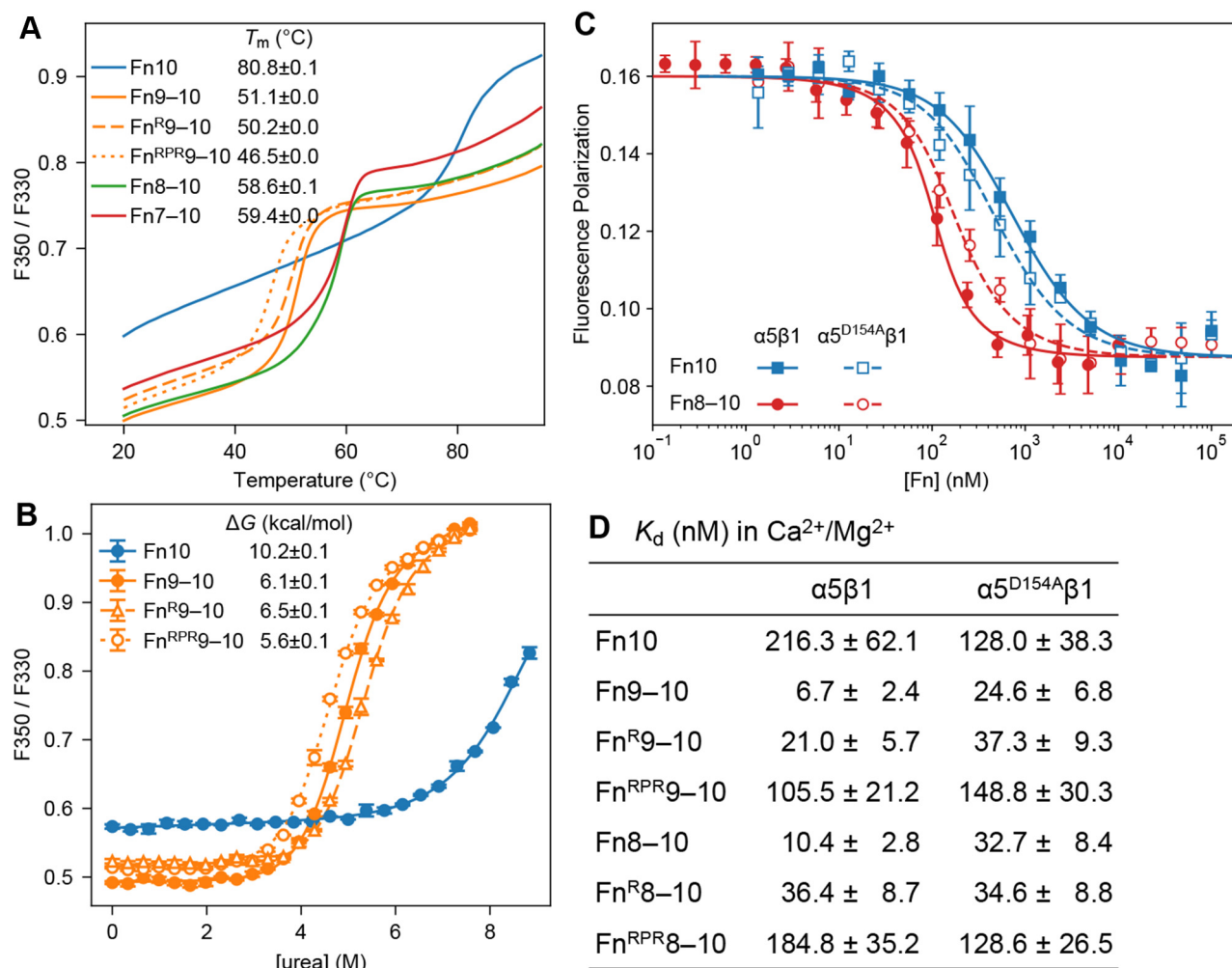
We next compared the importance of Fn domains 8, 9, and 10 and the synergy site for binding in  $\text{Ca}^{2+}/\text{Mg}^{2+}$  to a WT and mutant  $\alpha 5\beta 1$  integrin. The mutation is in a residue that interacts with the synergy site,  $\alpha 5$  D154A ( $\alpha 5^{\text{D154A}}\beta 1$ ) (Fig. 1E). Affinity of the  $\alpha 5\beta 1$  ectodomain for Fn was measured by

competition with a fluorescently labeled fibronectin-mimetic RGD peptide cyclized with a disulfide bond, ACRGDGWCG (cRGD) (28, 29), in fluorescence anisotropy. The affinities of WT  $\alpha 5\beta 1$  for Fn9–10 and Fn8–10 were within experimental error of one another; however, affinities of both Fn9–10 and Fn8–10 were substantially higher, by 20- to 30-fold, than that of Fn10 (Figs. 3, C and D and S2), consistent with the synergy site in Fn9 augmenting binding affinity for  $\alpha 5\beta 1$  (18–22). Thus, the addition of Fn9 to Fn10 raised affinity for  $\alpha 5\beta 1$  by  $\sim 25$ -fold over Fn10 alone, placing a quantitative value on the affinity enhancement by Fn9 and its synergy site. Furthermore, HDX showed that Fn9 was more stable in Fn7–10 than in Fn9–10 and  $T_m$  values showed that Fn7–10 and Fn8–10 were comparably stable and that Fn9–10 was less stable. However, because Fn8–10 and Fn9–10 have similar binding affinities for  $\alpha 5\beta 1$ , these results suggest that the stability of Fn9–10 is sufficient for maximal binding affinity for  $\alpha 5\beta 1$ .

To better understand the role of the synergy site, a single-residue mutation of Arg-1379 to Ala (Fn<sup>R</sup>) and a triple mutation of residues Arg-1374, Pro-1376, and Arg-1379 to Ala (Fn<sup>RPR</sup>) were created in Fn8–10 and Fn9–10. Previously, structures of Fn7–10 and the  $\alpha 5\beta 1$  headpiece bound to an RGD peptide, together with docking and mutation of acidic residues in the  $\alpha 5$   $\beta$ -propeller domain and Arg-1379 in the Fn9 domain, suggested that Asp-154 in the  $\alpha 5$   $\beta$ -propeller domain bound to Arg-1379 in the Fn9 domain (13) as later confirmed by cryo-EM (12) (Fig. 1E). We found similar 3-fold decreases in affinity of WT  $\alpha 5\beta 1$  for Fn<sup>R</sup>9–10 and Fn<sup>R</sup>8–10 and of WT Fn9–10 and Fn8–10 for  $\alpha 5^{\text{D154A}}\beta 1$  (Figs. 3D and S2). Furthermore, there was little or no further decrease in affinity of  $\alpha 5^{\text{D154A}}\beta 1$  for the Fn<sup>R</sup> mutants of Fn9–10 and Fn8–10, supporting the interaction of  $\alpha 5$  Asp-154 with Fn Arg-1379. The more drastic Fn<sup>RPR</sup> triple mutant lowered affinity by 15- to 17-fold of Fn9–10 and Fn8–10 for WT  $\alpha 5\beta 1$ . The decrease in affinity of the Fn8–10<sup>RPR</sup> and Fn9–10<sup>RPR</sup> mutants was similar for  $\alpha 5\beta 1$  and  $\alpha 5^{\text{D154A}}\beta 1$ , as expected from the interaction of  $\alpha 5$  Asp-154 with Fn Arg-1379 (13). However, the similarity in affinity of  $\alpha 5\beta 1$  for Fn<sup>RPR</sup>Fn9–10, Fn<sup>RPR</sup>8–10, and Fn10 (Fig. 3D) was unexpected. The  $\alpha 5\beta 1$ /Fn9–10 complex structure shows that Arg-1374 has no interaction with  $\alpha 5\beta 1$  and that Pro-1376 lacks a van der Waals interaction with  $\alpha 5\beta 1$  and buries only slightly less solvent-accessible surface area (15 Å<sup>2</sup>) than the Ala-1376 mutant (assuming the mutation does not perturb backbone conformation) (12). Furthermore, interactions between Fn and  $\alpha 5\beta 1$  extend beyond the synergy site to include, for example, a salt bridge between  $\alpha 5$  Lys-125 and Fn Asp-1373 (Fig. 1E). These results suggest that the Fn<sup>RPR</sup> mutation may alter the structure of Fn9 and largely disable its interaction with  $\alpha 5\beta 1$ .

Did the Fn<sup>RPR</sup> or Fn<sup>R</sup> mutations in Fn9 directly alter interactions with  $\alpha 5\beta 1$  or cause unfolding or a change in the structure of Fn9 so it no longer interacted? To examine unfolding, we measured the stabilities of the Fn<sup>RPR</sup> and Fn<sup>R</sup> mutants in thermal and urea denaturation (Fig. 3, A and B). The  $T_m$ s of Fn9–10 and Fn<sup>R</sup>9–10 were similar, while the  $T_m$  of Fn<sup>RPR</sup>9–10 was 4 °C lower. Unfolding in urea showed that the





**Figure 3. Stabilities and binding affinities of Fn fragments.** *A*, thermal unfolding of Fn fragments monitored by tryptophan fluorescence. *B*, chemical unfolding of Fn fragments monitored by tryptophan fluorescence. Data for Fn9-10, Fn<sup>R9</sup>-10, and Fn<sup>RPR9</sup>-10 were fit with shared  $m$  (fitting result  $m = 1.25 \pm 0.02$  kcal·l·mol<sup>-2</sup>). *C* and *D*, affinities measured in triplicate by fluorescence polarization with 100 nM  $\alpha 5\beta 1$  headpiece or  $\alpha 5\beta 1$  ectodomain, 10 nM FITC-cRGD probe, and competition with varying concentrations of Fn9-10 or Fn7-10 in the presence of 1 mM  $\text{Ca}^{2+}/\text{Mg}^{2+}$ . *C*, representative competitive binding curves; all conditions are shown in Fig. S2. *D*,  $K_d$  values with fitting errors.

folding free energy of Fn<sup>R9</sup>-10 was increased by 0.4 kcal/mol; thus, while removal of the sidechain of Arg-1379 decreases interaction with  $\alpha 5\beta 1$ , removal stabilizes Fn9-10 folding. In contrast, the folding free energy of Fn<sup>RPR9</sup>-10 was decreased by 0.5 kcal/mol relative to WT and by 0.9 kcal per mole relative to Fn<sup>R9</sup>-10. These differences are consistent with a change in the structure of Fn9 in the Fn<sup>RPR</sup> mutation.

In HDX experiments on Fn complexes with  $\alpha 5\beta 1$ , it is important to know the populations of the conformational states of bound and unbound  $\alpha 5\beta 1$  headpiece and to calculate the percent of the  $\alpha 5\beta 1$  headpiece bound to Fn. We used the  $\alpha 5\beta 1$  headpiece to simplify peptide analysis in HDX; HDX was performed initially with the ectodomain, but there were difficulties in reduction and digestion of the disulfide-rich lower leg of  $\beta 1$ . The headpiece contains the ligand-binding domains and lacks the lower legs of the ectodomain (Fig. 1A). In  $\text{Mg}^{2+}/\text{Ca}^{2+}$ , the  $\alpha 5\beta 1$  headpiece is 99.97% in the closed conformation and 0.03% in the open conformation based on previous accurate measurements of its conformational equilibria (8). To maximize integrin binding to fibronectin, all HDX measurements on  $\alpha 5\beta 1$

bound to fibronectin and cRGD were done in the presence of  $\text{Mn}^{2+}$  instead of  $\text{Mg}^{2+}/\text{Ca}^{2+}$ , which increases intrinsic affinity for ligand  $\sim 30$ -fold and also increases population of the high-affinity, open conformation (16). To determine the completeness of binding in  $\text{Mn}^{2+}$ , we measured the affinities of cRGD and Fn7-10 and Fn9-10 for the  $\alpha 5\beta 1$  headpiece in  $\text{Mn}^{2+}$ . The results with Fn7-10 and Fn9-10 were similar and showed affinities in  $\text{Mn}^{2+}$  of  $\sim 0.5$  nM for the basal ensemble and  $\sim 30$  nM for the closed state (Fig. S3). cRGD binds to the basal ensemble of the  $\alpha 5\beta 1$  headpiece in  $\text{Mn}^{2+}$  with an affinity of  $3.2 \pm 0.3$  nM. In  $\text{Mn}^{2+}$ , the intrinsic affinities of cRGD for the closed states of the  $\alpha 5\beta 1$  headpiece of  $44 \pm 3$  nM and the ectodomain of  $46 \pm 5$  nM are similar (16), in agreement with the previously established concept that integrin affinities are intrinsic to conformational states and independent of whether the integrin is on the cell surface or a particular fragment (8, 30). Using this concept, data from Fig. S3, other measurements (16), and the equations described in Experimental procedures, we calculated the extent of binding and the conformational composition of each of the HDX samples described in the following (Tables S2

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and S3). The results showed that the ligand-binding site on  $\alpha 5\beta 1$  was more than 99.66% occupied with all three ligands and that  $\alpha 5\beta 1$  was 93% open and 7% closed when bound to cRGD and 97 to 98% open and 1 to 2% closed when bound to Fn9–10 and Fn7–10.

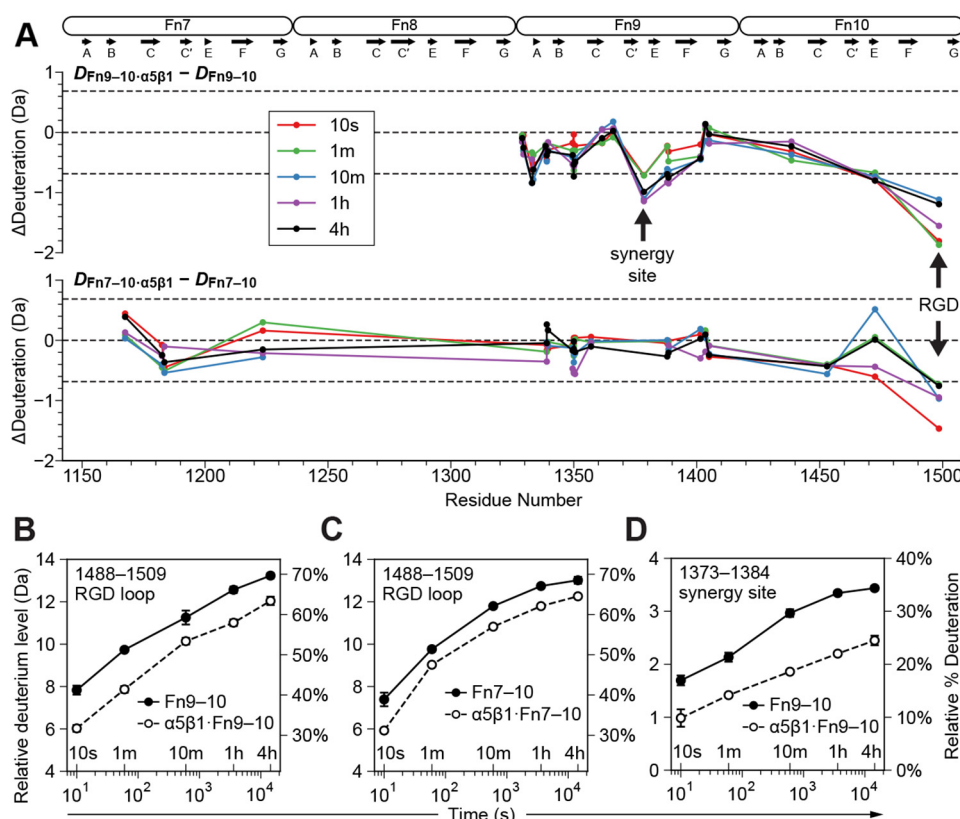
To map the effects on Fn of binding to  $\alpha 5\beta 1$ , we measured deuterium incorporation for Fn9–10 and Fn7–10 fragments in complex with  $\alpha 5\beta 1$  and compared those values to deuterium incorporation of Fn9–10 and Fn7–10 alone, under identical HDX MS conditions. Most Fn peptides did not show a meaningful difference in deuterium uptake ( $|\Delta D| > 0.7$  Da) between  $\alpha 5\beta 1$ -bound Fn and free Fn (Figs. 4 and S4). However,  $\alpha 5\beta 1$  protected RGD loop and synergy site peptides. The RGD-bearing loop (residues 1488–1509) in Fn9–10 and Fn7–10 incorporated less deuterium across all time points when bound to  $\alpha 5\beta 1$  (Fig. 4, B and C). The synergy site peptide, which was covered in Fn9–10 but not in sparsely covered Fn7–10 (Fig. 4A), also incorporated less deuterium at all time points in the presence of  $\alpha 5\beta 1$  (Fig. 4D).

### Dynamics of unliganded and liganded $\alpha 5\beta 1$ integrin

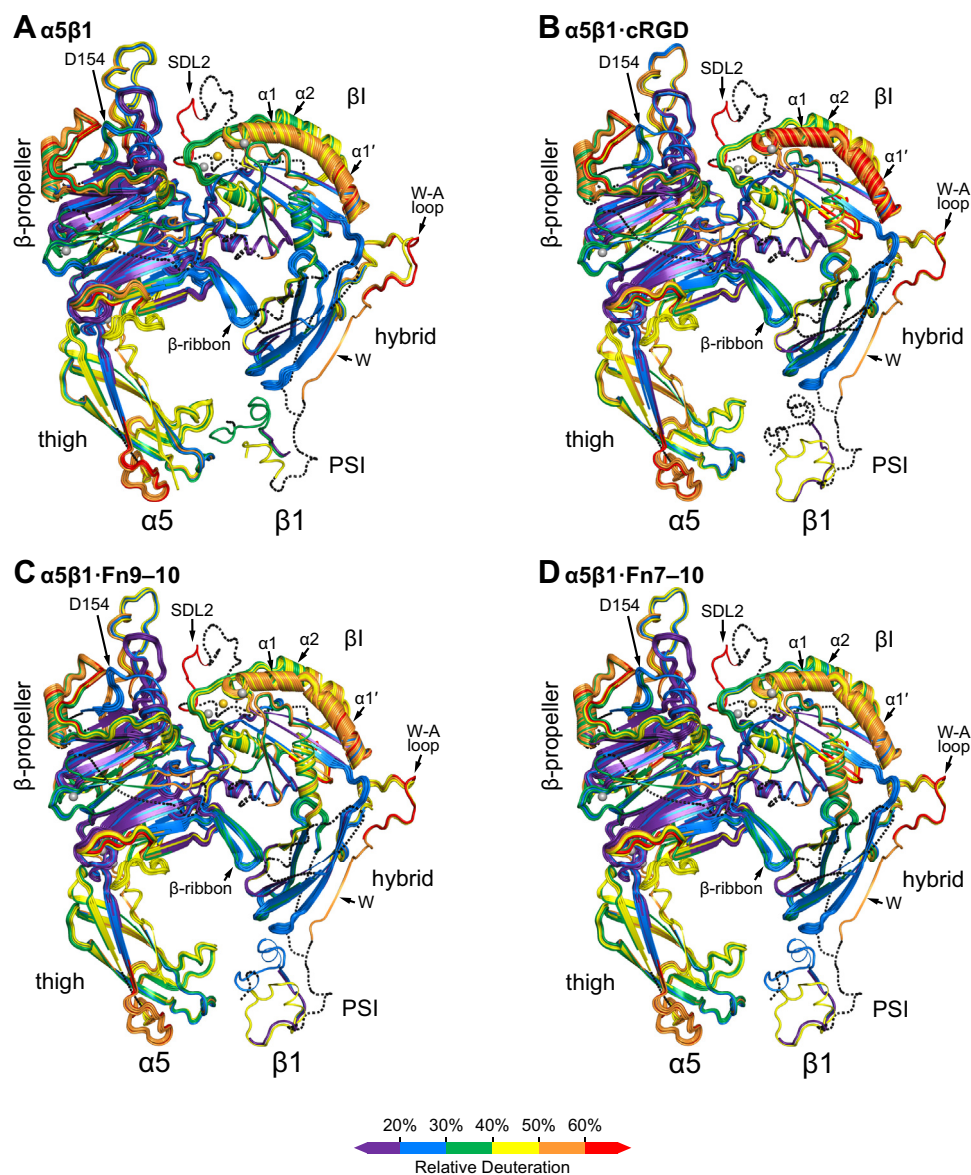
HDX MS was performed on the unliganded  $\alpha 5\beta 1$  headpiece and the headpiece in complex with cRGD peptide, Fn9–10, or Fn7–10 (Figs. S5–S8). For the unliganded  $\alpha 5\beta 1$  headpiece, deuterium uptake was below 50% after 4 h in most regions

(Figs. 5A and 6). In particular, the core of the  $\beta$ -propeller domain showed  $<30\%$  exchange even after 4 h, consistent with the fact that the  $\beta$ -strands that form the propeller blades are extensively hydrogen-bonded. Among the four large domains in the headpiece, the thigh domain showed more exchange overall than the other three domains (Fig. 5). Within the domains, a few isolated regions showed more extensive exchange ( $>50\%$  at 4 h). The W-A loop in the hybrid domain exchanged the most ( $>80\%$  at 4 h) (Figs. 5 and 6H), consistent with the disordered loop observed in some crystal structures (14). In the  $\beta I$  domain, the ligand-proximal  $\alpha 1$ -helix, the  $\alpha 1'$  helix, and the specificity-determining loop 2 (SDL2) exchanged extensively ( $>50\%$  at 4 h) (Figs. 5 and 6, I–K).

To map the effects of ligand binding on  $\alpha 5\beta 1$ , we measured  $\alpha 5\beta 1$  deuterium uptake using the same Fn9–10 and Fn7–10 complexes with  $\alpha 5\beta 1$  that were described in the previous section. Additionally, the  $\alpha 5\beta 1$  headpiece was complexed with cRGD. Comparing HDX of  $\alpha 5\beta 1$  in the ligand-bound form to the unbound form revealed deuterium uptake differences ( $\Delta D = D_{\text{bound } \alpha 5\beta 1} - D_{\text{free } \alpha 5\beta 1}$ ) both at and distal to the ligand-binding site. Changes in deuteration greater than 0.7 Da (increases, pink-dark red) or less than  $-0.7$  Da (decreases, cyan-blue) in peptides are shown on the structures in Figure 7. Altered HDX reflects both the effects on specific regions of  $\alpha 5\beta 1$  that bind to ligand and the effects of conformational change from the closed to the open conformation. Under the



**Figure 4. Alteration in Fn HDX upon binding of  $\alpha 5\beta 1$ .** A, differences in HDX of Fn9–10 and Fn7–10 with and without  $\alpha 5\beta 1$ -bound ( $\Delta D = D_{\text{bound Fn}} - D_{\text{free Fn}}$ ) are shown for each peptide plotted at the midpoint of its sequence position. The difference calculation was performed using the data shown in Figs. S1 and S4, for free and bound forms, respectively. All deuteration values and peptide sequences are found in the Supplementary Datafile. Horizontal dashed lines mark  $\Delta D = 0, \pm 0.7$  Da. For interpreting the HDX difference data, we chose a difference of 0.7 Da to mark differences that are clearly above the error of measurement and are likely meaningful. B–D, deuterium uptake curves for selected peptides.



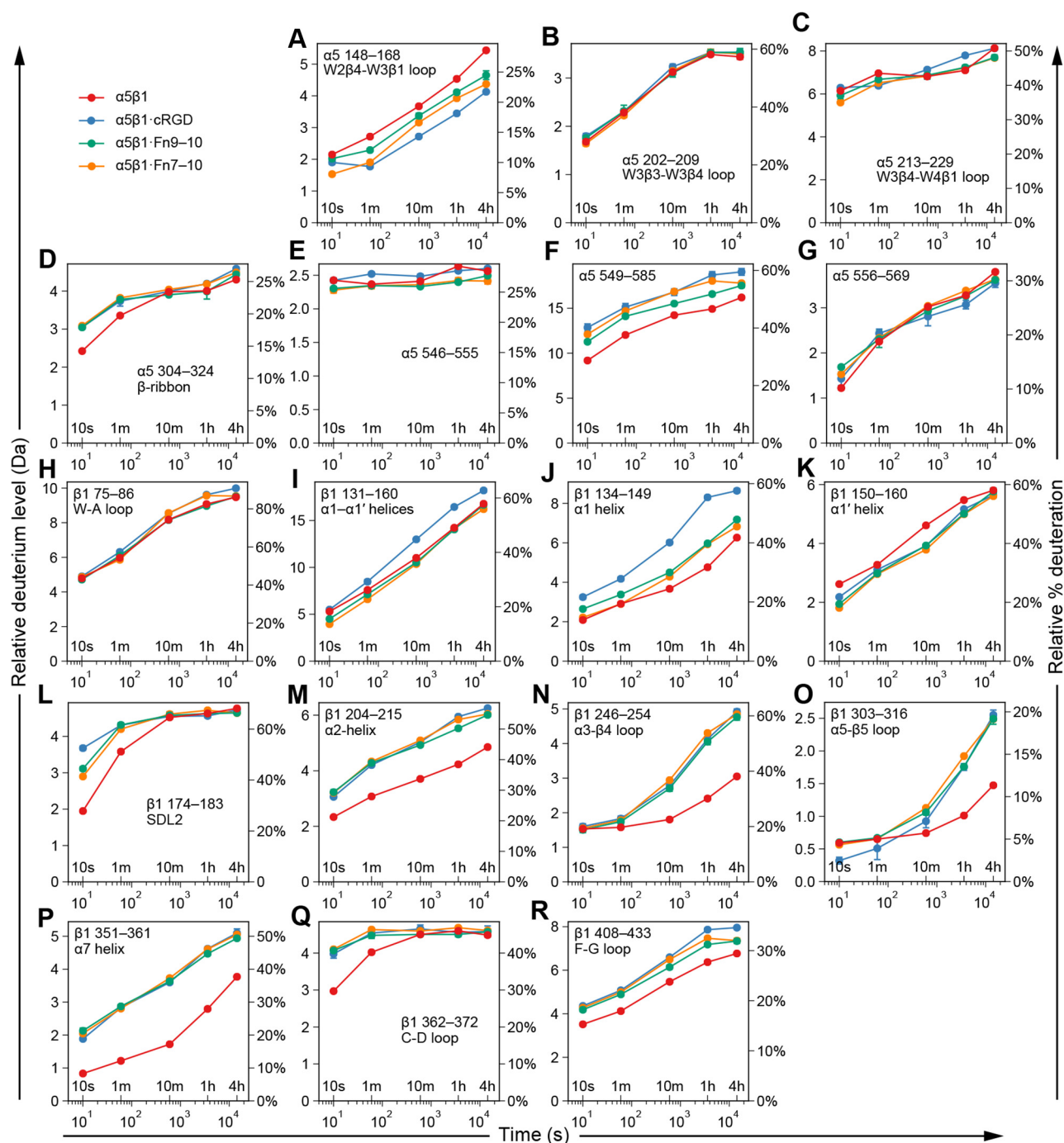
**Figure 5. HDX of  $\alpha 5\beta 1$  headpiece.** Exchange measurements in ligand-free (A), cRGD-bound (B), Fn9-10-bound (C) and Fn7-10-bound (D) states at 4 h are shown on the structure (PDB 3VI3) as *ribbon cartoons*, color coded according to the key. Regions not covered by HDX are shown in *black dotted lines*. All structures are shown in the closed conformation for comparison regardless of the actual conformations of the  $\alpha 5\beta 1$  headpiece in each state. All peptides are shown in this representation by dividing the ribbon representation into different segments for each overlapping peptide. The percent deuterated values of each colored peptide are provided in [Figs. S5–S8](#) and in the [Supplementary Datafile](#).

conditions of our HDX experiments in the presence of  $Mn^{2+}$ , the  $\alpha 5\beta 1$  headpiece changed from  $0.4 \pm 0.2\%$  open in the absence of ligand to 93 to 98% open in the presence of the three ligands ([Table S3](#)). All HDX mass spectra displayed a single binomial isotope distribution, indicative of a single population of molecules in solution, that increases in mass with longer deuteration time (EX2 kinetics ([31](#))). No peptides showed a bimodal distribution where the lower mass distribution reflects a more protected less deuterated species and the higher mass distribution a less protected more deuterated species, a hallmark of simultaneously existing populations in distinct conformational states (EX1 kinetics ([31](#))). These findings would be consistent with both the calculated predominance of the open integrin state in the presence of a ligand and the closed state in the absence of a ligand, as well as

interconversion between closed and open states on a timescale more rapid than our HDX experiments.

Interconversion between the open and closed conformations of integrin  $\alpha 5\beta 1$  is regulated by alterations in the  $\beta 1$  domain and its interface with the hybrid domain. The three regions of the integrin  $\beta 1$  domain that move the most in allostery are the  $\alpha 1$ - $\alpha 1'$ ,  $\alpha 2$ , and  $\alpha 7$  helices ([32](#)) ([Figs. 6, I–K, M and P](#) and [7, A, B and D](#)). All three ligands uniformly increased HDX of the  $\alpha 2$  and  $\alpha 7$  helices. In contrast, effects of the ligands differed on the  $\alpha 1$ - $\alpha 1'$  helix. A large number of peptides covered the combined  $\alpha 1$ - $\alpha 1'$  helix, including 131 to 160, which showed an increase in HDX by cRGD and not Fn9-10 or Fn7-10 ([Figs. 6I and S9](#)). Greater insights were provided by peptides 134 to 149 ( $\alpha 1$ -helix) and 150 to 160 ( $\alpha 1'$  helix) ([Fig. 6, J and K](#), respectively). cRGD enhanced exchange in  $\alpha 1$ ,

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**Figure 6. Deuterium uptake curves for selected peptides of  $\alpha 5\beta 1$  headpiece in the absence of ligand and presence of cRGD, Fn9-10, and Fn7-10.** A–R, deuteration of the indicated peptides is colored according to the key in the upper left. All deuteration values are found in the [Supplementary Datafile](#).

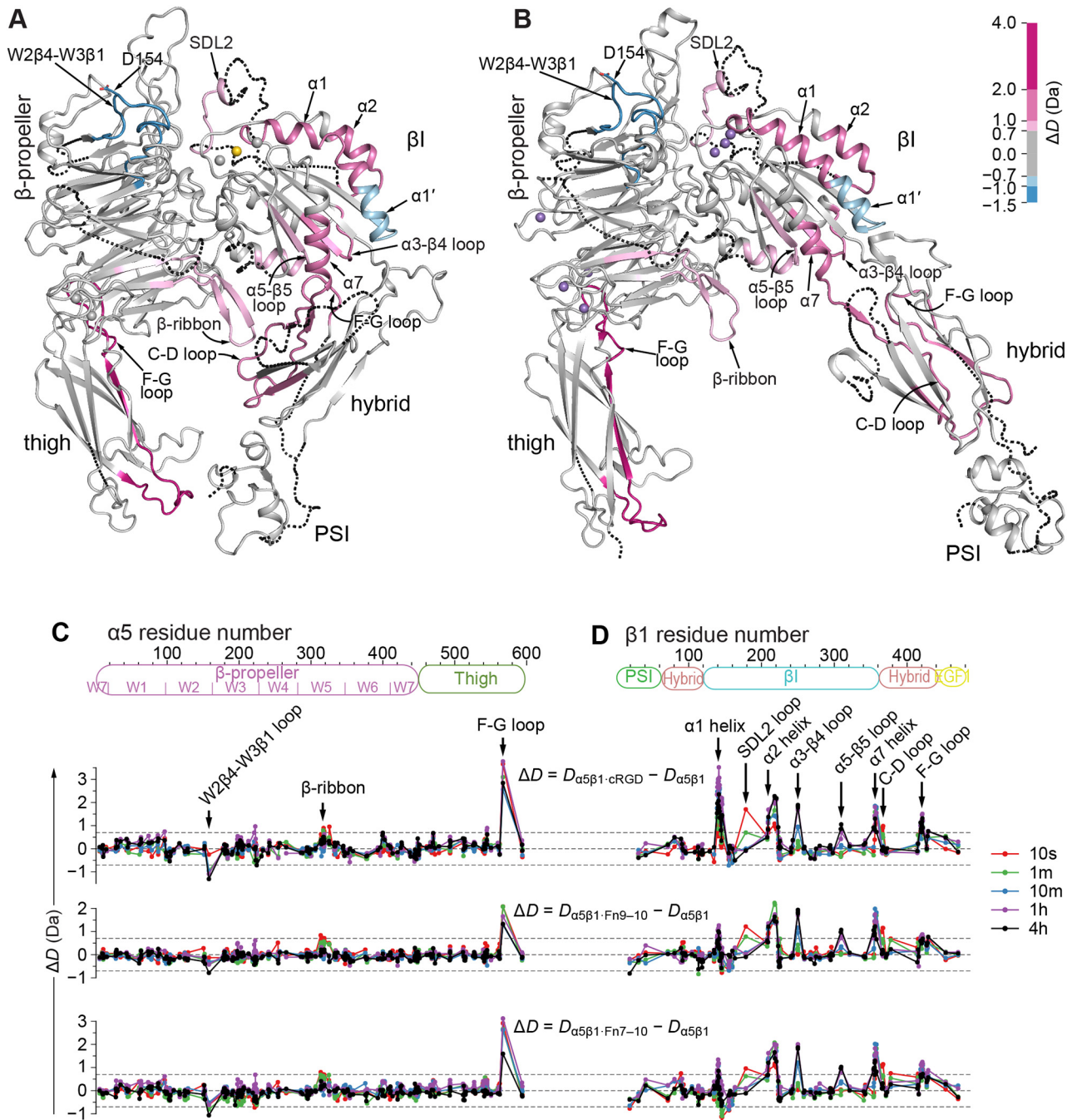
while Fn9-10 and Fn7-10 only slightly increased  $\alpha 1$  exchange. In contrast, all three ligands similarly and slightly decreased exchange in  $\alpha 1'$ . In the open conformation of the  $\beta I$  domain, the  $\beta$ -MIDAS motif that coordinates the MIDAS and ADMIDAS metal ions, which largely is within the  $\alpha 1$ -helix, moves and forms enhanced interactions with the MIDAS  $Mg^{2+}$  ion, the ADMIDAS  $Ca^{2+}$  ion, and the Asp of the RGD motif present in all three ligands (15, 32).

The increase in HDX of the  $\alpha 1$ -helix is the opposite of that expected from stabilization and burial by ligand binding. Instead, the increase in exchange in the  $\alpha 1$ ,  $\alpha 2$ , and  $\alpha 7$  helices

correlates with the lower stability of the  $\alpha 5\beta 1$  headpiece in the open conformation—by 4.7 kcal/mol compared to the closed conformation (8). The lesser increase in exchange of the  $\alpha 1$ -helix by the two Fn fragments than cRGD may be accounted by their larger burial of the  $\alpha 1$ -helix; for example, Tyr-1446 of the Fn10 domain hydrogen bonds to  $\beta I$  residue Asp-137, which is part of the  $\alpha 1$ -helix and coordinates the ADMIDAS  $Ca^{2+}$  ion (Fig. 1E).

Other regions of altered HDX in the  $\beta I$  subunit were in  $\beta I$  and hybrid domain interfaces. The  $\alpha 3$ - $\beta 4$  and  $\alpha 5$ - $\beta 5$  loops in the  $\beta I$  domain and F-G loop in the hybrid domain in the  $\beta I$ -





**Figure 7. Deuterium uptake differences between ligand-bound and free  $\alpha 5\beta 1$  headpiece.** A and B, deuterium uptake differences between Fn7-10-bound and free  $\alpha 5\beta 1$  headpiece ( $\Delta D = D_{\alpha 5\beta 1\text{-Fn7-10}} - D_{\text{free } \alpha 5\beta 1}$ ) are shown on the closed (A) and open (B)  $\alpha 5\beta 1$  headpiece structures (3VI4 and 7NWL, respectively) for comparison. Peptides are colored as shown in the key by their  $\Delta D$  at following time points:  $\beta$ -ribbon, SDL2 and C-D loops at 10 s;  $\alpha 1$  and  $\alpha 1'$  helices at 10 m; F-G ( $\alpha 5$  subunit), SDL2,  $\alpha 3$ - $\beta 4$ ,  $\alpha 5$ - $\beta 5$  and F-G ( $\beta 1$  subunit) loops and  $\alpha 2$  and  $\alpha 7$  helices at 1 h; W2 $\beta$ 4-W3 $\beta$ 1 loop at 4 h. Regions not covered by HDX are shown in black dotted lines. C and D,  $\Delta D = D_{\text{ligand bound-}\alpha 5\beta 1} - D_{\text{free } \alpha 5\beta 1}$  for each peptide is plotted at the midpoint of its sequence position. Horizontal dashed lines mark  $\Delta D = 0, \pm 0.7$  Da, as described in Figure 4. The difference calculation was performed using the data shown in Figs. S5–S8, for free and bound forms, respectively. All deuteration values and peptide sequences are found in the Supplementary Datafile.

hybrid domain interface all showed increased HDX in the presence of ligand (Figs. 6, N, O and R and 7, A, B and D). The C-D loop in the hybrid domain is in contact with a long  $\beta$ -ribbon in the  $\beta$ -propeller domain in the closed conformation that is broken in the open conformation, correlating with increased HDX in the presence of the three ligands (Figs. 1, C and D, 6Q, and 7, A, B and D). SDL2 shows an increase in

HDX at early time points in liganded  $\alpha 5\beta 1$  (Fig. 6L), correlating with its movement together with the contacting Tyr-133 sidechain in the  $\alpha 1$ -helix, which contacts Pro-1497 in the Fn10 RGD loop.

Ligand binding-dependent HDX signatures were fewer in  $\alpha 5$  than in the  $\beta 1$  subunit but provided important insights. The W2 $\beta$ 4-W3 $\beta$ 1 loop (peptide 148–168) in  $\alpha 5$ , which contains

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Asp-154 that binds to Fn9 synergy site residue Arg-1379, showed decreased HDX with both Fn9–10 and Fn7–10 at later time points, confirming the importance of the synergy site (Figs. 6A and 7, A–C). Interestingly, cRGD decreased exchange somewhat more in the same peptide. cRGD gains high affinity for  $\alpha 5\beta 1$  from the Trp residue in position 7 of its sequence, ACRGDGWCG, which was predicted to interact with  $\alpha 5$  Trp-157 by mutagenesis (28, 29). Indeed, a crystal structure shows a T-shaped interaction between Trp-7 of cRGD and  $\alpha 5$  Trp-157 with formation of two  $\pi$ – $\pi$  bonds (14), explaining the decrease in deuteration upon cRGD binding. In contrast, peptides containing Asp-227 in the  $\alpha 5$   $\beta$ -propeller W3 $\beta$ 4–W4 $\beta$ 1 loop, which forms bidentate hydrogen bonds to the Arg sidechain of the RGD moiety of the three ligands, did not show changes in HDX (Fig. 6C). This may be because Asp-227 lies in a cleft (Fig. 1E) and was already quite stable owing to a pre-existing hydrogen bond network that includes its sidechain and the presence of at least one mainchain–mainchain hydrogen bond in Asp-227 and its flanking residues from positions 223 to 231.

Two other regions of the  $\alpha 5$   $\beta$ -propeller showed higher exchange upon ligand binding. The  $\beta$ -ribbon in the  $\beta$ -propeller domain, which contacts the C-D loop in the hybrid domain in the closed but not open conformation, as already discussed above, showed increased deuterium uptake upon ligand binding at early time points (10 s and 1 m) (Figs. 6D and 7, A–C). Interestingly, increased exchange at the 10 s and 1 m and not later time points was seen for both the  $\beta$ -ribbon and C-D loop (Fig. 6, D and Q), suggesting that while ligand binding resulted in headpiece opening and broke cognate interactions between these regions at early time points, their interface was also susceptible to breathing motions in the closed conformation that resulted in exchange at later time points. A long peptide in the thigh domain (549–585) showed HDX that was increased by ligand binding by  $\sim 2$  to 3 Da at all time points (Figs. 6F and 7, A–C). Lack of differences in overlapping peptides 546 to 555 and 556 to 569 (Figs. 6, E and G and S9B) suggested that the increase in deuteration occurred in residues 570 to 585 in the thigh domain F-G loop. The F-G loop has an extensive interface with the  $\beta$ -propeller domain (Fig. 7, A and B). Studies of integrin ectodomains with multiple examples in crystal lattices have shown differences of up to 20° in thigh/ $\beta$ -propeller domain orientation (33). The thigh domain is close to the PSI domain in the closed conformation; loss of contact with PSI in the open conformation may alter thigh/ $\beta$ -propeller domain orientation and is one possible explanation for increased exchange of the thigh F-G loop in the open conformation. Another possibility is transmission of energy from the ligand binding interface through the relatively rigid  $\beta$ -propeller domain to its interface with the thigh domain.

### Discussion

The seminal observation by Ruoslahti *et al.* (34) that the RGD motif in fibronectin was sufficient to bind integrin  $\alpha 5\beta 1$ , and was also recognized by other integrins in other extracellular ligands, raised the question of how specificity was

obtained. Yamada *et al.* answered this question for  $\alpha 5\beta 1$  by demonstrating recognition of a distinct, synergistic site in Fn9 that neighbored the Fn10 domain bearing RGD (18, 19). Structures of  $\alpha 5\beta 1$  complexes by the groups of Takagi and Mizuno (12, 13) have clearly shown how the Fn9 domain is bound, but questions remain. For example, it has been suggested that synergy site mutations act indirectly by decreasing Fn9 stability, and a stabilizing L1408P mutation on the non-bound face of Fn9 was shown to increase  $\alpha 5\beta 1$  affinity for Fn9–10 (22).

We have determined equilibrium binding affinities for synergy site mutations and Fn fragments of different lengths and their stabilities using thermal and urea denaturation. Although our results on the Fn9 Arg-1379 interaction with  $\alpha 5$  Asp-154 are confirmatory, we believe that they are the first true affinity measurements. We find only a  $\sim 3$ -fold reduction in affinity with the Fn9 R1379A and  $\alpha 5$  D154A mutations alone or their combination. In contrast, competition of cell adhesion by R1379A Fn9–10 fragments showed a 70- to 100-fold reduction in potency (19, 21). Previous measurements by ELISA used washing and were thus inherently not in equilibrium; previous SPR measurements were not fit globally to demonstrate 1:1 Langmuir binding; furthermore, both methods use solid phases which introduce biases not found in solution binding assays. One study reported apparent affinities ( $EC_{50}$  values) in ELISA and SPR that differed internally from one another by 10-fold for some mutants and 5-fold for another; nonetheless, the relative differences between WT and R1379A Fn9–10 were close to the 3-fold difference found here (22). The three residues in Fn found most important for synergy were combined here in the triple mutant R1374A/P1376A/R1379A (Fn<sup>RPR</sup>) (19, 21). The Fn<sup>RPR</sup> mutant lowered affinity by 15- to 17-fold, almost as much as seen with the 20- to 30-fold decrease in affinity with omission of the Fn9 domain. The same Fn<sup>RPR</sup> mutant studied by the Takagi group was found to decrease  $EC_{50}$  by  $\sim 30$ -fold, in good agreement with our results (13).

Subsequent structure determination showed that Arg-1374 points away from  $\alpha 5$  and that Pro-1376 is on the periphery of the contact and buries only a small amount of solvent-accessible surface area (12), supporting the idea that the R1374A and P1376A mutations might lower Fn9 stability or alter its structure and thus indirectly affect affinity. Our stability measurements and the similar affinities for  $\alpha 5\beta 1$  of Fn<sup>RPR</sup>9–10 and Fn10 support this idea. We found single thermal and urea denaturation transitions for Fn7–10, Fn8–10, Fn9–10, and Fn10 fragments. Fn7 and Fn10 are very stable, and the single transitions in other fragments likely reflect unfolding of the less stable Fn8 and Fn9 domains (22, 26). The Fn<sup>R9</sup>–10 mutant was more stable than WT in urea, as also found for Fn<sup>R9</sup>–10 in guanidine (22). In contrast, the Fn<sup>RPR</sup> mutant was substantially less stable than WT, showing that only the R1374A and/or P1376A mutations are detrimental to stability. These results suggest that the predominant effect of the R1374A and P1376A mutations is to disrupt the structure of Fn9 so that it binds  $\alpha 5\beta 1$  less well.

In conclusion, it appears that previous attempts to define a synergy site within Fn9 identified both a specific contact of Fn9 Arg-1379 with  $\alpha 5$  Asp-154 that decreased affinity by 3-fold and other residues that decreased affinity substantially more by altering the folding or structure of Fn9. Although one group suggested that synergistic activity depended on structural stability, it is interesting that they found one exception to this trend: R1379A (Fig. 5 in (22)). It was noble to attempt to identify a synergy site motif in Fn9 that could be distilled down to a short amino acid sequence like RGD in Fn10 (18, 19, 21); however, our data and recent structures (12, 13) suggest that the folded structure of Fn9 and residues that are distant in sequence but close in structure are required for proper recognition by  $\alpha 5\beta 1$ . Thus, it appears more correct to think of Fn9 as being a synergistic domain rather than to think of Fn9 as containing a synergistic site that can be reduced to a sequence motif analogous to RGD.

Our HDX experiments showed integrin  $\alpha 5\beta 1$ -binding signatures in Fn7–10 and Fn9–10 only of peptides 1373 to 1384 in Fn9 and 1488 to 1509 in Fn10. It is important to point out that peptide 1373 to 1384 contains multiple residues within van der Waals distance of  $\alpha 5\beta 1$  including the sidechains Asp-1373, Arg-1379, Ser-1381, and the backbone of Ile-1382 and that these residues are in two  $\beta$ -strands and the loop between them and thus require an intact Fn9 domain for their proper 3-dimensional organization into a binding surface (12). Peptide 1488 to 1509 contains only three residues in van der Waals contact with  $\alpha 5\beta 1$ , Arg-1493, Asp-1495, and Pro-1497; these residues are at the tip of the long RGD loop and therefore have little dependence on the remainder of the Fn10 domain for their 3-dimensional organization. These differences correlate with the domain-like and sequence motif-like characters of  $\alpha 5\beta 1$  binding by Fn domains 9 and 10, respectively.

In  $\alpha 5\beta 1$ , we saw an HDX signature for binding of Asp-154 to Arg-1379 in Fn9 in the decreased exchange of  $\alpha 5$  peptide 148 to 168 (W2 $\beta$ 4-W3 $\beta$ 1 loop). We did not see a signature for binding of Arg-1493 in the RGD motif of Fn10 to Asp-227 in the  $\alpha 5$   $\beta$ -propeller domain. As discussed in Results, the lack of decreased HDX may be because the Asp-227 sidechain and the mainchain of residues 223 to 231 in the polypeptide chain are secured by hydrogen bonds, and thus, mainchain exchange may already be low prior to Fn binding.

An unexpected increase of large magnitude in HDX was seen in the thigh domain F-G loop, which is in intimate contact with the  $\alpha 5$   $\beta$ -propeller domain. The thigh domain may act as a chaperone for folding of integrin  $\beta$ -propellers because no integrin has ever been successfully biosynthesized and expressed as a recombinant  $\alpha\beta$  heterodimer containing only the  $\beta$ -propeller domain in the  $\alpha$ -subunit, despite the ability to proteolytically remove the thigh domain after biosynthesis with full retention of ligand-binding activity (32). The  $\alpha 5$   $\beta$ -propeller domain is knitted together by association of residues 1 to 12 containing  $\beta$ -propeller  $\beta$ -strand 4 with  $\beta$ -strands 1 to 3 formed by residues 410 to 452 into  $\beta$ -sheet (blade) 7 of the 7-bladed  $\beta$ -propeller domain (35). The thigh F-G loop lines the interface with  $\beta$ -sheet 7 of the  $\beta$ -propeller domain and

contacts both the N-terminal segment prior to  $\beta$ -strand 1, the connection to the thigh domain through the segment after  $\beta$ -strand 4, and the loop between  $\beta$ -strands 2 and 3. Thus, the thigh F-G loop may chaperone the  $\beta$ -propeller by helping to splice together its N- and C-terminal portions. Movement of the PSI domain in the upper  $\beta$ -leg away from close proximity to the thigh domain and its impact on motions at the thigh- $\beta$ -propeller interface at the F-G loop is one possible explanation for its increased HDX as mentioned in the Results section. Another possible explanation would be transmission of motion through the  $\beta$ -propeller domain from its ligand-binding interface with the  $\beta I$  domain to the thigh F-G loop; specific pathways for transmission of allostery and motion through domains have been observed in model systems (36).

Binding of Fn7–10, Fn9–10, and cRGD peptide to  $\alpha 5\beta 1$  strikingly altered HDX in all  $\beta I$  subunit regions with conformational change upon opening (12, 15, 32), including the  $\beta I$  domain  $\alpha 1$  and  $\alpha 1'$  helices. In opening, these helices move toward the ligand-binding site and the  $\alpha 1$  helix contacts ligand, including forming mainchain hydrogen bonds to the sidechain of the Asp of RGD. Upon opening, the  $\alpha 1$  and  $\alpha 1'$  helices, which are separated by a bend in the closed conformation, fuse to form a single straight helix. Binding of cRGD increased exchange in the  $\alpha 5\beta 1$   $\beta I$  domain  $\alpha 1$  helix substantially more than Fn7–10 and Fn9–10, consistent with burial of  $\alpha 1$  helix residues D137–E140 by non-RGD portions of Fn including formation of a hydrogen bond from Fn Tyr-1446 to the Asp-137 sidechain; the other carboxyl oxygen of the Asp-137 sidechain coordinates the ADMIDAS  $\text{Ca}^{2+}$  ion. It appeared that conformational change upon binding to RGD in all ligands substantially increased exchange in the  $\alpha 1$ -helix and that this was partially offset by stabilizing interactions formed by non-RGD portions of the Fn10 domain in Fn7–10 and Fn9–10 that lowered exchange. On the other hand, exchange in the  $\alpha 1'$  portion of the merged  $\alpha 1/\alpha 1'$ -helix in the  $\beta I$  domain was decreased by binding of all ligands, perhaps by helix merger.

All other regions of the  $\alpha 5\beta 1$  headpiece that alter conformation upon headpiece opening showed ligand binding-induced increases in HDX. The  $\alpha 2$ - and  $\alpha 7$ -helices in the  $\beta I$  domain lie on opposite sides of the  $\alpha 1$ -helix and show piston-like movements in allostery. Each showed dramatic increases in deuteration after ligand binding, which stabilized almost complete conversion of ligand-bound  $\alpha 5\beta 1$  to the open conformation as shown by energy landscape measurements and calculations. Two loops each in the  $\beta I$  domain and in the hybrid domain showed more deuteration after ligand binding. Three of these loops were at the interface between the  $\beta I$  and hybrid domains, which undergoes a large change in inter-domain orientation upon opening. Regions of the  $\beta$ -propeller and hybrid domains that touch in the closed but not open conformations also showed increased HDX. The HDX results thus define many regions that are less stable in the open than the closed conformations. Measurements of the free energy on the closed and open states of the  $\alpha 5\beta 1$  headpiece fragment show an increase of 4.7 kcal/mol in the open state (8). Our findings thus highlight critical regions in the  $\alpha 5\beta 1$  headpiece

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where this increase in energy in the open conformation is likely to be stored.

In conclusion, our results add much to our understanding of how integrin  $\alpha 5\beta 1$  binds fibronectin and how stabilization of the open conformation by ligand binding alters integrin dynamics. Affinity measurements and stability measurements on WT and mutant fibronectin fragments show that both the Fn9 and Fn10 domains make important contributions to affinity for  $\alpha 5\beta 1$ . A well-folded Fn9 domain and its Arg-1379 sidechain are required for full affinity; however, other residues that were thought to be part of a synergy site appear to stabilize folding of Fn9 rather than to contribute specific contacts as previously suggested. Owing to the prescient modeling of Fn- $\alpha 5\beta 1$  interaction based on an  $\alpha 5\beta 1$  RGD peptide crystal structure (13) and a cryoEM structure of a fibronectin fragment bound to  $\alpha 5\beta 1$  (12), our work added little information on where fibronectin and  $\alpha 5\beta 1$  bind to one another.

Comparison of the  $\alpha 5\beta 1$  headpiece alone, which was almost completely in the closed conformation, to the fibronectin-bound  $\alpha 5\beta 1$  headpiece, which was almost completely in the open conformation, revealed interesting information on changes in integrin dynamics upon conformational change. Binding of the Fn10 domain to the  $\beta 1$  domain  $\alpha 1$ -helix backbone and sidechain was accompanied by an increase in  $\alpha 1$ -helix dynamics. Normally, HDX decreases in binding sites; therefore, the increase in HDX was attributed to the known conformational change in the  $\alpha 1$ -helix between the closed and open states in  $\alpha 5\beta 1$ . This was supported by the greater increase in HDX in  $\alpha 5\beta 1$  when bound to a cyclic RGD peptide, which induces the same open state of  $\alpha 5\beta 1$  (8) yet buries less of the  $\alpha 1$ -helix in its complex. Merger of the  $\alpha 1$ -helix with the  $\alpha 1'$ -helix in the open conformation was accompanied by a decrease in  $\alpha 1'$ -helix HDX. All other regions involved in conformational change showed increased HDX, including  $\alpha$ -helices that undergo connecting rod-like movements in the  $\beta 1$  domain, loops in the  $\beta 1$  domain and the hybrid domain at their shape-shifting interface, and contacts between the  $\alpha$  and  $\beta$ -subunits. The increase in HDX showed that these regions were more dynamic, *i.e.*, had higher energy, in the open than in the closed conformation. In agreement, measurements of the free energy of the closed and open states of the same  $\alpha 5\beta 1$  headpiece fragment show an increase of 4.7 kcal/mol in the open state (8). Thus, our HDX measurements highlight, at least in part, regions in the  $\alpha 5\beta 1$  headpiece where this increase in energy in the open conformation is stored. We believe it is interesting that this energy is distributed in multiple regions throughout the  $\beta 1$  and hybrid domains rather than concentrated. This distribution might serve to lower the energy of the transition state between the two states, thus increasing the likelihood of the transition and the kinetics of conformational change. Distribution might also serve to decrease the likelihood of denaturation of a specific portion or the entirety of a domain. These results thus provide important insights into integrin conformational change that are orthogonal and complementary to insights from previous structural and thermodynamic studies.

## Experimental procedures

### Proteins and cRGD peptide

$\alpha 5\beta 1$  headpiece and ectodomain (mature residues  $\alpha 5$  F1–L609/ $\beta 1$  Q1–E481 and  $\alpha 5$  F1–Y954/ $\beta 1$  Q1–D708, respectively) were expressed in HEK 293S GnTI<sup>-/-</sup> cells and purified as described (14). The  $\alpha 5$  D154A mutant of  $\alpha 5\beta 1$  ectodomain ( $\alpha 5^{\text{D154A}}\beta 1$ ) was expressed in Expi293F GnTI<sup>-/-</sup> cells and purified in the same way. Fibronectin fragments Fn10, Fn9–10, Fn8–10, and Fn7–10 (mature residues V1416–T1509, G1326–T1509, T1265–T1509, and P1142–T1509, respectively) and their associated synergy site mutants R1379A (Fn<sup>R</sup>) and R1374A/P1376A/R1379A (Fn<sup>RPR</sup>) were expressed in *E. coli* BL21(DE3) cells and purified as described (37). The cyclic peptide ACRGDGWCG (>95% pure) was synthesized by GenScript. To form  $\alpha 5\beta 1$ -Fn9–10 and  $\alpha 5\beta 1$ -Fn7–10 complexes,  $\alpha 5\beta 1$  headpiece was incubated with 2-fold molar excess of Fn9–10 or Fn7–10 for at least 20 min at 23 °C in 20 mM Tris buffer pH 7.4, 150 mM NaCl, 2 mM MnCl<sub>2</sub>, and 0.2 mM CaCl<sub>2</sub>. The complexes were then isolated by size-exclusion chromatography.

### Fluorescence polarization

Saturation binding was performed with 10 nM FITC-cRGD probe and varying concentrations of  $\alpha 5\beta 1$  headpiece or ectodomain. Competitive binding was performed with 100 nM  $\alpha 5\beta 1$  headpiece or  $\alpha 5\beta 1$  ectodomain, 10 nM FITC-cRGD probe, and varying concentrations of Fn9–10 or Fn7–10. Binding was in 20 mM Tris buffer pH 7.4, 150 mM NaCl, supplemented either with 1 mM MgCl<sub>2</sub> and 1 mM CaCl<sub>2</sub> or with 2 mM MnCl<sub>2</sub> and 0.2 mM CaCl<sub>2</sub>, as indicated. The mixture was allowed to equilibrate at 22 °C for 2 h before recording FP on a Synergy NEO HTS multi-mode microplate reader (Biotek). Experiments were performed in triplicate unless otherwise indicated.

### Thermal stability

Thermal denaturation was measured with a Prometheus NT.Plex (NanoTemper Technologies). Fn7–10, Fn8–10, and Fn9–10 (1 mg/ml) in 20 mM Tris buffer, pH 7.4, 150 mM NaCl were heated from 20 °C to 95 °C at a rate of 1 °C/min. Intrinsic tryptophan fluorescence was excited at 275 nm, and emission was monitored at 350 nm and 330 nm. The ratio of fluorescence intensities (F350/F330) is plotted as a function of temperature.  $T_m$  is defined as the temperature at the inflection point of the curve.

### Chemical denaturation

Fn10, Fn9–10, Fn<sup>R</sup>9–10, and Fn<sup>RPR</sup>9–10 (0.2 mg/ml) were equilibrated in 20 mM Tris pH 7.4, 150 mM NaCl and indicated urea concentrations for 48 h at 20 °C. Intrinsic tryptophan fluorescence were measured at 330 nm and 350 nm upon excitation at 275 nm on a Prometheus NT.Plex (NanoTemper Technologies). The ratio of fluorescence intensities (F350/F330) was fit as a function of urea

concentration to a 2-state transition model using the linear extrapolation method (38):

$$F350 / F330 = \frac{\alpha_N + \beta_N c + (\alpha_D + \beta_D c) \exp\left(-\frac{\Delta G - mc}{RT}\right)}{1 + \exp\left(-\frac{\Delta G - mc}{RT}\right)}$$

where  $\Delta G$  is the unfolding free energy in the absence of urea;  $c$  is the concentration of urea;  $m$  is the cooperativity of transition from the native state (N) to the denatured state (D);  $R$  is the gas constant;  $T$  is absolute temperature; and  $\alpha_N$ ,  $\beta_N$ ,  $\alpha_D$ , and  $\beta_D$  are the  $y$ -intercept ( $\alpha$ ) and slope ( $\beta$ ) of the baseline of the native (N) and denatured states (D), respectively. Data for Fn9–10, Fn<sup>R</sup>9–10, and Fn<sup>RPR</sup>9–10 were fit with shared  $m$  because their increase in solvent-exposed surface upon unfolding is similar (39).

### Hydrogen deuterium exchange mass spectrometry

HDX MS studies were performed using methods modified from those reported previously (40). In addition to the descriptions below, comprehensive experimental details and parameters are provided in Table S1 and the Supplementary Datafile, in the recommended (41) tabular format. Relative deuterium levels and percent deuteration values for all peptides described in both figures and text are provided in the Supplementary Datafile. The HDX MS data have been deposited to the ProteomeXchange Consortium *via* the PRIDE partner repository (42) with the dataset identifier PXD031508.

Deuterium exchange was measured for the following samples:  $\alpha 5\beta 1$  headpiece (74.5  $\mu$ M), Fn9–10 (68.6  $\mu$ M), Fn7–10 (65.2  $\mu$ M),  $\alpha 5\beta 1$  (59.6  $\mu$ M) + cRGD (119.2  $\mu$ M) mixture,  $\alpha 5\beta 1$ ·Fn9–10 complex (47.3  $\mu$ M), and  $\alpha 5\beta 1$ ·Fn7–10 complex (54.4  $\mu$ M). Samples (2  $\mu$ l) were diluted 15-fold into 20 mM Tris, 150 mM NaCl, 2 mM MnCl<sub>2</sub>, and 0.2 mM CaCl<sub>2</sub>, 99% D<sub>2</sub>O (pD 7.4) at 21 °C. At deuterium exchange time points from 10 s to 4 h, an aliquot was quenched by adjusting the pH to 2.5 with an equal volume of 4 M GnHCl, 200 mM sodium phosphate, 0.5 M tris(2-carboxyethyl)phosphine hydrochloride (TCEP-HCl), and H<sub>2</sub>O and digested offline with pepsin for 5 min on ice prior to UPLC separation.

Mass spectrometry (MS) analyses were performed with a Synapt-G2-Si coupled to a nanoAcquity HDX Manager (Waters) (43). PLGS 3.0 and DynamX 3.0 were used to identify the peptides and to measure deuterium incorporation. All comparison experiments were done under identical experimental conditions such that deuterium levels were not corrected for back exchange and are therefore reported as relative (23). The error of measuring the mass of each peptide was  $\pm 0.15$  Da or less in this instrumental setup. Each experiment was performed in at least duplicate (see Table S1). All replicates of a given dataset were merged to a single DynamX file, and the error bars in uptake graphs indicate the spread of the data as provided by the DynamX software, which in most cases also includes measurements

from more than one charge state of any given peptide in any given replicate.

### Equilibrium populations of $\alpha 5\beta 1$ headpiece

The percentage of  $\alpha 5\beta 1$  headpiece bound to ligand and the percentages of  $\alpha 5\beta 1$  in the closed or open conformations in the HDX samples were calculated according to the following equations.

$$C \rightleftharpoons O \quad K_{\text{conf}}^{C \rightleftharpoons O} = \frac{[O]}{[C]} \quad (1)$$

$$C \cdot L \rightleftharpoons C + L \quad K_d^C = \frac{[C][L]}{[C \cdot L]} \quad (2)$$

$$O \cdot L \rightleftharpoons O + L \quad K_d^O = \frac{[O][L]}{[O \cdot L]} \quad (3)$$

$$C \cdot L + O \cdot L \rightleftharpoons C + O + L \quad K_d^{\text{ens}} = \frac{[C \cdot L] + [O \cdot L]}{([C] + [O])[L]}$$

$$= \frac{1}{K_d^C} \left( \frac{1}{1 + K_{\text{conf}}^{C \rightleftharpoons O}} \right) + \frac{1}{K_d^O} \left( \frac{K_{\text{conf}}^{C \rightleftharpoons O}}{1 + K_{\text{conf}}^{C \rightleftharpoons O}} \right) \quad (4)$$

$$[C] + [C \cdot L] + [O] + [O \cdot L] = [\alpha 5\beta 1]_{\text{total}} \quad (5)$$

$$[L] + [C \cdot L] + [O \cdot L] = [L]_{\text{total}} \quad (6)$$

$$\% \text{ bound } \alpha 5\beta 1 = \frac{[C \cdot L] + [O \cdot L]}{[\alpha 5\beta 1]_{\text{total}}} \quad (7)$$

$$\% \text{ closed complex} = \frac{[C \cdot L]}{[\alpha 5\beta 1]_{\text{total}}} \quad (8)$$

$$\% \text{ open complex} = \frac{[O \cdot L]}{[\alpha 5\beta 1]_{\text{total}}} \quad (9)$$

Equation 1 describes the conformational equilibrium between the closed (C) and open (O) conformations of  $\alpha 5\beta 1$  headpiece in the basal conformational ensemble. A ligand (L) can bind each conformation in the ensemble to form the closed complex (C·L) and the open complex (O·L) with intrinsic affinities  $K_d^C$  and  $K_d^O$ , respectively (Equations 2 and 3). Equation 4 relates the affinity of the basal ensemble ( $K_d^{\text{ens}}$ ) to the intrinsic affinities ( $K_d^C$  and  $K_d^O$ ) and the conformational equilibrium ( $K_{\text{conf}}^{C \rightleftharpoons O}$ ), which provides a way to determine  $K_{\text{conf}}^{C \rightleftharpoons O}$  from experimentally measurable affinities.  $K_d^{\text{ens}}$  and  $K_d^C$  were experimentally measured here (Fig. S3).  $K_d^O$  for cRGD or

## Dynamics of $\alpha 5\beta 1$ , fibronectin, and their complex

Fn in the presence of  $Mn^{2+}$  was difficult to measure accurately by fluorescence polarization because ligand binding/dissociation rates for the open conformation were too slow for the system to reach equilibrium. Instead, we estimated  $K_d^O$  based on our previous affinity measurements for  $\alpha 5\beta 1$  (8, 16), which found that  $K_d^O$  was  $\sim 2000$  to  $7000$  times smaller than  $K_d^C$  (Table S2).

Equations 5 and 6 are according to the law of conservation of mass. When known concentrations of  $\alpha 5\beta 1$  headpiece and ligand are mixed, the equilibrium concentrations of each species ( $[C]$ ,  $[O]$ ,  $[L]$ ,  $[C-L]$  and  $[O-L]$ ) can be solved simultaneously using numeric methods from Equations 2–6 in which  $K_d^C$ ,  $K_d^O$ ,  $K_d^{ens}$ ,  $[\alpha 5\beta 1]_{total}$ , and  $[L]_{total}$  were known inputs. The percentage of ligand-bound  $\alpha 5\beta 1$  headpiece was then calculated by Equation 7. The percentages of closed and open complexes were calculated by Equations 8 and 9, respectively. The results are listed in Table S3.

### Data availability

All manuscript data can be found in the main text, figures, and supplemental files. The raw HDX MS data have been deposited to the ProteomeXchange Consortium *via* the PRIDE partner repository with the dataset identifier PXD031508.

**Supporting information**—This article contains supporting information (8, 16, 44, 45).

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**Conflict of interest**—The authors declare that they have no conflicts of interest with the contents of this article.

**Abbreviations**—The abbreviations used are: BC, bent-closed; EC, extended-closed; ECM, extracellular matrix; EO, extended-open; Fn, fibronectin; HDX, hydrogen-deuterium exchange.

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