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 αIβ3·T289C/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 αIβ3·V332C/M352C 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 conformational change and ligand binding are linked (4). The low affinity integrin conformation is highly bent, with the headpiece that contains the ligand binding domains in an extensive interface with the tailpiece that contains the ligand binding domains in an extended conformation. After priming or ligand binding, a structural change around the C-terminal 7-helix of the β subunit I-like domain causes activation, supporting some type of conformational change (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 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 β3 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 β 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 domains (4, 8). Recently, we mutationally introduced N-glycosylation sites into the interface of the hybrid and I-like domains to stabilize the open headpiece. The wedge-open mutants exhibited constitutively high affinity for ligand and adopted an extended conformation (9).

We have proposed that the change in affinity at the ligand binding site in the I-like domain around its metal ion-dependent adhesion site (MIDAS) is communicated to the interface with the hybrid domain on the opposite end of the I-like domain by axial displacement in the C-terminal direction of the I-like domain α7-helix (4, 8, 9) (Fig. 1, B and C). The β subunit I-like domain is inserted in the hybrid domain, and thus these domains have two interconnections. A piston-like movement at the I-like α7-helix connection and pivoting about the other connection would yield a swing at the I-like-hybrid domain interface approximating that seen in electron microscopy studies (4, 8). One basis for proposing this mechanism for communicating a change in affinity to the I-like domain MIDAS is that the structurally homologous I domain inserted in some integrin α subunits undergoes a similar piston-like movement of its C-terminal α7-helix, which regulates the affinity of its MIDAS for ligand (2).
the β6-αI loop and α7-helix. The α1 (13–16) and α25 (17) I domains have been locked into closed, intermediate, or open conformers with low, intermediate, or high affinity for ligand, respectively. Crystal structure studies on the mutant α1 I domains confirmed alterations in the disulfide-constrained, high affinity, open conformation of the α6-α7 domain corresponding precisely in the critical β6-α7 loop and MIDAS loops to the open conformation of the wild-type α1 and α25 I domains seen when this conformation was stabilized in crystals by ligand or ligand-like lattice contacts (18, 19). The studies reported here on the β1 I-domain show that disulfide bonds mutationally introduced into the β6/α7 region lock integrins 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 β1 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 347–533 of Protein Data Bank accession number 1LVJ (6) as template and aligning them with residues 108–333 and 340–346 of the model sequence, respectively. This corresponded to a 7-residue, 2-turn displacement of the α7-helix along its helical axis; residues 334–339 were left untemplated.

Plasmid Construction, Transient Transfection, and Immunoprecipitation—Plasmids coding for full-length human αIIb and β3 were subcloned into pEF/V5-HisA or pcDNA3.1/Myc-His template and aligning them with residues 108–333 and 340–346 of the model sequence, respectively. This corresponded to a 7-residue, 2-turn displacement of the α7-helix along its helical axis; residues 334–339 were left untemplated.

Labeling of Free Cysteines and Western Blotting—Transiently transfected 293T cells were incubated with 60 μg/ml PT25-2 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).

RESULTS

Expression and Ligand Binding Activity of αIIbβ3 on CHO-K1 Transfectants—The plasmids described above coding for αIIb 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 αIIbβ3. 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 Ca2+, or 1 mM Ca2+ plus 10 μM/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 transduced CHO-K1 cells were labeled with 2′,7′-bis-(carboxyethyl)-5(and-6)-carboxyfluorescein (Molecular Probes, Eugene, OR) and suspended to 10^6/ml in HBS supplemented with 5.5 mM glucose, 1% BSA, and either 1 mM EDTA, 5 mM Ca2+, or 5 mM DTT plus 5 mM Ca2+. 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 fluorescence concentration analyzer (Idexx, Westbrook, ME). Bound cells were expressed as a percentage of total input cells.

LiberS 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 αIIbβ3 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.

Expression of Wild Type and Mutant αIIbβ3 Receptors and Formation of Disulfide Bonds—Wild type and mutant β3 subunits were co-transfected with wild type αIIb in 293T cells and subjected to immunostaining and flow cytometry (Fig. 2A and B). The wild type and mutant receptors were recognized equally well by mAb to epitopes constitutively present on the α IIb and β3 subunits, including AP3 (anti-β3), 10E5 (anti-αIIb), HA5 (anti-αIIb), and AP2 (anti-αIIbβ3 complex-specific) (Fig. 2A), suggesting that the two mutant receptors adopted a native fold on the cell surface. However, the mutant β3/V332C/M335C receptor was recognized weakly by anti-β3 mAb 7E3 (Fig. 2B). The 7E3 mAb recognizes residues in the specificity-determining β2-β3 loop near the β3 I-like MIDAS (24). Since the single cysteine mutants β3/V332C and β3/M335C 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 $\beta_3^{T329C/A347C}$ was well recognized by 7E3 (data not shown) (see Fig. 4A).

Nonreducing SDS-PAGE of $^{35}S$-labeled, immunoprecipitated receptors showed that the $\alpha_{III}B_{\beta_3}$ subunits migrated similarly (Fig. 2C), whereas the mutant $\beta_3^{T329C/A347C}$ and $\beta_3^{V332C/M335C}$ subunits (Fig. 2C, lanes 6 and 7) migrated slightly faster than wild type $\beta_3$ (Fig. 2C, lane 5). By contrast, all $\beta_3$ single cysteine mutants migrated similarly to wild-type $\beta_3$ (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 $\beta_3^{T329C/A347C}$ and $\beta_3^{V332C/M335C}$ mutants form a disulfide bond.

To confirm disulfide bond formation, free sulfhydryls were labeled with the maleimide-containing reagent, biotin-BMCC.
The cysteines introduced in the V332C/M335C and T329C/A347C 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 determined. As an additional control, wild-type β₃ on the transfectants was treated with 5 mM DTT for 30 min at 37 °C. The avidin/anti-myc ratios for the wild type, wild type with DTT treatment, β₃/V332C, β₃/V332C/M335C, β₃/M335C, and β₃/T329C/A347C subunits were 0.05, 0.82, 0.20, 0.04, 0.19, and 0.04, respectively. If β₃/V332C is assumed to have one additional free cysteine sulfhydryl compared with wild type, then β₃/M335C also has 1.0 additional free cysteine, β₃/V332C/M335C and β₃/T329C/A347C have no additional free β₃ cysteines, and wild type α₁β₃ treated with DTT has 5.2 free cysteines. By contrast, there are a total of 54 cysteines in wild type β₃.

Ligand Binding Properties of 293T Transfectants with Disulfide-locked Receptors—Binding to soluble fibrinogen was first examined using two-color flow cytometry (4) in transiently transfected 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 α₁β₃/T329C/A347C did not bind soluble fibrinogen even in the presence of PT25-2 (Fig. 3A). Furthermore, the putative locked open mutant α₁β₃/V332C/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 α₁β₃/V332C/M335C mutant was abolished by two blocking α₁mAbs, HA5 and 10E5, but neither blocked nor further activated by the activating 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 Transfectants—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, α₁β₃/T329C/A347C, and α₁β₃/T329C/A347C/V332C/M335C. The transfectants were recognized equally well by a panel of mAbs to constitutively expressed α₁, β₃, and α₁β₃ epitopes, with the exception of 7E3 mAb (Fig. 4A). Mutant α₁β₃/T329C/A347C/V332C/M335C 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 α₁β₃/T329C/A347C did not bind fibrinogen 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 Cys₃₃₂–Cys₃₄₃ disulfide bond locked the I-like domain in the closed conformation, and this constraint was released by DTT treatment. By contrast, mutant α₁β₃/V₃₃₂C/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 α₁β₃/V₃₃₂C/M₃₃₅C mutant in Ca²⁺; probably because the Cys₃₃₂–Cys₃₄₃ disulfide bond was stable under nondenaturing 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 mimetic 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 α₁β₃/T329C/A347C did not adhere even at the highest coating concentration of fibrinogen, whereas DTT treatment yielded binding of α₁β₃/V₃₃₂C/M₃₃₅C indistinguishable 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 α₁β₃/V₃₃₂C/M₃₃₅C 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, resulting 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 PMI-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 extents the binding of the mAbs LIBS1, LIBS6, and PMI-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 lowered the affinity state of the α₁β₃/V₃₃₂C/M₃₃₅C mutant but did not completely abolish the binding of 7E3.
peptide resulted in maximal exposure of LIBS epitopes, with or without DTT treatment. By contrast, LIBS exposure in the low affinity αIIIβ₃ T329C/A347C 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 αIIIβ₃ V332C/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 αIIIβ₃ V332C/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, Cys329/Cys347 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 β subunit in the mature αIIIβ₃ complex labeled under native conditions. This might appear to contradict a recent report that resting and activated conformers of αIIIβ₃ isolated from outdated human platelets...
Constraining β₃ I-like Domain Conformation

The α₃β₃₃/V₃323C/M₃353C 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 Mn²⁺. The functional effects of disulfide bond formation were completely reversible by DTT reduction, with DTT-treated α₃β₃₃/V₃323C/M₃353C behaving identically to DTT-treated wild type α₃β₃. We conclude that in crystal structures determined to date, the β₃ 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 Ala₃⁴⁷ relative to that of Thr²⁸⁸ is required. The position of Thr²⁸⁸ is largely fixed by its location within a β-strand with numerous backbone hydrogen bonds to the central I-like domain β-sheet, and therefore the data are most consistent with a movement of the β₇-helix containing Ala₃⁴⁷.

Results with the α₃β₃₃/V₃323C/M₃353C mutant specifically support a conformational change in the β₆-α₇ loop as an activation mechanism. The β₆-α₇ loops of integrin β I-like and α I domains have a different number of residues, and it is therefore difficult to model rearrangement of the I-like β₆-α₇ loop. It is also not clear whether downward movement of the α₇-helix in β I-like domains would involve one or two turn displacements as found in the intermediate and open conformations of I domains, respectively (16). Therefore, it is difficult to know whether the change in the β₆-α₇ loop induced by disulfide formation between Cys₃₃² and Cys₃₃⁵ will accurately mimic physiologic rearrangement of this loop. Nonetheless, the position of residue 332 is largely fixed by its position in the β₆-strand and the backbone hydrogen bonds between the β₆ and β₇ strands. Therefore, the backbone rearrangement required to form the Cys₃₃²-Cys₃₃⁵ disulfide bond is almost certain to come from a downward displacement of the β₆-α₇ loop, bringing Cys₃₃⁵ into position to form the disulfide bond with Cys₃₃² that was directly demonstrated here by chemical labeling studies. The α₃β₃₃/V₃323C/M₃353C 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 α₃β₃₃/V₃323C/M₃353C mutant was not reversed by reduction. It is likely that the Cys₃₃²-Cys₃₃⁵ disulfide bond is resistant to reduction, like most wild type β₃ 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 α₁ 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 α₃β₃₃/V₃323C/M₃353C mutant did not constitutively express activation epitopes, but these were induced upon treatment with RGD peptide and Mn²⁺. An analogous result was obtained with an α₁ I domain locked in the high affinity state with a disulfide bond (14). Subsequent crystal structure studies on the isolated, high affinity α₁ I domain demonstrated that the C-terminal α₇-helix had indeed been displaced downward by the disulfide bond introduced into the β₆-α₇ loop, although there was some deformation of the α₇-helix by the mutation (16). The intact, high affinity α₁β₃ heterodimer remained in the bent conformation, and extension was activated by Mn²⁺, as revealed by mAb to LIBS or activation epitopes. The interpretation for the α₁ I domains is that the α₇-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 α₇-helix in the I domain is not necessarily transmitted to other integrin domains (14). Simi-

contain 2.6 and 4.4 free cysteines per β₃ subunit labeled under denaturing conditions, respectively, and that disulfide exchange is involved in integrin activation (25). However, early studies on α₃β₃ either isolated from platelets and labeled under denaturing conditions or labeled with sulphydryl reagents under native conditions on intact platelets found no free sulphydryls in the α₃β₃ or β₃ subunits, whereas the one free sulphydryl in the cytoplasmic domain of platelet GpIb was detected (26, 27). Therefore, our results are in good agreement with the earlier studies.

The disulfide formed in the β₃₃/V₃323C/M₃353C mutant stabilized the low affinity state of α₃β₃. Thr²⁸⁸ and Ala₃⁴⁷ are located within the beginning portion of the β₆-strand and within the last portion of the α₇-helix, respectively, much closer to the “bottom” of the I-like domain that connects to the hybrid domain than to the “top” of the I-like domain that binds ligand. There is no significant difference in the positions of these residues between the unliganded and liganded α₃β₃ crystal structures (5, 6), and their positions are such that when substituted with cysteine, disulfide bond formation should result in little or no local structural rearrangement. The Cys₃₃²-Cys₃₃⁵ disulfide bond locked Cys₃₃²/M₃₃⁵ in the low affinity conformation, as shown by lack of binding to soluble fibrinogen or PAC-1 mAb, with or without activation. It was interesting that α₃β₃₃/V₃323C/M₃353C was also incapable of mediating adhesion of transfectants to fibrinogen on substrates, which does not require activation. In this assay, binding to ligand may occur as a consequence of the high local concentration of ligand, and ligand binding may drive the shift in the equilibrium from the low to the high affinity conformation of the receptor.

The α₃β₃₃/V₃323C/M₃353C mutant was also incapable of mediating adhesion of transfectants to fibrinogen on substrates, which does not require activation. In this assay, binding to ligand may occur as a consequence of the high local concentration of ligand, and ligand binding may drive the shift in the equilibrium from the low to the high affinity conformation of the receptor.

FIG. 5. Exposure of LIBS epitopes. Wild-type and mutant CHO transfectants were incubated in the presence of 5 mM Ca²⁺, 1 mM Mn²⁺ plus 100 μM GRGSDSP (RGD) peptide, 5 mM DTT plus 5 mM Ca²⁺, or 5 mM DTT plus 1 mM Mn²⁺ and 100 μM GRGDSP (RGD) peptide for 30 min at room temperature and then with mAbs on ice for 30 min. Binding was determined with FITC anti-IgG as described under “Materials and Methods.”
larly, after introduction here of the Cys\textsuperscript{332}–Cys\textsuperscript{355} 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 Mn\textsuperscript{2+} to activate LIBS epitope exposure in the α\textsubscript{IIb}β\textsubscript{3}V\textsuperscript{332C/M335C} mutant.

Taken together, the results with the α\textsubscript{IIb}β\textsubscript{3}T\textsuperscript{329C/A347C} and α\textsubscript{IIb}β\textsubscript{3}V\textsuperscript{332C/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 α\textsubscript{IIb}β\textsubscript{3} 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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