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The C-Terminal αI Domain Linker as a Critical Structural Element in the Conformational Activation of αI Integrins*

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The activation of α/β heterodimeric integrins is the result of highly coordinated rearrangements within both subunits. The molecular interactions between the two subunits, however, remain to be characterized. In this study, we use the integrin $\alpha_L\beta_2$ to investigate the functional role of the C-linker polypeptide that connects the C-terminal end of the inserted (I) domain with the β-propeller domain on the α subunit and is located at the interface with the βI domain of the β chain. We demonstrate that shortening of the C-linker by eight or more amino acids results in constitutively active $\alpha_L\beta_2$ in which the αI domain is no longer responsive to the regulation by the βI domain. Despite this intersubunit uncoupling, both I domains remain individually sensitive to intrasubunit conformational changes induced by allosteric modulators. Interestingly, the length and not the sequence of the C-linker appears to be critical for its functionality in α/β intersubunit communication. Using two monoclonal antibodies (R7.1 and CBR LFA-1/1) we further demonstrate that shortening of the C-linker results in the gradual loss of combinational epitopes that require both the αI and β-propeller domains for full reactivity. Taken together, our findings highlight the role of the C-linker as a spring-like element that allows relaxation of the αI domain in the resting state and controlled tension of the αI domain during activation, exerted by the β chain.

Integrins are a large family of α/β heterodimeric cell surface receptors that mediate interactions with other cells or the extracellular matrix. They are important therapeutic targets in a wide range of diseases, including cardiovascular and immune disorders (1). Integrin activation is dynamically regulated by signals from within the cell in a process termed inside-out signaling. In addition, outside-in signaling induced by ligand binding directs signals from the extracellular domains to the cytoplasm. This bidirectional signaling is associated with highly coordinated domain rearrangements in both the α and the β subunits (2).

Several studies indicate that inside-out signaling converts integrins from a bent conformation with a closed headpiece into an extended conformation with an open headpiece and thereby activates ligand binding (2) (Fig. 1, *A*–C). Central to ligand recognition are von Willebrand factor type A domains that are present in all integrin β subunits (termed β inserted (βI) domains) and in some α subunits (termed α inserted (αI) domains). In integrins that lack αI domains, the activated βI domain directly interacts with the ligand through a metal ion-dependent adhesion site (MIDAS).[3] In αI integrins, the αI domain serves as the ligand-binding domain instead of the nearby βI domain. Interestingly, in these integrins, the βI domain appears to regulate the activation of the αI domain and, thus, ligand binding. In the current model, an invariant Glu residue (Glu-310 in α_L) located at the C-terminal end of the αI domain is thought to bind to the MIDAS of the active βI domain (Fig. 1*C*). This interaction is hypothesized to lead to an axial displacement of the α7 helix of the αI domain in the C-terminal direction, as seen in crystal structures of isolated αI domains (3). As a consequence, the αI MIDAS is turned into a high-affinity, ligand-binding state (2).

Additional support for a structural link between the αI and βI domains comes from studies that characterize small molecule inhibitors of the integrin $\alpha_L\beta_2$ at a molecular level (4). One class of $\alpha_L\beta_2$ inhibitors, termed αI allosteric inhibitors, has been shown to bind underneath the C-terminal α7 helix of the isolated αI domain and stabilize it in the closed, low-affinity state (4). On the basis of this finding it is hypothesized that these inhibitors lock the integrin in its inactive form by preventing the downward axial shift of the α7-helix required for αI domain/βI domain interactions (4) (Fig. 1*A*). Another class of $\alpha_L\beta_2$ allosteric inhibitors, termed α/β I allosteric inhibitors, appear to bind to the MIDAS of the βI domain (4, 5). Thereby they are hypothesized to competitively antagonize the binding of α_L Glu-310 to the βI domain. As a result, the αI domain remains in an inactive state, whereas the βI domain together with the "leg" region of the integrin is stabilized in a pseudo-liganded, active state, as shown by the induction of activation-dependent epitopes and induction of the extended conformation with the open headpiece (as seen with electron microscopy) (6) (Fig. 1*D*).

Crystal structures of the αI integrin $\alpha_X\beta_2$ revealed unanticipated flexibility of the αI domain (7). One possible function of this flexibility would be to enable two βI domain conformational states to couple with three αI domain states (7). A key

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[3] The abbreviations used are: MIDAS, metal ion-dependent adhesion site; ICAM-1, intercellular adhesion molecule-1; DMSO, dimethyl sulfoxide; MFI, mean fluorescence intensity; LFA-1, lymphocyte function-associated antigen-1.

question remaining to be answered is which structural features enable the conformational flexibility of the αI domain needed for the activation of I domain integrins. Linkers connect the N termini (N-linker) and C termini (C-linker) of the αI domain to the β-propeller domain in which the αI domain is inserted (7). The role of the N-linker appears to be limited by its short length of three residues. In contrast, the C-linker, which follows the α7-helix of the αI domain and contains the invariant Glu-310, is ten residues long and is flexible, as shown by weak electron density in the crystal structure (7).

Here we use the leukocyte integrin $\alpha_L\beta_2$ to test the hypothesis that the C-linker acts as a spring-like element that, when mutationally shortened, activates the αI domain. $\alpha_L\beta_2$ is selectively expressed on all leukocytes and is among the best characterized of αI integrins (8). The ligands of $\alpha_L\beta_2$ are members of the Ig superfamily, including intercellular adhesion molecule-1 (ICAM-1). The $\alpha_L\beta_2$/ICAM-1 interaction plays a major role in inflammatory and immune responses by regulating cell adhesion, leukocyte trafficking, and T cell costimulation (8). The present study provides important insights into how the C-linker regulates αI integrin adhesiveness.

EXPERIMENTAL PROCEDURES

Antibodies, Small Molecules, and Recombinant ICAM-1—The sources of the mouse anti-human αL mAbs TS2/4, TS1/22, CBR LFA-1/1, and the mouse anti-human β2 mAb CBR LFA-1/2 have been described previously (9, 10). The mouse anti-human β2 mAb KIM127 was a kind gift from Martyn Robinson at Celltech (11). The mouse anti-human αL mAb R7.1 was provided by Robert Rothlein (Boehringer-Ingelheim Pharmaceuticals, Ridgefield, CT) (12). LFA878 was obtained from Novartis Pharma, Basel, Switzerland. XVA143 was synthesized according to example 345 of the patent (13) and was obtained from Paul Gillespie (Hoffmann-La Roche, Inc., Nutley, NJ). LFA878 and XVA143 were dissolved in DMSO at 10 mM or 1 mM, respectively, and stored at −20 °C. Recombinant ICAM-1 D1-D5 was produced as described (14) using a C-terminal His tag and purification by nickel-nitrilotriacetic acid-agarose.

Cell Culture—Human embryonic kidney 293T cells were cultured in Dulbecco's modified Eagle's medium supplemented with 2 mM L-glutamine, 10% fetal bovine serum, nonessential amino acids, and penicillin-streptomycin at 37 °C with 5% CO_2 (all reagents from Invitrogen). The day before transfection low-passage 293T cells were transferred into 24-well plates.

cDNA Constructs and Transient Transfections—cDNA of WT αL was inserted into pcDNA3.1/Hygro(-) (Invitrogen) and used as template for mutagenesis. αL C-linker deletion and swap mutants were generated by overlap extension PCR (15). For the αL C-linker deletion mutants Δ 8 and Δ 10, the mutant Δ 6 was used as a template. Human-mouse αL chimeras in expression vector AprM8 and I-less $\alpha_L\beta_2$ (lacking residues 129–308) in the same expression vector were described previously (9, 16). The chimeras were named according to the species origin of their segments. For example, h217m248h indicates that residues 1–217 are from human (h) αL, residues from 218 to 248 are from mouse (m) αL, and residues from 249 to the C terminus are from human αL. αL I domain expressed on the cell surface with N-terminal or C-terminal transmembrane domains have been described previously (17, 18). Resequencing of these vectors demonstrated that they contain αL residues Val-130 to Val-339 and Gly-128 to Tyr-307, respectively. All constructs were confirmed by sequencing. 293T cells (80% confluent) were transfected with empty vector (mock) or cotransfected with mutant αL and wild-type (WT) β2 plasmids (either inserted into pcDNA3.1/Hygro(-), AprM8, or pcDNA3.1(+)) using Lipofectamine 2000 according to the manufacturer's instructions. Two days after transfection the cells were harvested for flow cytometric analysis, adhesion, and binding assays.

Immunofluorescence Flow Cytometry—Immunofluorescence flow cytometry was performed as described previously (19). Briefly, transfected 293T cells were detached and washed once in 20 mM HEPES (pH 7.3) containing 150 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, and 1.5% BSA (assay buffer A). Cells were then resuspended in assay buffer A containing 10 μg/ml primary antibody and incubated on ice for 30 min. mAb KIM127 was used at a concentration of 7 μg/ml and incubated at 37 °C for 20 min. After a washing step, the cells were exposed to FITC-conjugated goat anti-mouse IgG (Invitrogen) diluted 1:500 in assay buffer A for 20–30 min on ice. After two washing steps, cells were resuspended in cold assay buffer A and analyzed on a FACScan (BD Biosciences). Mean fluorescence intensities were calculated using the CellQuest software.

Cell Adhesion to ICAM-1—The cell adhesion assay was performed in V-bottom 96-well plates (Corning) as described previously (20). Briefly, the plates were coated with 10 μg/ml recombinant human ICAM-1 or 10 μg/ml BSA as a control in 20 mM Tris (pH 8), 150 mM NaCl, and 2 mM $MgCl_2$ at 4 °C overnight (or 37 °C for 2 h) and then blocked with 20 mM Tris (pH 7.5) containing 150 mM NaCl, 1.5% BSA, and 5 mM glucose (assay buffer B) at 37 °C for 2 h. The transfected 293T cells were detached, resuspended in assay buffer B, and labeled with 1–2 μg/ml 2′,7′-bis-(carboxyethyl)-5-(and-6)-carboxyfluorescein acetoxymethyl ester (BCECF AM) (Invitrogen) at 37 °C for 30 min in the dark. After this labeling step, the cells were washed once and resuspended in assay buffer B containing 1 mM $CaCl_2$ and 1 mM $MgCl_2$ (resting condition) or 1 mM $CaCl_2$, 1 mM $MgCl_2$, and activating mAbs (10 μg/ml KIM127 and 10 μg/ml CBR LFA-1/2) or 1 mM $MnCl_2$ alone (activating conditions). After incubation at 37 °C for 25 min in the dark, cells were vigorously pipetted up and down and added to the ICAM-1- and BSA-coated plates (the cell number varied from 3000 to 10,000 cells/well from experiment to experiment). The plates were immediately centrifuged at 100 × *g* for 10 min (Beckman CS centrifuge, brake off). After centrifugation, nonadherent cells that accumulated in the center of the V bottom were quantified using the Fluoroskan Ascent microplate fluorometer (Thermo Scientific) with the "small beam" setting and filter sets allowing excitation at 485 nm and quantification of emission at 535 nm. The percentage of cell adhesion was calculated according to the following formula:

$$\left[1 - \frac{FI_{ICAM\text{-}I}}{FI_{BSA}}\right] \times 100 = \%\ \text{of adhesive cells} \qquad \text{(Eq. 1)}$$

where $Fl_{ICAM\text{-}1}$ is the fluorescent signal (arbitrary units) when cells bind to ICAM-1 (low signal) and Fl_{BSA} is the

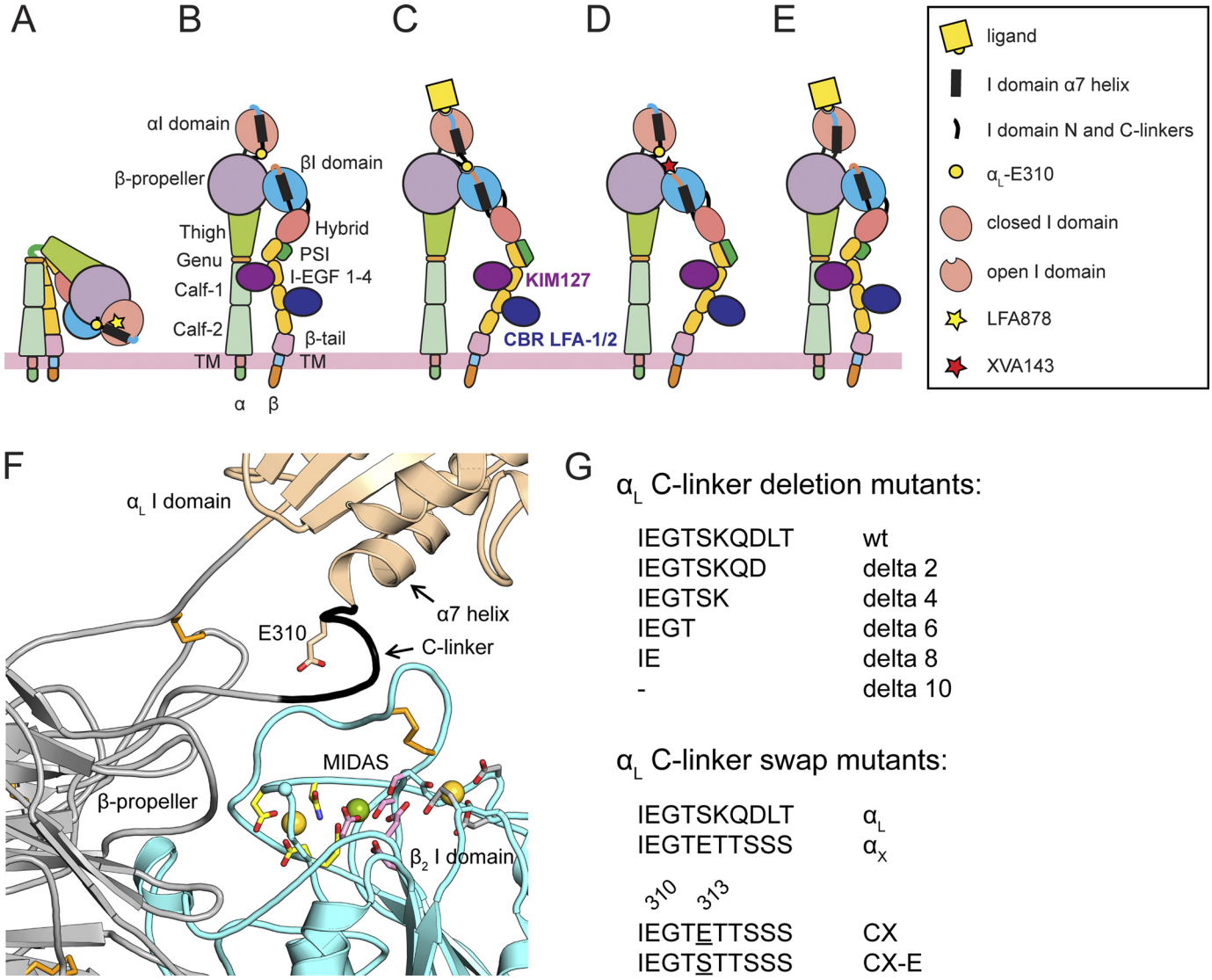

FIGURE 1. **Model of conformational activation of $\alpha_L\beta_2$ with the C-linker acting as a spring-like element (modified from (2)).** *A*, bent conformation of WT $\alpha_L\beta_2$ with closed headpiece, stabilized by the αI allosteric antagonist LFA878 (low affinity). *B*, extended conformation of WT $\alpha_L\beta_2$ with closed headpiece and epitopes of mAbs KIM127 and CBR LFA-1/2 exposed (low affinity (23)). TM, transmembrane; PSI, plexin-semaphorin-integrin. *C*, extended conformation of WT $\alpha_L\beta_2$ with open headpiece and ligand bound (high affinity). *D*, extended conformation of WT $\alpha_L\beta_2$ with open headpiece but closed α I domain induced by α/β I allosteric inhibitor XVA143. *E*, $\alpha_L\beta_2$ C-linker mutant Δ 10 shown in its extended conformation with a constitutively active α I domain that is no longer responsive to regulation by the β I domain. F, close-up of the $\alpha_L\beta_2$ C-linker region. The C-linker is shown in *black*. Upon activation, residue Glu-310 (shown in stick) is hypothesized to bind to the MIDAS Mg^{2+} ion shown as a *green sphere*. The homology model is based on the $\alpha_X\beta_2$ crystal structure (7). *G*, C-linker deletions Δ 2 to Δ 10 and replacement of the $\alpha_L\beta_2$ C-linker by $\alpha_X\beta_2$ C-linker residues.

fluorescent signal in absence of ICAM-1 and presence of BSA (high signal).

Binding of Multimeric Soluble ICAM-1—The binding of soluble ICAM-1 was assessed as described previously (21). Transfected 293T cells were detached using 20 mM HEPES (pH 7.3) supplemented with 150 mM NaCl and 5 mM glucose (assay buffer C) and transferred into V-bottom 96-well plates (Corning). The cells were washed in assay buffer C and resuspended in assay buffer C containing 2 mM $CaCl_2$ and 2 mM $MgCl_2$ (50 μl/well). Multimeric ICAM-1 complexes were prepared by mixing human ICAM-1/Fc (R&D Systems) with affinity-purified goat anti-human IgG (H+L)-FITC antibodies (Invitrogen) (1:10 w/w) and incubated at room temperature for 30 min. The ICAM-1 complexes were diluted 1:6 in assay buffer C and added to the plates (50 μl/well), yielding a final concentration of 1 mM for each cation. The cells were incubated at room temperature for 30 min, washed in assay buffer C containing 1 mM $CaCl_2$/1 mM $MgCl_2$ and subjected to immunofluorescence flow cytometry. As a control, soluble multimeric human myeloma IgG1κ complexes with anti-human IgG were prepared as described above and exposed to the transfected cells.

RESULTS

Design and Cell Surface Expression of $\alpha_L\beta_2$ C-linker Mutants—We designed five $\alpha_L\beta_2$ mutants in which the C-linker of the αI domain (residues 309–318) (Fig. 1*F*) was shortened by 2, 4, 6, 8, or 10 amino acids (Fig. 1*G*). In Δ 10, the C-linker is completely removed, and thus, this mutant is the only mutant that lacks the invariant Glu-310 residue thought to be important for αI/βI domain communication (Fig. 1*F*). The C-linker of $\alpha_L\beta_2$ was also

replaced with the C-linker of $\alpha_X\beta_2$ (CX mutant) to differentiate the importance of its sequence as compared with its length (Fig. 1*G*). Wild-type (WT) and mutated α subunits were coexpressed with WT β2 in 293T cells. Immunofluorescence flow cytometry using mAb TS2/4, which binds the αL β-propeller domain and requires β2 association for reactivity, demonstrated that all $\alpha_L\beta_2$ C-linker deletion mutants were correctly assembled and expressed at WT levels (Fig. 2*A*). In contrast, swapping the $\alpha_L\beta_2$ C-linker for the $\alpha_X\beta_2$ C-linker reduced cell-surface expression by 2-fold (Fig. 2*A*). However, expression at WT level was achieved when αX-Glu-313 of the CX mutant was replaced by the corresponding αL C-linker Ser residue (mutant CX-E) (Figs. 1*G* and 2*A*).

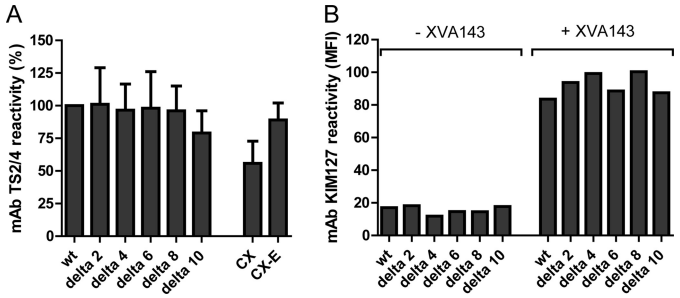

FIGURE 2. **Reactivity of $\alpha_L\beta_2$ C-linker mutants with mAb TS2/4 and KIM127.** *A*, the reactivity of mAb TS2/4 with WT or mutant $\alpha_L\beta_2$ transiently expressed on the surface of 293T cells was determined by immunofluorescence flow cytometry. Each *bar* represents the mean ± S.D. of four independent experiments. The binding to mock-transfected 293T cells was subtracted before calculating the mean values. *B*, the binding of mAb KIM127 to transiently expressed WT and mutant αLβ2 was measured in the presence of 0.1% DMSO (-XVA143) or 1 μM XVA143 (+XVA143). Results are expressed as MFI. The binding of KIM127 to mock-transfected 293T cells has been subtracted from the MFI values. A representative experiment of two independent experiments is shown.

Impact of C-linker Shortening on Global Conformation of the Mutants—To assess the global conformation of the deletion mutants, we tested the binding of mAb KIM127. The epitope of this activation-dependent antibody involves residues on the β2 subunit that are masked in the bent (inactive) and exposed in the extended (active) conformation (22). Further, the KIM127 epitope is known to be induced by α/β I allosteric inhibitors such as XVA143 (5). Under resting conditions in the presence of Ca^{2+} and Mg^{2+}, only basal binding of mAb KIM127 to the mutants was noted (Fig. 2*B*). This result suggests that the mutants are largely in the bent conformation in the absence of activating agents. Interestingly, KIM127 epitope exposure in the mutants was induced by XVA143 to a degree comparable with the WT receptor (Fig. 2*B*). These results show that despite C-linker shortening, all mutants are basally bent and are able to bind XVA143 and undergo conversion to an extended conformation.

Impact of C-linker Shortening or Swapping on $\alpha_L\beta_2$ Function—The function of the mutants was studied by investigating the adhesion of 293T cell transfectants to immobilized ICAM-1 using the V well assay format. Under activating conditions in the presence of Mn^{2+}, the mutants Δ 2 and Δ 10 adhered comparably to the WT, whereas adhesion of 293T cells expressing

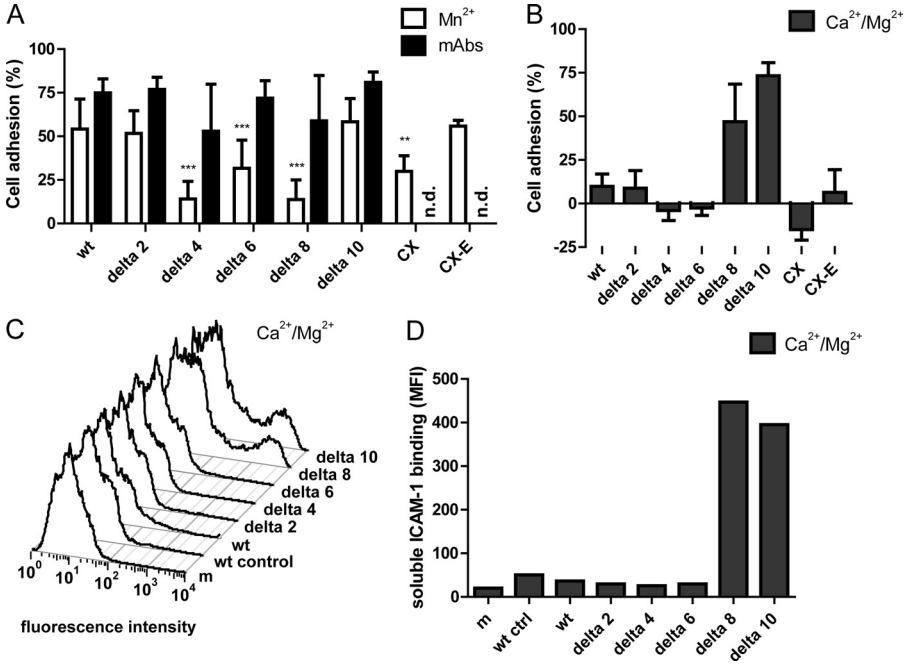

FIGURE 3. **Ligand-binding activity of $\alpha_L\beta_2$ C-linker mutants under activating or resting conditions.** *A*, adhesion of fluorescently labeled 293T cell transfectants to immobilized ICAM-1 induced by Mn^{2+} or by activating mAbs (KIM127 & CBR LFA-1/2) was quantified using the V-bottom adhesion assay (activating conditions). Percentage of adhesive cells was calculated as described under "Experimental Procedures." Each *bar* represents the mean value ± S.D. of four independent experiments run in triplicates (Mn^{2+}) or three independent experiments run in duplicates or triplicates (mAbs). The adhesion of mock-transfected cells varied from 12% to 36% and was subtracted before calculating the mean values. **, $p < 0.01$; ***, $p < 0.001$, paired two-tailed Student's *t* test comparisons to WT. *n.d.*, not determined. *B*, the adhesion of transfectants to ICAM-1 was quantified in presence of 1 mM $CaCl_2$/1 mM $MgCl_2$ using the V-bottom assay (resting conditions). Each *bar* represents the mean value ± S.D. of three to five independent experiments run in triplicates. The binding of mock-transfected cells varied from 2% to 23% and was subtracted before calculating the mean values. *C* and *D*, the binding of soluble multimeric ICAM-1 complexes to 293T cell transfectants was measured in the presence of 1 mM $CaCl_2$ and 1 mM $MgCl_2$ using flow cytometry (resting conditions). Results are expressed as a histogram (*C*) and MFI values (*D*). *m*, mock-transfected cells; *wt ctrl*, binding of soluble multimeric human myeloma IgG1κ to WT $\alpha_L\beta_2$-expressing cells; *WT*, wild-type $\alpha_L\beta_2$; *Δ 2* to *10*, αL C-linker deletion mutants; *CX* and *CX-E*, αL C-linker swap mutants.

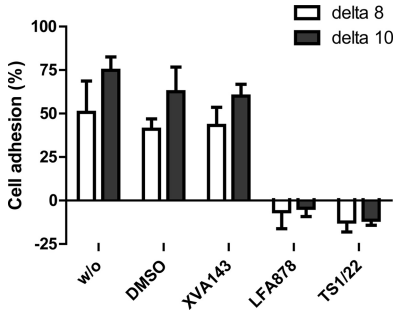

FIGURE 4. **Effect of $\alpha_L\beta_2$ inhibitors on the adhesion of C-linker deletion mutants Δ 8 and Δ 10.** The adhesion of 293T cells expressing the constitutively active mutants Δ 8 and Δ 10 to immobilized ICAM-1 was quantified in the absence (*w/o*) and presence of DMSO (0.1%), XVA143 (1 μM), LFA878 (10 μM), or TS1/22 (10 μg/ml) using the V-bottom assay. The experiment was performed under resting conditions. The percentage of adhesive cells was calculated as described under "Experimental Procedures." Each *bar* represents the mean value ± S.D. of one to three independent experiments run in triplicates. The adhesion of 293T cells transfected with empty vector varied from −7 to 25% and was subtracted before calculating the mean values.

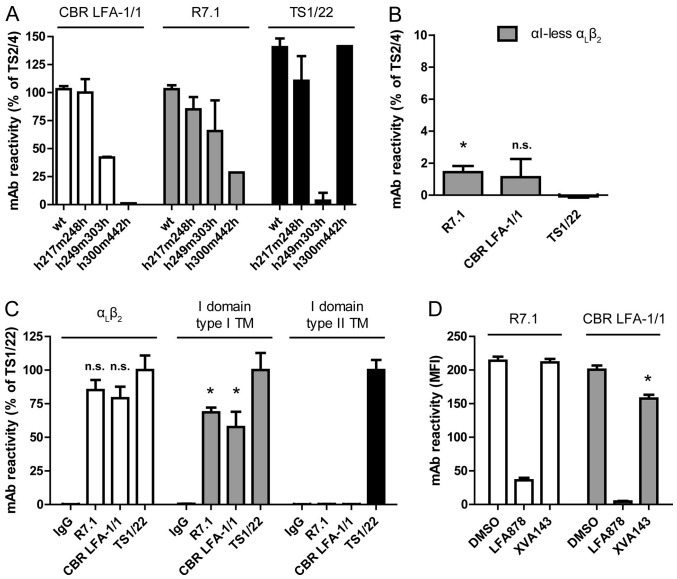

FIGURE 5. **Characterization of the binding sites of mAbs R7.1 and CBR LFA-1/1.** *A*, mapping studies using $\alpha_L\beta_2$ mouse-human chimeras. 293T cells transiently transfected with $\alpha_L\beta_2$ mouse-human chimeras were stained with mAbs CBR LFA-1/1, R7.1, TS1/22 (control mAb), and TS2/4 (as a measure for cell surface expression) and subjected to flow cytometry. The specific MFI values were determined by subtracting the MFI of mock-transfected cells. The binding of CBR LFA-1/1, R7.1, and TS1/22 was expressed as a percentage of mAb TS2/4 binding. Reactivity of mAb TS2/4 to chimeras h217m248h, h249m303h, and h300m442h was 78%, 24%, and 97% of the reactivity to WT $\alpha_L\beta_2$, respectively. Each *bar* represents the mean value ± S.D. of two independent experiments. *B*, binding to αI-less $\alpha_L\beta_2$. 293T cells transiently transfected with αI-less $\alpha_L\beta_2$ were stained with mAbs R7.1, CBR LFA-1/1, TS1/22, and TS2/4 and subjected to flow cytometry. The binding of the antibodies to mock-transfected 293T cells was subtracted from the MFI values. Results are expressed as percentage of mAb TS2/4 control. Each *bar* represents the mean value ± S.D. of triplicates. *, $p < 0.05$, paired two-tailed Student's *t* test comparison to TS1/22. *n.s.*, not significant. *C*, binding of mAbs to 293T cells transiently transfected with WT $\alpha_L\beta_2$ or with αI domains with type I or type II transmembrane (*TM*) domains fused to the C- or N-terminus, respectively, measured by flow cytometry. Results are expressed as a percentage of mAb TS1/22 MFI and are mean ± S.D. of three independent experiments. *, $p < 0.05$, paired two-tailed Student's *t* test comparisons to TS1/22 binding. *n.s.*, not significant. *D*, effect of allosteric $\alpha_L\beta_2$ inhibitors on R7.1 and CBR LFA-1/1 epitope expression. 293T cells transiently transfected with wt $\alpha_L\beta_2$ were stained with mAb R7.1 and CBR LFA-1/1 in the presence of DMSO (0.1%), LFA878 (10 μM), or XVA143 (1 μM). Binding of the antibodies was quantified by flow cytometry. The specific MFI values were determined by subtracting the MFI of mock-transfected cells. Each *bar* represents the mean ± S.D. of triplicates. *, $p < 0.05$, paired two-tailed Student's *t* test comparison to DMSO control.

mutants Δ 4, 6, or 8 was significantly lower despite normal expression (Figs. 3*A* and 2*A*). In contrast, all mutants bound at WT levels when mAb CBR LFA-1/2 and mAb KIM127 were used to stimulate adhesion (Fig. 3*A*). CBR LFA-1/2 is an activating antibody that binds to the leg region of the β2 subunit and maximally activates $\alpha_L\beta_2$ in combination with higher concentrations of mAb KIM127 (22, 23). The CX-E transfectants were activated by Mn^{2+} to levels observed for WT transfectants (Fig. 3*A*). The reduced adhesion of the CX mutant in presence of Mn^{2+} is explained by lower cell surface expression (Figs. 3*A* and 2*A*).

Under resting conditions, in presence of physiological concentrations of Mg^{2+} and Ca^{2+}, the C-linker mutants Δ 8 and Δ 10 constitutively adhered to immobilized ICAM-1, whereas the rest of the mutants exhibited weak or no adhesion to ICAM-1 (Fig. 3*B*). The level of constitutive adhesion was comparable with the level observed for activated WT $\alpha_L\beta_2$ (Fig. 3, *A* and *B*). Selective binding of soluble ICAM-1 complexes to the Δ 8 and Δ 10 mutants confirmed their constitutively active phenotype (Fig. 3, *C* and *D*).

Susceptibility of the Constitutively Active Mutants to Small Molecule Antagonists and Inhibitory Antibodies—Interestingly, the adhesion of the constitutively active mutants Δ 8 and Δ 10 to immobilized ICAM-1 was not affected by the α/β I allosteric inhibitor XVA143 (Fig. 4), although the compound clearly binds to the mutants, as shown by induction of KIM127 epitope exposure (Fig. 2*B*). This result suggests that the active state of the αI domain is no longer regulated by the βI domain in these mutants. In contrast, inhibitors that bind underneath the α7-helix of the αI domain, such as LFA878, abolished the pro-adhesive state of the Δ 8 and Δ 10 mutants, demonstrating that shortening of the C-linker does not irreversibly activate the αI domain (Fig. 4). As expected, the control mAb TS1/22 completely blocked the binding of the two mutants to ICAM-1 (Fig. 4). TS1/22 was mapped to the ligand binding region of the αI domain (residues Gln-266 and Ser-270) and competitively inhibits $\alpha_L\beta_2$ (24, 25).

Characterization of mAb R7.1 and CBR LFA-1/1 Epitopes—To further understand the impact of C-linker shortening on neighboring regions, we characterized two antibodies that recognize epitopes that appear to include both the αI and β-propeller domains. mAbs R7.1 and CBR LFA-1/1 each inhibit the function of human $\alpha_L\beta_2$ and are specific for the α_L subunit (9, 25, 26). CBR LFA-1/1 reacts with a cell surface-expressed fragment containing α_L residues 130–338 that includes α_L I domain residues 130–308 (17, 25). Similarly, mAb R7.1 binds to a purified fragment containing the αI domain (26). Species-specific residues recognized by these mouse anti-human antibodies were mapped within intact $\alpha_L\beta_2$ using mouse-human chimeras. Loss of reactivity with the h300m442h chimera showed that human residues 301–442 were absolutely required for CBR LFA-1/1 (Fig. 5*A*) in agreement with the previously described requirement for residues 301–359 (9). Furthermore, human αI domain residues 250–303 were also required for full CBR LFA-1/1 reactivity (Fig. 5*A*). The h300m442h chimera

reduced R7.1 reactivity by 71%, showing that a portion of its epitope maps to residues 301–442 (Fig. 5*A*). TS1/22, mapping to αI domain residues Gln-266 and Ser-270, was used as a control antibody that did not bind to the chimera h249m303h (Fig. 5*A*).

The reactivity of the mAbs was further checked with αI-less $\alpha_L\beta_2$, which lacks α_L residues 129–308, *i.e.* the αI domain (16). Reactivity of both CBR LFA-1/1 and R7.1 was greatly decreased with αI-less $\alpha_L\beta_2$ compared with wild-type $\alpha_L\beta_2$ (Fig. 5*B*). However, the binding of mAb R7.1 was significantly above the reactivity of the control mAb TS1/22 ($p = 0.012$) whose epitope exclusively maps to the αI domain (Fig. 5*B*). Reactivity of CBR LFA-1/1 with the αI-less construct was also greater than TS1/22, although this difference did not reach significance (Fig. 5*B*).

Furthermore, the binding of CBR LFA-1/1 and R7.1 to type I or type II-anchored αI domains was assessed. The type I construct (C-terminal anchorage of the I domain) includes αL residues 130–339, *i.e.* almost all the αI domain (129–308), the C-linker (309–318), and an adjacent β-propeller segment (319–339), whereas the type II construct (N-terminal anchorage) contains residues 128–307, *i.e.* the αI domain only (17, 18). CBR LFA-1/1 and R7.1 recognized type I- but not type II-anchored αL I domains (Fig. 5*C*). The binding of the antibodies to the type I TM-anchored αI domain was slightly but significantly reduced relative to TS1/22 (Fig. 5*C*). These results suggest that the C-linker and/or the β-propeller domain, in addition to the αI domain, contribute to the CBR LFA-1/1 and R7.1 epitopes.

Next we explored the effect of LFA878, an αI allosteric antagonist, and XVA143, an α/β I allosteric antagonist, on R7.1 and CBR LFA-1/1 reactivity. It has been described previously that αI and α/β allosteric inhibitors reduce or do not affect the binding of R7.1 to $\alpha_L\beta_2$, respectively (26, 27). The effects of the compounds on R7.1 binding were as described previously (Fig. 5*D*). Similarly to R7.1, CBR LFA-1/1 reactivity was reduced in the presence of LFA878. However, in contrast to R7.1, CBR LFA-1/1 reactivity was marginally (but significantly ($p = 0.021$)) influenced by the α/β I allosteric antagonist XVA143 (Fig. 5*D*). Taken together, these unique properties indicated that the mAbs were well suited for the characterization of the C-linker mutants.

Impact of C-linker Shortening on mAb R7.1 and CBR LFA-1/1 Reactivity—Shortening of the $\alpha_L\beta_2$ C-linker progressively decreased the binding of the R7.1 and CBR LFA-1/1 antibodies to different degrees (Fig. 6). The CBR LFA-1/1 epitope was already affected in the Δ 2 mutant and almost lost in the Δ 4 mutant, whereas the epitope of mAb R7.1 was still preserved in Δ 2, was affected in Δ 4, and almost lost in Δ 6. Intriguingly, the binding of both antibodies could be reconstituted by the insertion of the $\alpha_X\beta_2$ C-linker (CX mutant) and its modified version (CX-E mutant), which differ from the $\alpha_L\beta_2$ C-linker in six and five amino acids, respectively (Figs. 6 and 1B). Thus, the data suggest that these C-linker residues, 313–318, do not directly contribute to the R7.1 or CBR LFA-1/1 epitopes. In summary, our data show that shortening of the C-linker results in the gradual (yet differential) loss of two combinational epitopes that require both the αI domain and the C-linker/β-propeller domain for full reactivity.

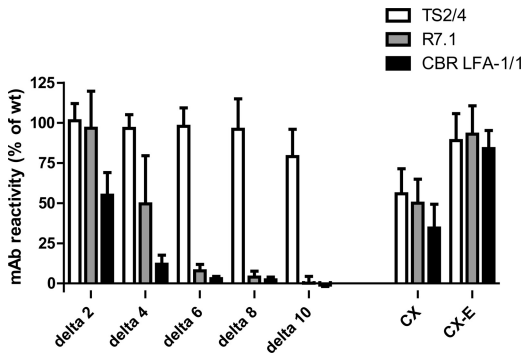

FIGURE 6. **Reactivity of mAbs with C-linker mutants.** 293T cells transiently transfected with $\alpha_L\beta_2$ deletion and swap mutants were stained with mAbs TS2/4 (as a measure for cell surface expression), mAbs R7.1, and CBR LFA-1/1 and subjected to flow cytometry. The reactivity of the antibodies is expressed as a percentage of MFI with the WT. Each *bar* represents the mean value ± S.D. of more than three independent experiments. The binding to mock-transfected 293T cells was subtracted before calculating the mean values.

DISCUSSION

The C-linker is defined as the C-terminal connection between the αI domain and the β-propeller domain, which belongs structurally to neither the αI nor the β-propeller domains. The C-linker is located at the interface with the βI domain, and mutations in the C-linker including Glu-310, Lys-314, and Leu-317 have been shown to regulate I domain affinity (28, 29). In this study, we systematically addressed the functional role of the C-linker of the αI domain integrin $\alpha_L\beta_2$ by mutational analysis. Stepwise shortening of the C-linker neither impaired expression of $\alpha_L\beta_2$ nor its adhesive function induced by activating antibodies. However, differences in the level of adhesion were observed when the divalent cation Mn^{2+} was used as a stimulus. The mechanism of β2 integrin activation by Mn^{2+} is not well understood, and these differences may suggest two distinct mechanisms that are differentially affected by C-linker shortening.

Most interestingly, our studies demonstrate that substantial shortening of the C-linker by 8 and 10 amino acids constitutively activates $\alpha_L\beta_2$. As evidenced by mutant Δ 10, which lacks residue Glu-310, this constitutive activation does not require the putative interaction of αL Glu-310 with the βI MIDAS (Fig. 1*E*). In agreement with this assumption, Δ 8 and Δ 10 were resistant to inhibition by the α/β I allosteric inhibitor XVA143. This class of inhibitor is thought to block the binding of αL residue Glu-310 to the βI MIDAS (5). However, the Δ 8 and Δ 10 mutants only marginally expressed the KIM127 activation epitope on the β chain, which is normally exposed upon αI/βI domain interaction (25). These results suggest that a "pull" triggered by shortening of the C-linker is sufficient to keep the αI domain in a high-affinity conformation independent of a regulative function of the β2 chain. Similarly, the previously described αL-E310C/β2-A210C double mutant is constitutively active in the absence of Glu-310, does not express activation epitopes on the β chain, and is resistant to XVA143 inhibition (although binding of the inhibitor to the mutant was demonstrated) (30). However, in the latter case, the pull keeping the I domain of αL-E310C/β2-A210C in a high-affinity state is exerted by a disulfide connecting C-linker residue 310 to a residue in a βI MIDAS-coordinating loop (30). Intriguingly, αI

allosteric inhibitors that bind underneath the α7-helix of the αI domain were still able to convert the constitutively active state of the αL-E310C/β2-A210C mutant into an inactive state (30). The same property was observed for the C-linker Δ 8 and Δ 10 mutants in this study.

Inhibition by αI allosteric antagonists demonstrates that there is still enough "play" in the Δ 8 and Δ 10 C-linker mutants to enable the reversion of the open αI domain conformation to the closed conformation. Because in Δ 8 and Δ 10 the C-linker is largely or completely removed, respectively, this play must come from somewhere else. This remaining play may come both from changes in αI domain orientation with respect to the β-propeller and βI domains and from unwinding of the C-terminal portion of the α7-helix. Unwinding of the C-terminal portion is feasible because crystal structures of isolated α_L I domains complexed with this class of inhibitors show interaction with residues located only in the N-terminal portion of the α7-helix (31). For example, the main contacts of LFA878 are formed with α7-helix residues Glu-301, Leu-302, and Lys-305 (31). Interestingly, the constitutively active $\alpha_L\beta_2$ mutant α_L-K287C/α_L-K294C is resistant to inhibition by αI allosteric inhibitors (5, 25). This latter mutant contains an αI domain which, in contrast to the above described mutants, is "locked" in the high-affinity form by a disulfide bond introduced prior to the α7-helix in the β6-α7 loop (25). However, similarly to the C-linker mutants and αL-E310C/β2-A210C, there was only little expression of the KIM127 epitope in this mutant (25).

Thus, constitutive activation of the αI domain via C-linker shortening or introduction of disulfide bonds is not associated with a global conformational change from the bent to the extended form. Does this mean that bent $\alpha_L\beta_2$ can be adhesive? Most likely this is not the case. All of the mutants described above express the KIM127 epitope to a certain degree, suggesting that some receptors are transiently extended and sampling extended conformational space. Even wild-type LFA-1 has basal adhesive activity. Taken together, our findings demonstrate that the αI domains of the C-linker mutants Δ 8 and Δ 10 are able to respond to αI allosteric inhibitors similarly to WT $\alpha_L\beta_2$. Furthermore, α/β I allosteric inhibitors that bind to the MIDAS of the βI domain were still able to convert the β2 chain of the mutants from an inactive into an active conformation, as shown by the exposure of the KIM127 activation epitope. These observations demonstrate that intrachain conformational change within the αI domain and within the βI and hybrid domains is still preserved in the C-linker mutants, whereas communication between the αI and βI domains is disrupted.

Conformational changes because of C-linker shortening were also detectable by the anti-αL mAbs R7.1 and CBR LFA-1/1. Our study indicates that the epitopes of these antibodies involve both the αL I domain and the β propeller domain. Two results indicate that CBR LFA-1/1 requires the αI domain for binding: deletion of the αI domain (129–308) led to greatly reduced reactivity, and mapping studies demonstrate that αI domain residues 250–303 contribute to the epitope recognized by CBR LFA-1/1. The lack of reactivity with cell surface expressed αI domain alone shows that another component is required to constitute the CBR LFA-1/1 epitope. The reactivity of the antibodies with a cell surface-expressed αL fragment containing the αI domain, the C-linker, and part of the β-propeller domain (residues 130–339) indicates that residues of the C-linker and β-propeller region may constitute this second component. This notion is supported by mapping studies that show that residues 301- 442 (in this study) and residues 301–359 (as reported previously (9)) are involved in the epitope. The observation that CBR LFA-1/1 only marginally binds to the latter region in the absence of the αI domain in I-less $\alpha_L\beta_2$ are in line with this interpretation. Moreover, the dual specificity is consistent with the previous observation that CBR LFA-1/1 had properties intermediate between antibodies to the αI and β-propeller domains in its requirement for folding of these domains for immunoprecipitation (32). R7.1 similarly required both the αI and β-propeller domains for full reactivity but recognizes an overlapping but distinct epitope on the basis of mapping the human residues for which it is specific with chimeras.

C-linker shortening gradually reduced expression of the R7.1 and CBR LFA-1/1 epitopes, with the CBR-LFA-1/1 epitope more sensitive to shortening than the R7.1 epitope. Both epitopes were abolished after complete C-linker deletion. Intriguingly, the epitopes of R7.1 and CBR LFA-1/1 could be reconstituted by inserting the C-linker of an integrin that is not recognized by the antibodies, *i.e.* $\alpha_X\beta_2$. The sequence exchanged is identical to the smallest segment of residues that was deleted to almost completely abrogate antibody binding (*i.e.* Δ 6). In other words, deletion of residues 313–318, SKQDLT, in Δ 6 abolished reactivity, and replacement with residues ETTSSS in CX-E restored activity. Thus, R7.1 and CBR LFA-1/1 do not appear to recognize this portion of the C-linker directly. Instead, they appear to recognize a combinatorial epitope comprised of portions of the αI and β-propeller domains and possibly conserved linker residues 309–312. Thus, the length but not the sequence of C-linker residues 313–318 is important for enabling a specific orientation between the segments recognized by the antibodies.

Consistent with the assumption that R7.1 and LFA CBR-1/1 are sensitive to conformational changes in the C-linker region, binding of both antibodies was perturbed by allosteric inhibitors that target this area. Notably, CBR LFA-1/1 blocks ligand binding by wild-type $\alpha_L\beta_2$ but not by the disulfide-locked high-affinity mutant, suggesting inhibition by an allosteric mode of action (25). Our studies establish R7.1 and CBR LFA-1/1 as a new class of $\alpha_L\beta_2$ antibodies sensitive to conformational change across the important interface between the αI and β-propeller domains and as important tools to study conformational alterations within the C-linker region of $\alpha_L\beta_2$. In contrast, the allosteric inhibitory $\alpha_L\beta_2$ antibodies TS2/14 and 25-3-1 have been mapped to species-specific residues contained wholly within the α_L I domain in the β5-α6 loop and α6 helix (24).

Functionally, the replacement of the $\alpha_L\beta_2$ C-linker by the $\alpha_X\beta_2$ C-linker, altering residues 313–318, resulted in mutants with properties comparable with the WT. This result indicates that the length of the C-linker is more critical for its function than the sequence. In support of this notion we also found that single point mutations in the C-linker region (K314A and L317A) did not affect expression and function of $\alpha_L\beta_2$ in 293T cells (data not shown). It is not known why this normal pheno-

type in 293T cells differs from the constitutively active phenotype of the same mutants reported in COS-7 cells (28). It is noteworthy, however, that a similar discrepancy between COS-7 cells and another cell line has been reported with an $\alpha_L\beta_2$ receptor mutant before (33). Our findings suggest that the C-linker functions as a spring-like element that allows relaxation of the αI domain in the resting state and controlled tension of the αI domain during activation. In agreement with this notion, the crystal structure of the αI domain integrin $\alpha_X\beta_2$ in the bent, inactive conformation reveals that the C-linker is not in contact with the surrounding regions (7). The C-linker polypeptide on the α subunit is not the only spring-like element in integrins that fine-tunes an equilibrium between the resting and active states. Recently, it has been demonstrated in β3 integrins that the length of a loop expressed on the β subunit at the integrin "knee" modulates the equilibrium between low- and high-affinity states of these integrins (34). In fact, disordered regions such as the C-linker are quite common in eukaryotic proteins. They can be found in transcription factors and cell signaling molecules (35). A recent mechanistic model suggests that intramolecular site-to-site allosteric coupling is optimized when intrinsic disorder is present in the domains containing one or both of the coupled sites (36). Thus, it appears to be an emerging theme that polypeptide stretches such as the C-linker in $\alpha_L\beta_2$ are critical elements for conformational flexibility and functionality. It is intriguing to speculate that such disordered regions and their vicinity could become new targets for drug development and therapeutic intervention.

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REFERENCES

1. Cox, D., Brennan, M., and Moran, N. (2010) *Nat. Rev. Drug Discov.* **9,** 804–820
2. Luo, B. H., Carman, C. V., and Springer, T. A. (2007) *Annu. Rev. Immunol.* **25,** 619–647
3. Shimaoka, M., Xiao, T., Liu, J. H., Yang, Y., Dong, Y., Jun, C. D., McCormack, A., Zhang, R., Joachimiak, A., Takagi, J., Wang, J. H., and Springer, T. A. (2003) *Cell* **112,** 99–111
4. Shimaoka, M., and Springer, T. A. (2003) *Nat. Rev. Drug Discov.* **2,** 703–716
5. Shimaoka, M., Salas, A., Yang, W., Weitz-Schmidt, G., and Springer, T. A. (2003) *Immunity* **19,** 391–402
6. Nishida, N., Xie, C., Shimaoka, M., Cheng, Y., Walz, T., and Springer, T. A. (2006) *Immunity* **25,** 583–594
7. Xie, C., Zhu, J., Chen, X., Mi, L., Nishida, N., and Springer, T. A. (2010) *EMBO J.* **29,** 666–679
8. Evans, R., Patzak, I., Svensson, L., De Filippo, K., Jones, K., McDowall, A., and Hogg, N. (2009) *J. Cell Sci.* **122,** 215–225
9. Huang, C., and Springer, T. A. (1995) *J. Biol. Chem.* **270,** 19008–19016
10. Huang, C., Zang, Q., Takagi, J., and Springer, T. A. (2000) *J. Biol. Chem.* **275,** 21514–21524
11. Robinson, M. K., Andrew, D., Rosen, H., Brown, D., Ortlepp, S., Stephens, P., and Butcher, E. C. (1992) *J. Immunol.* **148,** 1080–1085
12. Argenbright, L. W., Letts, L. G., and Rothlein, R. (1991) *J. Leukocyte Biol.* **49,** 253–257
13. Fotouhi, N., Gillespie, P., Guthrie, R., Pietranico-Cole, S., and Yun, W. (October 12, 1999) Patent Cooperation Treaty International Application WO0021920
14. Song, G., Lazar, G. A., Kortemme, T., Shimaoka, M., Desjarlais, J. R., Baker, D., and Springer, T. A. (2006) *J. Biol. Chem.* **281,** 5042–5049
15. Higuchi, R., Krummel, B., and Saiki, R. K. (1988) *Nucleic Acids Res.* **16,** 7351–7367
16. Yalamanchili, P., Lu, C., Oxvig, C., and Springer, T. A. (2000) *J. Biol. Chem.* **275,** 21877–21882
17. Lu, C., Shimaoka, M., Ferzly, M., Oxvig, C., Takagi, J., and Springer, T. A. (2001) *Proc. Natl. Acad. Sci. U.S.A.* **98,** 2387–2392
18. Astrof, N. S., Salas, A., Shimaoka, M., Chen, J., and Springer, T. A. (2006) *Biochemistry* **45,** 15020–15028
19. Lu, C., Oxvig, C., and Springer, T. A. (1998) *J. Biol. Chem.* **273,** 15138–15147
20. Weetall, M., Hugo, R., Friedman, C., Maida, S., West, S., Wattanasin, S., Bouhel, R., Weitz-Schmidt, G., and Lake, P. (2001) *Anal. Biochem.* **293,** 277–287
21. Salas, A., Shimaoka, M., Phan, U., Kim, M., and Springer, T. A. (2006) *J. Biol. Chem.* **281,** 10876–10882
22. Lu, C., Ferzly, M., Takagi, J., and Springer, T. A. (2001) *J. Immunol.* **166,** 5629–5637
23. Chen, X., Xie, C., Nishida, N., Li, Z., Walz, T., and Springer, T. A. (2010) *Proc. Natl. Acad. Sci. U.S.A.* **107,** 14727–14732
24. Lu, C., Shimaoka, M., Salas, A., and Springer, T. A. (2004) *J. Immunol.* **173,** 3972–3978
25. Lu, C., Shimaoka, M., Zang, Q., Takagi, J., and Springer, T. A. (2001) *Proc. Natl. Acad. Sci. U.S.A.* **98,** 2393–2398
26. Kelly, T. A., Jeanfavre, D. D., McNeil, D. W., Woska, J. R., Jr., Reilly, P. L., Mainolfi, E. A., Kishimoto, K. M., Nabozny, G. H., Zinter, R., Bormann, B. J., and Rothlein, R. (1999) *J. Immunol.* **163,** 5173–5177
27. Welzenbach, K., Hommel, U., and Weitz-Schmidt, G. (2002) *J. Biol. Chem.* **277,** 10590–10598
28. Huth, J. R., Olejniczak, E. T., Mendoza, R., Liang, H., Harris, E. A., Lupher, M. L., Jr., Wilson, A. E., Fesik, S. W., and Staunton, D. E. (2000) *Proc. Natl. Acad. Sci. U.S.A.* **97,** 5231–5236
29. Yang, W., Shimaoka, M., Chen, J., and Springer, T. A. (2004) *Proc. Natl. Acad. Sci. U.S.A.* **101,** 2333–2338
30. Yang, W., Shimaoka, M., Salas, A., Takagi, J., and Springer, T. A. (2004) *Proc. Natl. Acad. Sci. U.S.A.* **101,** 2906–2911
31. Weitz-Schmidt, G., Welzenbach, K., Dawson, J., and Kallen, J. (2004) *J. Biol. Chem.* **279,** 46764–46771
32. Huang, C., and Springer, T. A. (1997) *Proc. Natl. Acad. Sci. U.S.A.* **94,** 3162–3167
33. Lupher, M. L., Jr., Harris, E. A., Beals, C. R., Sui, L. M., Liddington, R. C., and Staunton, D. E. (2001) *J. Immunol.* **167,** 1431–1439
34. Smagghe, B. J., Huang, P. S., Ban, Y. E., Baker, D., and Springer, T. A. (2010) *J. Biol. Chem.* **285,** 32954–32966
35. Fink, A. L. (2005) *Curr. Opin Struct. Biol.* **15,** 35–41
36. Hilser, V. J., and Thompson, E. B. (2007) *Proc. Natl. Acad. Sci. U.S.A.* **104,** 8311–8315

The C-Terminal αI Domain Linker as a Critical Structural Element in the Conformational Activation of αI Integrins

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