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Locking the β₃ Integrin I-like Domain into High and Low Affinity Conformations with Disulfides

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Although integrin α subunit I domains exist in multiple conformations, it is controversial whether integrin β subunit I-like domains undergo structurally analogous movements of the α7-helix that are linked to affinity for ligand. Disulfide bonds were introduced into the β₃ integrin I-like domain to lock its β6-α7 loop and α7-helix in two distinct conformations. Soluble ligand binding, ligand mimetic mAb binding and cell adhesion studies showed that disulfide-bonded receptor α₃β₃T329C/A347C was locked in a low affinity state, and dithiothreitol treatment restored the capability of being activated to high affinity binding; by contrast, disulfide-bonded α₃β₃V332C/M359C was locked in a high affinity state. The results suggest that activation of the β subunit I-like domain is analogous to that of the α subunit I domain, i.e. that axial movement in the C-terminal direction of the α7-helix is linked to rearrangement of the I-like domain metal ion-dependent adhesion site into a high affinity conformation.

Integrins are large heterodimeric adhesion molecules that convey signals bidirectionally across the plasma membrane (1, 2). Both integrin subunits are type I transmembrane proteins with large extracellular domains. Priming of the extracellular domain for ligand binding (i.e., increasing its affinity for ligand) is initiated by moving apart the α and β subunit cytoplasmic domains and probably separation of the transmembrane domains as well (3). Conversely, binding of ligand can also initiate cytoplasmic domain separation (3); the equilibria relating these domains and probably separation of the transmembrane domains and cytoplasmic domains are linked to integrin activation. Disulfide bonds are structurally analogous to those that occur in solution x-ray scattering studies and exposure of epitopes on the inner side of the hybrid domain in the presence of ligand (11, 12) support the direct observations of hybrid domain swing-out (4, 8). There is controversy concerning this proposed mechanism. Soaking of a ligand-mimetic Arg-Gly-Asp (RGD) peptide into integrin α₃β₃ crystals, in which α₃β₃ was constrained in the bent conformation by lattice contacts, induced β6-α7 loop and α7-helix movements, but not α7-helix displacement (5). It was therefore suggested that α I and β I-like domains are activated by distinct mechanisms. Demonstration of movement of an epitope in the α1-helix was used to support the hypothesis that the mechanism of I-like domain activation differs from that of the I domain (10). On the other hand, conformational change at this region would not contradict C-terminal α7-helix movement, and the mutation L358A in the α7-helix of the β₁ I-like domain causes activation, supporting some type of conformational change around the α7-helix upon ligand binding (11). Furthermore, solution x-ray scattering studies and exposure of epitopes on the inner side of the hybrid domain in the presence of ligand (11, 12) support the direct observations of hybrid domain swing-out (4, 8).

Here, we directly test the hypothesis that specific rearrangements occur in the β6-α7 loop and α7-helix of β₃ I-like domains that are structurally analogous to those that occur in α I domains and are linked to integrin activation. Disulfide bonds have previously been introduced into α I domains to constrain
the β6-α7 loop and α7-helix. The α3 (13–16) and α2 (17) I domains have been locked into closed, intermediate, or open conformations with low, intermediate, or high affinity for ligand, respectively. Crystall structure studies on the mutant α1 I domains confirmed alterations in the β6-α7 loop corresponding to α7-helix displacements of one and two turns of helix in the intermediate and open conformations, respectively (18). The disposition of this high affinity, open confirmation of the α1 I domain corresponds precisely in the critical β6-α7 loop and MIDAS loops to the open confirmation of the wild-type α3 and α2 I domains seen when this conformation was stabilized in crystals by ligand or ligand-like lattice contacts (19, 18). The studies reported here on the β I-like domain show that disulfide bonds mutationally introduced into the β6/α7 region lock intermediates that lack I domains into two distinct affinity states. The data uniquely support the proposal that downward movement of the α7-helix induces I-like domain activation and demonstrate that α I and β I-like domains are activated by structurally analogous mechanisms.

MATERIALS AND METHODS

High Affinity I-like Domain Model—The model was built with the Segmod module (20) of GeneMine version 3.5 using residues 108–333 and 347–353 of Protein Data Bank accession number 1JZV (2) as template and aligning them with residues 108–333 and 340–346 of the model sequence, respectively. This corresponds to a 7-residue, 2-turn displacement of the α7-helix along its helical axis; residues 334–339 were left unnoted. Plassid Construction, Transient Transfection, and Immunoprecipitation—Plassids coding for full length human α1Ib, and β3 were subcloned into pEF/V5-HisA or pcDNA3.1/Myc-His (Invitrogen) for 30 min, washed and 2°C for 1 h and subjected to SDS 7.5% PAGE and fluorography.

Labeling of Free Cysteines and Western Blotting—Transiently transfected 293T cells, treated with or without 5 mM DTT at 37°C for 30 min in TBS containing 1 mM CaCl2, followed by washing with TBS plus 1 mM CaCl2 three times, were labeled with 400 μM 1-biotinamido-4 (1-male-imidoethyl-cyclohexane)-carboxamidobutane (biotin-BMCC) (Pierce) at room temperature for 30 min, washed with TBS plus 1 mM CaCl2 three times, and lysed with TBS with 1% Triton X-100 and 0.1% Nonidet P-40 were immunoprecipitated with 1 μg of anti-β3 mAb AP3 and protein G-Sepharose at 4°C for 1 h and subjected to nonreducing SDS 7.5% PAGE and fluorography (22).

Expression and Ligand Binding Activity of α1Ibβ3 on CHO-K1 Transfectants—The plasmids described above coding for α1Ib and β3 were introduced into CHO-K1 cells using calcium phosphate precipitates (21). Transfectants were selected with 5 mg/ml G418. After 2 weeks, the cells were stained with AP3 mAb and subjected to fluorescence-activated cell sorting to obtain lines expressing the desired level of α1Ibβ3. Prior to ligand binding, transfected cells were suspended in HBS supplemented with 5.5 mM glucose and 1% BSA and incubated with 1 mM EDTA, 5 mM CaCl2, or 1 mM CaCl2 plus 10 μg/ml PT25-2, with or without 5 mM DTT, at 20°C for 30 min. Staining with fluorescein-labeled human fibrinogen and the ligand mimetic PAC-1 mAb (Becton Dickinson, San Jose, CA) was measured as described (4).

Cell Adhesion to Immobilized Fibrinogen—Cell adhesion was assayed as transfected CHO-K1 cells were labeled with 2′,7′-bis(carboxyethyl)-5(6)-carboxyfluorescein (Molecular Probes, Eugene, OR) and suspended to 107/ml in HBS supplemented with 5.5 mM glucose, 1% BSA, and either 1 mM EDTA, 5 mM CaCl2, or 5 mM DTT plus 5 mM CaCl2. Cell suspensions were incubated in wells that had been coated with different concentrations of fibrinogen followed by blocking with 1% BSA. After incubation at 37°C for 1 h, unbound cells were washed off after three resuspensions with a multichannel pipette. The fluorescence of input cells and bound cells in each well was quantitated on a fluorescent concentration analyzer (Idexx, Westbrook, ME). Bound cells were expressed as a percentage of total input cells.

LIHS Expression—Anti-LIBS mAbs LIBS-1, LIBS-6, and PMI-1 were kind gifts of M. H. Ginsberg (Scripps Research Institute, La Jolla, CA). LIBS expression was measured as described (9). In brief, CHO-K1 cells stably expressing wild type or mutant α1Ibβ3 in HBS supplemented with 5.5 mM glucose and 1% BSA were incubated under different conditions as indicated in the legend to Fig. 5 for 30 min at 20°C. LIBS mAbs were added to a final concentration of 10 μg/ml, and cells were incubated on ice for another 30 min before staining with FITC-conjugated anti-mouse IgG and flow cytometry.

RESULTS

Design of I-like Domains Locked in Low Affinity and High Affinity Conformations—We hypothesized that in both the unliganded and liganded α1Ibβ3 structures (5, 6), the C-terminal α7-helix of the β3 I-like domain is in a position stabilizing a closed, low affinity conformation; therefore, these structures were used to design low affinity mutants. An open, high affinity conformation was modeled assuming that the α7-helix was displaced in the C-terminal axial direction by two α-helical turns (see “Materials and Methods”). The distance between Cβ atoms of Thr239 and Ala247 in the unliganded and RGD-liganded α1Ibβ3 structures is 4.4 and 4.9 Å, respectively, whereas it is 9.6 Å in the hypothesized high affinity model. Therefore, the mutant β3V323C/M335C was expected to form a disulfide bond in the low but not the high affinity conformation and to be stabilized in the low affinity, closed conformation (Fig. 1, D and F). On the other hand, the distance between the Cβ atoms of Val312 and Met335 in the unliganded and RGD-ligand α1Ibβ3 structures is 10.0 and 8.3 Å, respectively; whereas it is 3.7 Å in the hypothesized high affinity model. Therefore, the mutant β3V323C/M335C was expected to form a disulfide bond in the high but not the low affinity conformation (Fig. 1, E and G) and to be stabilized in the high affinity, open conformation.

Expression of Wild Type and Mutant α1Ibβ3 Receptors and Formation of Disulfide Bonds—Wild type and mutant β3 subunits were co-transfected with wild type α1Ib in 293T cells and subjected to immunostaining flow cytometry (Fig. 2, A and B). The wild type and mutant receptors were recognized equally well by mAb to epitopes constitutively present on the α1Ib and β3 subunits, including AP3 (anti-β3), 10E5 (anti-α1Ib), HA5 (anti-α1Ib), and AP2 (anti-α1Ibβ3 complex-specific) (Fig. 2A), suggesting that the two mutant receptors adopted a native fold on the cell surface. However, the mutant β3V323C/M335C receptor was recognized weakly by anti-β3 mAb 7E3 (Fig. 2B). The 7E3 mAb recognizes residues in the specificity-determining β3-β3 loop near the β3 I-like MIDAS (24). Since the single cysteine mutants β3V312C and β3M335C were recognized by 7E3 (Fig. 2B),
the conformational change induced by the disulfide bond formed between V332C and M335C (see below) appears to diminish the 7E3 epitope. By contrast, mutant β3\textsuperscript{V332C/M335C} was well recognized by 7E3 (data not shown) (see Fig. 4A).

Nonreducing SDS-PAGE of \textsuperscript{35}S-labeled, immunoprecipitated receptors showed that the α\textsubscript{IIb} subunits migrated similarly (Fig. 2C), whereas the mutant β3\textsubscript{V332C/A347C} and β3\textsubscript{V332C/M335C} subunits (Fig. 2C, lanes 6 and 7) migrated slightly faster than wild type β3 (Fig. 2C, lane 5). By contrast, all β3 single cysteine mutants migrated similarly to wild-type β3 (Fig. 2C, lanes 1–4). In general, disulfide bonds increase the mobility of proteins in SDS-PAGE, and these results suggest that the cysteines introduced into the β3\textsubscript{V332C/A347C} and β3\textsubscript{V332C/M335C} mutants form a disulfide bond.

To confirm disulfide bond formation, free sulfhydryls were labeled with the maleimide-containing reagent, biotin-BMCC. The idea was that introducing a single cysteine should increase labeling, whereas introducing two cysteines would not increase labeling if they formed a disulfide bond. Transfectants were treated with biotin-BMCC, lysed, immunoprecipitated with AP3 mAb to α\textsubscript{IIb}β\textsubscript{3}, and subjected to SDS-PAGE and fluorography. Segments of straight lines drawn through the β3 bands in lanes 1–5 and lanes 6 and 7 are shown between lanes to emphasize the difference in migration. D, labeling of free cysteines. Transfected cells were treated with (wild-type only) or without 5 mM DTT at 37 °C for 30 min and labeled with biotin-BMCC at room temperature for 30 min. Immunoprecipitates with AP3 mAb to α\textsubscript{IIb}β\textsubscript{3} were treated with (wild-type only) or without 5 mM DTT at 37 °C for 30 min and labeled with biotin-BMCC at room temperature for 30 min. Immunoprecipitates with AP3 mAb to detect biotin-BMCC or anti-myc mAb to detect the myc-tagged β3 subunit.

The idea was that introducing a single cysteine should increase labeling, whereas introducing two cysteines would not increase labeling if they formed a disulfide bond. Transfectants were treated with biotin-BMCC, lysed, immunoprecipitated with AP3 mAb to α\textsubscript{IIb}β\textsubscript{3}, and subjected to SDS-PAGE and blotting with avidin (Fig. 2D). The β3 subunit was fused at its C terminus to a myc tag, and blotting with a myc mAb was used as a control for β3 loading. Whereas wild-type α\textsubscript{IIb}β\textsubscript{3} showed almost no labeling (Fig. 2D, lane 2), the α\textsubscript{IIb}β\textsubscript{3} single cysteine mutants V332C and M335C showed marked labeling (Fig. 2D,
fectants was treated with 5 mM DTT for 30 min at 37 °C

.../H9252/avidin/anti-

10E5, but neither blocked nor further activated by the activat-

10E5. Binding to soluble fibrinogen was first

10E5, -like Domain Conformation

lanes 4 and 6). The cysteines introduced in the V332C/M335C and T329CA347C mutants clearly formed disulfides, because labeling was at the same level as the wild type (Fig. 2D, lanes 5 and 7), whereas it would have been twice that of the single cysteine mutants if disulfides had not formed. To estimate the number of free cysteines per β3 subunit, the ratio of the intensity of avidin binding to that of anti-myc binding was deter-

Fig. 3. Ligand binding activity of mutant α1β3 integrins on 293T cells. A, soluble fibrinogen binding of 293T cell transfectants in the presence of 5 mM Ca²⁺ (white bar) or 1 mM Ca²⁺ plus 10 g/ml PT25-2 (black bar) at room temperature for 30 min. B, effect of mAbs. Soluble fibrinogen binding in the presence of 5 mM Ca²⁺ was measured in presence of activating LIBS antibody AP5 or blocking antibodies HA5 and 10E5. The control is with X63 IgG1. Fibrinogen binding was mea-

uted with two-color immunofluorescence as described under “Materials and Methods” and is expressed as the mean fluorescence intensity of fibrinogen staining as a percentage of mean fluorescence intensity of staining with AP3 mAb.

Ligand Binding Properties of 293T Transfectants with Disul-

dife-locked Receptors—Binding to soluble fibrinogen was first examined using two-color flow cytometry (4) in transiently trans-

fected 293T cells, in which wild type α1β3 basally has low affinity for ligand. Wild type α1β3 bound fibrinogen when stimulated with the activating mAb PT25-2 but not basally in Ca²⁺ (Fig. 3A). Each of the four single cysteine mutants behaved similarly to the wild type receptor (Fig. 3A). By contrast, the putative locked closed, double cysteine mutant α1β3T329C/A347C did not bind soluble fibrinogen even in the presence of PT25-2 (Fig. 3A). Furthermore, the putative locked open mutant α1β3V332C/M335C bound soluble fibrinogen even in Ca²⁺, and the addition of PT25-2 mAb did not further increase binding (Fig. 3A). Constitutive binding in Ca²⁺ by the α1β3V332C/M335C mutant was abolished by two blocking α9 mAbs, HA5 and 10E5, but neither blocked nor further activated by the activat-

ing β3 mAb AP5 (Fig. 3B), confirming that the high affinity binding of the transfected cells was specific.

Functional Properties of Mutant Receptors in CHO-K1 Trans-

fectants—To further examine the disulfide-locked receptors, stable CHO-K1 transfectants were established, and clones were selected that expressed similar quantities of wild-type α1β3, α1β3T329C/A347C, and α1β3T329C/A347C. The transfect-

ants were recognized equally well by a panel of mAb to constitutively expressed α1β3, β3, and α1β3 epistopes, with the exception of 7E3 mAb (Fig. 4A). Mutant α1β3T329C/A347C blunted but did not completely abolish the binding of 7E3.

CHO-K1 transfectants expressing the wild type receptor did not bind soluble fibrinogen or PAC-1 in Ca²⁺ but bound when stimulated by activating mAb PT25-2 (Fig. 4, B and C). Treatment with 5 mM DTT at 20 °C for 30 min slightly increased ligand binding to wild type α1β3 in Ca²⁺, but this binding was much less than that seen with PT25-2 mAb with or without DTT treatment. Mutant α1β3T329C/A347C did not bind fibrino-

gen or PAC-1 basally, and binding was not stimulatable with PT25-2. However, DTT treatment restored the ability of PT25-2 to stimulate fibrinogen and PAC-1 binding (Fig. 4, B and C), suggesting that the Cy²³⁴⁻—Cy³³⁴⁻ disulfide bond locked the I-like domain in the closed conformation, and this constraint was released by DTT treatment. By contrast, mutant α1β3V332C/M335C showed high binding to soluble fibrinogen and PAC-1, and binding was not further increased by activation. DTT treatment did not reduce fibrinogen or PAC-1 binding of the α1β3V332C/M335C mutant in Ca²⁺, probably because the Cy³³²⁻—Cy³³⁵⁻ disulfide bond was stable to reduction under non-denaturing conditions like the vast majority of the native disulfides in β3.

The affinity state of disulfide-bonded mutants was further tested in cell adhesion assays on immobilized fibrinogen. High affinity is required for binding to soluble ligand or ligand mi-

metic mAbs. In contrast, wild type α1β3 can mediate cell adhesion to immobilized fibrinogen in the absence of activation, as long as high coating concentrations above 1 μg/ml of fibrinogen are used (Fig. 4D), consistent with our previous report (9). DTT treatment slightly increased the avidity of the wild type receptor, as shown by a shift in the dose-response curve. In contrast, mutant α1β3T329C/A347C did not adhere even at the highest coating concentration of fibrinogen, whereas DTT treatment yielded binding of α1β3T329C/A347C indistinguish-

able from that of the DTT-treated wild type receptor, suggesting that DTT treatment could release the disulfide bond, which locked the receptor in the low avidity state. On the other hand, the high affinity α1β3T329C/A347C mutant adhered to immobilized fibrinogen at coating concentrations as low as 0.3 μg/ml, and DTT treatment did not alter its binding avidity, consistent with the results for soluble ligand binding.

Ligand-induced Binding Site (LIBS) Epitope Expression—

Priming and ligand binding alter the conformation of α1β3, result-

ing in the exposure of so-called LIBS. Such epitopes are buried in the bent conformation in interfaces between the head-piece and tail-piece and between the α leg and β leg and are exposed in the extended conformation (4, 7). To probe the conformational state of the α1β3 mutants, binding of a panel of anti-LIBS mAbs was determined. The mAbs LIBS1 (anti-β3; residues 420–690), LIBS6 (anti-β3; residues 602–690), and PUM-1 (anti-α1; residues 844–859) bound poorly to the cells stably expressing wild-type α1β3 in Ca²⁺ but bound maximally to α1β3 activated with Mn²⁺ and RGD peptide (Fig. 5). DTT treatment for 30 min at 20 °C increased to different ex-

tent the binding of the mAbs LIBS1, LIBS6, and PUM-1 to wild type α1β3 (Fig. 5), consistent with the ability of the DTT to partially activate the receptor. Activation with Mn²⁺ and RGD
peptide resulted in maximal exposure of LIBS epitopes, with or without DTT treatment. By contrast, LIBS exposure in the low affinity α₃β₃ mutant was either blunted or absent in response to RGD plus Mn²⁺ (Fig. 5). This suggests that the low affinity, disulfide-bonded, mutant receptor is in the overall bent conformation and is largely resistant to Mn²⁺/RGD activation. DTT treatment of this mutant rescued expression of LIBS1, LIBS6, and PMI-1 epitopes in response to Mn²⁺ and RGD peptide. For the open mutant α₁β₃V332C/M335C, the exposure of LIBS1, LIBS6, and PMI-1 epitopes behaved similarly to wild type. The LIBS mAbs bound poorly to the mutant in Ca²⁺, but Mn²⁺/RGD fully exposed the epitopes. Therefore, the high affinity mutant is in an overall bent conformation. These findings suggest that the high affinity ligand binding of mutant α₁β₃V332C/M335C is due to local conformational change within the I-like domain. Other LIBS mAbs including D3 (anti-β₃, residues 422–490) and AP5 (anti-β₃, residues 1–6) gave similar results (data not shown).

**DISCUSSION**

We have tested the hypothesis that axial displacement of the C-terminal, α7-helix of the I-like domain in integrin β subunits regulates affinity for ligand by a mechanism analogous to that previously demonstrated for I domains in integrin α subunits. As reviewed in the Introduction, there is controversy as to whether the position of the α7-helix visualized in crystal structures corresponds to a low or high affinity conformation and whether the position of the α7-helix moves during conformational regulation of affinity. We present two independent tests of the hypothesis that the conformation of the α7-helix seen in crystal structures stabilizes integrins in a low affinity conformation and that the helix is displaced during activation: 1) a disulfide designed to lock the α7-helix in the same position as in crystal structures should stabilize the low affinity state; 2) a disulfide designed to displace the β6-α7 loop in the C-terminal, axial direction should activate the high affinity state. Both experimental tests support the hypothesis, with the first test providing particularly strong support because the functional effects of disulfide formation were reversible upon reduction, and a crystal structure rather than a hypothetical model was available for designing where the cysteines were introduced.

The formation of each of the introduced disulfide bonds, Cys¹⁴⁸₃–Cys¹⁴⁹⁷ to stabilize the low affinity conformation and Cys³³²–Cys³³⁵ to stabilize the high affinity conformation, was directly demonstrated by a shift in mobility in nonreducing SDS-PAGE and by quantitating free sulfhydryls with biotinylation. An interesting sidelight is that we find a background level of ~0.3 free sulfhydryls per β₃ subunit in the mature α₁β₃ complex labeled under native conditions. This might appear to contradict a recent report that resting and activated conformers of α₁β₃ isolated from outdated human platelets...
The \( \alpha_{IIb} \beta_3 \text{T329C/A347C} \) mutant remained in the bent conformation as shown by lack of activation epitope exposure. It was also largely resistant to activation epitope exposure by RGD peptide and Mn\(^{2+}\). The functional effects of disulfide bond formation were completely reversible by DTT reduction, with DTT-treated \( \alpha_{IIb} \beta_3 \text{T329C/A347C} \) behaving identically to DTT-treated wild type \( \alpha_{IIb} \beta_3 \). We conclude that in crystal structures determined to date, the \( \beta_3 \) I-like domain is in the low affinity state, and for conversion to the high affinity state, a substantial movement in the position of the side chain of Ala\(^{347}\) relative to that of Thr\(^{329}\) is required. The position of Thr\(^{329}\) is largely fixed by its location within a \( \beta \)-strand with numerous backbone hydrogen bonds to the central I-like domain \( \beta \)-sheet, and therefore the data are most consistent with a movement of the \( \alpha7 \)-helix containing Ala\(^{347}\).

Results with the \( \alpha_{IIb} \beta_3 \text{V332C/M335C} \) mutant specifically support a conformational change in the \( \beta6-\alpha7 \) loop as an activation mechanism. The \( \beta6-\alpha7 \) loops of integrin \( \beta \)-I-like and \( \alpha \)-I domains have a different number of residues, and it is therefore difficult to model rearrangement of the I-like \( \beta6-\alpha7 \) loop. It is also not clear whether downward movement of the \( \alpha7 \)-helix in \( \beta \)-I-like domains would involve one or two turn conformations as found in the intermediate and open conformations of I domains, respectively (16). Therefore, it is difficult to know whether the change in the \( \beta6-\alpha7 \) loop induced by disulfide formation between Cys\(^{332}\) and Cys\(^{335}\) will accurately mimic physiologic rearrangement of this loop. Nonetheless, the position of residue 332 is largely fixed by its position in the \( \beta6 \)-strand and the backbone hydrogen bonds between the \( \beta6 \) and \( \beta6 \) strands. Therefore, the backbone rearrangement required to form the Cys\(^{332}\)-Cys\(^{335}\) disulfide bond is almost certain to come from a downward displacement of the \( \beta6-\alpha7 \) loop, bringing Cys\(^{335}\) into position to form the disulfide bond with Cys\(^{332}\) that was directly demonstrated here by chemical labeling studies. The \( \alpha_{IIb} \beta_3 \text{V332C/M335C} \) mutant was constitutively active in soluble ligand binding assays and appeared to be maximally activated. The mutant was also highly active in adhesion to fibrinogen. The activity of the \( \alpha_{IIb} \beta_3 \text{V332C/M335C} \) mutant was not reversed by reduction. It is likely that the Cys\(^{332}\)-Cys\(^{335}\) disulfide bond is resistant to reduction, like most wild type \( \beta3 \) disulfides; however, we cannot rule out the possibility that both the disulfide bond and the combination of two free cysteines at positions 332 and 335 are activating, although each single cysteine is not. A converse result was obtained in a similar study on \( \alpha1 \)-I domains; a disulfide designed to stabilize the high affinity conformation was reversible by DTT, whereas a disulfide designed to stabilize the low affinity conformation was not reversible with DTT (13, 14, 17).

The high affinity \( \alpha_{IIb} \beta_3 \text{V332C/M335C} \) mutant did not constitute express activation epitopes, but these were induced upon treatment with RGD peptide and Mn\(^{2+}\). An analogous result was obtained with an \( \alpha1 \)-I domain locked in the high affinity state with a disulfide bond (14). Subsequent crystal structure studies on the isolated, high affinity \( \alpha1 \)-I domain demonstrated that the C-terminal \( \alpha7 \)-helix had indeed been displaced downward by the disulfide bond introduced into the \( \beta6-\alpha7 \) loop, although there was some deformation of the \( \alpha7 \)-helix by the mutation (16). The intact, high affinity \( \alpha1 \beta3 \) heterodimer remained in the bent conformation, and extension was activated by Mn\(^{2+}\), as revealed by mAb to LIBS or activation epitopes. The interpretation for the \( \alpha1 \)-I domains is that the \( \alpha7 \)-helix should not be viewed as a rigid rod but rather as a spring or a rope; in other words, some looping out may occur so that a downward movement of the \( \alpha7 \)-helix in the I domain is not necessarily transmitted to other integrin domains (14). Simi-
larly, after introduction here of the Cys332–Cys335 disulfide between the β6-strand and the β6-α7 loop, the local conformational change in this loop did not appear to be transmitted to a change in orientation between the I-like and hybrid domains, as revealed by lack of LIBS epitope exposure. This suggests that the α7-helix in the β I-like domain may also behave as a spring or rope, with conformational change in the β6-α7 loop not necessarily communicated to downward movement at the bottom of the α7-helix, perhaps because of bulging out of the α7-helix or the connection between the β6-α7 loop and the α7-helix. Movements of the adjacent β I-like α1- and α2-helices have also been implicated in integrin activation (5, 10), and concerted movements of the α1-, α2-, and α7-helices may be required for full linkage of the affinity state of the β I-like MIDAS to the I-like domain interface with the hybrid domain and explain the ability of RGD peptide plus Mn2+ to activate LIBS epitope exposure in the α1β1\(\text{V332C/M335C}\) mutant.

Taken together, the results with the α1β1\(\text{V329C/A347C}\) and α1β1\(\text{V332C/M335C}\) mutations strongly support the importance of β I-like domain α7-helix movement in integrin affinity regulation. Both hypothesis-driven mutations in the β I-like domain had the predicted effect. One, designed to displace the β6-α7 loop downward, indeed activated ligand binding. The other, designed to hold the β6-strand and α7-helix together near the end of the α7-helix, indeed maintained α1β1 in the low affinity state and demonstrated that this relative arrangement of these two secondary structure elements, visualized in crystal structures (5, 6), corresponds to the low affinity state. Together, the results with the two mutations support the hypothesis that C-terminal α7-helix displacement increases affinity for ligand but do not rule out the possibility that additional movements are also involved in linking the high affinity state of the ligand binding site to movements at the I-like domain interface with the hybrid domain and LIBS epitope exposure.

Integrins are important therapeutic targets in many inflammatory and vascular disorders. The rational design of mutations that allosterically stabilize high affinity or low affinity conformations of integrins demonstrates marked advances in our understanding of the molecular basis of affinity regulation. This progress also holds out the promise that drugs might be designed that stabilize the low affinity conformation of integrins, in contrast to the current generation of “ligand-mimetic” integrin antagonists that stabilize the high affinity conformation.

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REFERENCES