

Intersubunit signal transmission in integrins by a receptor-like interaction with a pull spring

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Contributed by Timothy A. Springer, December 31, 2003

The function of some multidomain proteins is regulated by inter-domain communication. We use second-site suppressor cysteine mutations to test a hypothesis on how the inserted (I)-like domain in the integrin β -subunit regulates ligand binding by the neighboring I domain in the integrin α -subunit [Huth, J. R., Olejniczak, E. T., Mendoza, R., Liang, H., Harris, E. A., et al. (2000) *Proc. Natl. Acad. Sci. USA* 97, 5231–5236; and Alonso, J. L., Essafi, M., Xiong, J. P., Stehle, T. & Arnaout, M. A. (2002) *Curr. Biol.* 12, R340–R342]. The hypothesis is that an interaction between the β I-like metal ion-dependent adhesion site (MIDAS) and an intrinsic ligand in the linker following the α I domain, Glu-310, exerts a pull that activates the α I domain. Individual mutation of α I linker residue Glu-310 or β MIDAS residues Ala-210 or Tyr-115 to cysteine abolishes I domain activation, whereas the double mutation of α I-E310C with either β 2-A210C or β 2-Y115C forms a disulfide bond that constitutively activates ligand binding. The disulfide-bonded mutant is resistant to small molecule antagonists that bind to the β I-like domain near its interface with the α I domain and inhibit communication between these domains but remains susceptible to small molecule antagonists that bind underneath the I domain α 7-helix and certain allosteric antagonistic antibodies. Thus, the α 7-helix and its linker are better modeled as a pull spring than a bell rope. The results suggest that α I residue Glu-310, which is universally conserved in all I domain-containing integrins, functions as an intrinsic ligand for the β I-like domain, and that when integrins are activated, the β I-like MIDAS binds to Glu-310, pulls the spring, and thereby activates the α I domain.

Integrins are a large family of adhesion receptors that regulate cell migration and tissue organization and transduce signals bidirectionally across the plasma membrane. They are the most structurally complicated adhesion molecules yet known, with noncovalently associated α - and β -transmembrane subunits containing five and eight distinctive domains, respectively, in their extracellular segments. Half of vertebrate integrin α -subunits and all β -subunits contain von Willebrand factor-type A domains, termed inserted (I) and I-like domains, respectively (1–3). Both I and I-like domains have an α/β -fold with a central β -sheet surrounded by α -helices and a metal ion-dependent adhesion site (MIDAS) at the C-terminal ends of the central β -strands, i.e., the “top” face (1, 4–6). In integrins that lack I domains, I-like domains directly mediate ligand binding: a metal at the MIDAS coordinates to an acidic residue in the ligand (7). In I domain-containing integrins such as α 1 β 2, the I domain binds the acidic residue of the ligand through its MIDAS (4, 8–10), whereas the I-like domain regulates binding by the I domain (11). However, the molecular mechanism of I domain regulation by the I-like domain remains unknown.

The I domain is inserted in the integrin α -subunit between blades 2 and 3 of the β -propeller domain (12). The I domain C-terminal α 7-helix and the linker connecting it to the β -propeller domain are crucial for regulation of ligand binding. Downward movement of the α 7-helix activates the I domain (8, 9, 13–15). Mutations in the α 7-helix and linker may either activate or inactivate the I domain (16–19). A liganded crystal structure of integrin α v β 3, which lacks an α I domain, shows that

the acidic Asp side chain of a ligand-mimetic peptide Arg-Gly-Asp is bound to the MIDAS of the β 3 I-like domain, whereas the Arg side chain binds to loops of the α v β -propeller, at a site equivalent to where the I domain is inserted into the α L β -propeller domain (6). Because a Glu residue in the linker between the I and β -propeller domains corresponding to Glu-310 in α L is absolutely conserved in all I domain-containing integrins, and mutation of this residue in α L (16) or α M (20) abolishes I domain activation, it previously has been proposed that Glu-310 might interact with the metal in the β 2 MIDAS in a way that mimics ligand binding by integrins that lack I domains (Fig. 1A) (1, 16, 20). However, a large number of explanations are possible for the negative effect of mutation of α L Glu-310, and no evidence for an interaction with the β I-like domain has been presented. In this article, by constructing second-site revertant mutations (21), we test the hypothesis that when activated, the β 2 I-like domain MIDAS binds α L residue Glu-310 in the linker between the I domain and the β -propeller domain and exerts a downward pull on the α 7-helix of the I domain that activates the I domain (Fig. 1A).

Materials and Methods

Cell Lines, Antibodies, and Small Molecule Inhibitors. cDNAs of wild-type α L and β 2 were inserted into pcDNA3.1/Hygro(+) or pcDNA3.1(+) and used as the template for mutagenesis. The α L and β 2 mutations were generated by using QuikChange XL Site-Directed Mutagenesis Kit (Stratagene). All constructs were verified by DNA sequencing. 293T cells were transfected with Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. K562 cells were transfected by electroporation and selected with 1 mg/ml G418 (22). mAbs to human α L and β 2 are as described (11). mAbs m24 (23) and KIM127 (24) were kind gifts of N. Hogg (Imperial Cancer Research Fund, London) and M. Robinson (Celltech, Slough, U.K.), respectively. mAbs were used as 10 μ g/ml purified IgG or 1:200 ascites. LFA703 (25, 26) was kindly provided by Novartis Pharma (Basel). XVA143 (27) was synthesized according to example 345 of the patent (28) and was obtained from Paul Gillespie (Roche, Nutley, NJ).

Cell Adhesion Assay. Binding of fluorescently labeled transfectants to immobilized intercellular adhesion molecule-1 (ICAM-1) was as described (22). Briefly, soluble ICAM-1 (domains 1–5) was immobilized at 10 μ g/ml on microtiter plates. Binding of the 293T transient transfectants to immobilized ICAM-1 was determined in 2.5% FBS/L15 medium. Binding of K562 stable transfectants to immobilized ICAM-1 was determined in Hepes/NaCl/Glucose/BSA (20 mM Hepes, pH 7.5/140 mM NaCl/2 mg/ml glucose/1% BSA) supplemented as indicated with diva-

Abbreviations: MIDAS, metal ion-dependent adhesion site; I, inserted; ICAM-1, intercellular adhesion molecule-1; HA, high-affinity.

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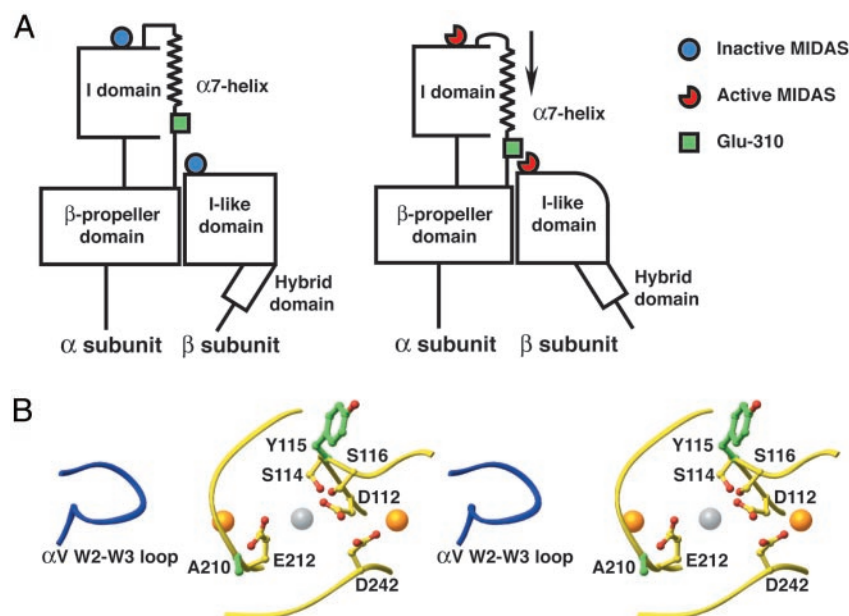


Fig. 1. The structural hypothesis. (A) Schematic. It is hypothesized that α_L -Glu-310 acts as an intrinsic ligand that binds to the β_2 -subunit I-like domain MIDAS and, thus, axially displaces the I domain $\alpha 7$ -helix in the C-terminal direction, reshapes the $\beta 6$ - $\alpha 7$ loop, and activates the α_L I domain MIDAS (1, 16, 20). C-terminal axial displacement of the $\alpha 7$ -helix and $\beta 6$ - $\alpha 7$ loop reshaping is known to result in a 10,000-fold increase in affinity for ligand of the α_L I domain (9). Swing-out of the hybrid domain is depicted as demonstrated for $\alpha_V\beta_3$ and $\alpha_5\beta_1$ (35, 36). (B) Stereo diagram of the MIDAS loops of the β_2 and β_3 I-like domains (yellow) and the W2-W3 loop of the α_V β -propeller domain (blue). The structure shown is that of liganded $\alpha_V\beta_3$ (6); all side chains shown are identical in β_2 and β_3 , and numbering is that of β_2 . Mutated residues are shown in green; MIDAS-coordinating residues are shown in yellow; O atoms are red. The ligand-induced metal-binding site (LIMBS), MIDAS, and adjacent to MIDAS (ADMIDAS) metal ions are gold, silver, and gold spheres, respectively, from left to right.

lent cations and DTT. After incubation at room temperature for 30 min, unbound cells were washed off and bound cells were quantitated (22).

Binding of Soluble ICAM-1. Binding of soluble ICAM-1-IgA/Fc fusion protein complexed with affinity-purified, FITC anti-human IgA was measured by immunofluorescence flow cytometry (29).

Cell Surface Biotinylation and Immunoprecipitation. Cell surface biotinylation and immunoprecipitation were as described (29).

Results

Design and Cell Surface Expression of $\alpha_L\beta_2$ Second-Site Reversion Mutants. α_L - and β_2 -subunits were coexpressed in transient 293T or stable K562 transfectants, and adhesion to ICAM-1 immobilized on substrates or binding to soluble multimeric fluorescent ICAM-1 was measured (Fig. 2). Mutation α_L -E310C abolished binding to ICAM-1 similarly to α_L -E310A, confirming a crucial role for Glu-310 in I domain activation (Fig. 2A) (16). To search for a site in the β_2 -subunit where a cysteine could be introduced that would suppress the α_L -E310C mutation by formation of an intersubunit disulfide bond, the liganded $\alpha_V\beta_3$ structure (7) was examined for a residue around the I-like domain MIDAS that was close to the loop between blade 2 and blade 3 of the β -propeller, where the I domain is inserted in α_L (12). Metal coordinating residues and buried residues were excluded. By using these criteria, residue β_2 -A210 was selected, which is in the MIDAS loop that bears the metal-coordinating residue Glu-212 (Fig. 1B).

We expected that α_L -E310 would bind to the β_2 MIDAS as part of a larger intersubunit interface, and that exposed residues in β_2 MIDAS loops also would contribute to this interface. β_2 -A210 is nearby the MIDAS coordinating residue β_2 -E212, and we therefore hoped that the mutation β_2 -A210C might by

itself inactivate $\alpha_L\beta_2$. Indeed, the β_2 -A210C mutation abolished binding to ICAM-1 (Fig. 2A), showing a crucial role for a non-metal-coordinating β_2 MIDAS loop residue in $\alpha_L\beta_2$ activation and suggesting that residues in the vicinity of the β_2 MIDAS, including β_2 -Ala-210, might interact with the I domain C-terminal linker.

Formation of an Intersubunit Disulfide Bond Between α_L -E310C and β_2 -A210C Constitutively Activates Integrin $\alpha_L\beta_2$. To directly test the hypothesis that an interaction between residues in the vicinity of α_L linker residue E310 and β_2 MIDAS residue A210 activates $\alpha_L\beta_2$, the α_L -E310C and β_2 -A210C mutants were cotransfected. Despite the abolition of binding by the individual substitutions in the α_L -E310C/ β_2 and α_L / β_2 -A210C heterodimers, the α_L -E310C/ β_2 -A210C double mutant heterodimer was fully activated (Fig. 2A, C, and D). By contrast, the α_L -E310A/ β_2 -A210C double mutant was inactive. Immunofluorescent flow cytometry showed all α_L -E310 and β_2 -A210 single and double mutants were as well expressed as wild-type $\alpha_L\beta_2$ in both 293T and K562 transfectants (Fig. 2E, bottom line, and data not shown). These results demonstrate second-site reversion between mutations at residues α_L -E310 and β_2 -A210 when each residue is mutated to cysteine. Immunoprecipitation from K562 transfectants and reducing and nonreducing SDS/PAGE demonstrated that the α_L -E310C/ β_2 -A210C heterodimer, but not the wild-type $\alpha_L\beta_2$ heterodimer, is covalently linked with a disulfide bond (Fig. 2B), with an efficiency of formation of 80%. β I-like domains contain a specificity-determining loop with disulfide-bonded cysteines that locate ≈ 12 Å from the I-like MIDAS and 16 Å from β_2 -A210 (7). To rule out any possible interaction with the engineered disulfide, these cysteines were mutated to Ala in the β_2 -C169A/C176A mutant. Although α_L / β_2 -C169A/C176A/A210C was inactive, α_L -E310C/ β_2 -C169A/C176A/A210C was constitutively active in binding to ICAM-1 and bound as well as α_L -E310C/ β_2 -A210C and activated wild-type $\alpha_L\beta_2$ after correc-

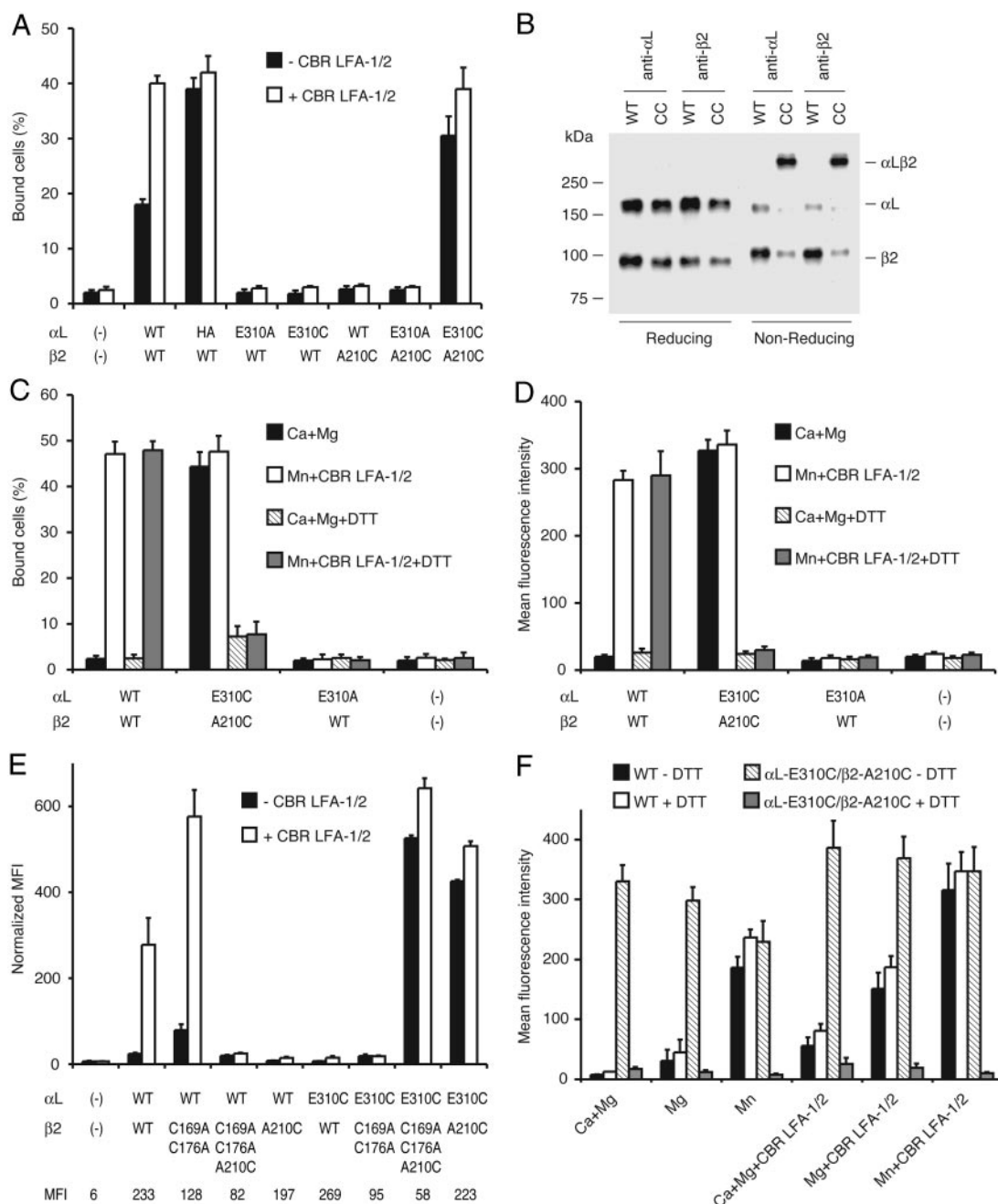


Fig. 2. Second-site suppressor α_L -E310C and β_2 -A210C mutations constitutively activate $\alpha_L\beta_2$ by forming a disulfide bond. (A) Binding of 293T cell transfectants to immobilized ICAM-1. Adhesion to ICAM-1 of cells transfected with the indicated α_L and β_2 cDNA was determined in the absence (black bars) or presence (white bars) of activating mAb CBR LFA-1/2 at 37°C. HA, high-affinity α_L K287C/K294C I domain mutant (8). (B) Immunoprecipitation. K562 transfectants expressing wild-type $\alpha_L\beta_2$ (WT) and α_L -E310C/ β_2 -A210C (CC) were surface-labeled with biotin, and lysates were immunoprecipitated by either α_L antibody (TS2/4) or β_2 antibody (May.017). Immunoprecipitates were subjected to reducing and nonreducing SDS/7.5% PAGE, transferred to nitrocellulose, and Western-blotted with horseradish peroxidase-streptavidin and enhanced chemiluminescence. (C–F) Binding of K562 stable (C, D, and F) or 293T transient (E) transfectants to ICAM-1 substrates (C) and soluble ICAM-1 complexes (D–F). Binding was assayed in HEPES/NaCl/glucose/BSA (K562 transfectants) or 2.5% FBS/L15 medium (293T transfectants) supplemented with 1 mM CaCl_2 /1 mM MgCl_2 , 2 mM MgCl_2 , or 2 mM MnCl_2 plus 10 $\mu\text{g}/\text{ml}$ CBR LFA-1/2 or 2 mM DTT as indicated at room temperature. In E, binding of ICAM-1 by different 293T transfectants was normalized to their $\alpha_L\beta_2$ cell surface expression by multiplying by the ratio of the specific mean fluorescence intensity (MFI) of TS2/4 mAb binding to wild-type and mutant $\alpha_L\beta_2$. MFI of TS2/4 mAb before subtraction of the MFI of untransfected cells to obtain specific MFI is shown in the bottom row of E. Binding by the mock transfectant was not normalized.

tion for the lower expression of heterodimers containing the β_2 -C169A/C176A mutation (Fig. 2E). Furthermore, α_L -E310C/ β_2 -C169A/C176A/A210C formed a disulfide-linked heterodimer (data not shown).

Suppression between the α_L -E310C and β_2 -A210C mutations did more than restore wild-type ligand binding, it also resulted

in constitutive activation. In 293T transfectants in the absence of activation, the α_L -E310C/ β_2 -A210C double mutant was more active than wild-type $\alpha_L\beta_2$ and appeared maximally activated, as shown by lack of further activation by CBR LFA-1/2 mAb to the β_2 I-EGF3 domain and comparison to $\alpha_L\beta_2$ with a mutant high-affinity (HA) α_L I domain (Fig. 2A). In K562 transfectants,

Table 1. Inhibition by α_L I and β_2 I-like domain antibodies of multimeric ICAM-1 binding to $\alpha_L\beta_2$ mutants

mAb	Epitope		Inhibition, %		
			Wild-type $\alpha_L\beta_2$	α_L -E310C/ β_2 -A210C	HA $\alpha_L\beta_2$
TS2/6	α_L I domain	154–183	97 $\pm$ 2	96 $\pm$ 2	97 $\pm$ 1
May.035	α_L I domain	K197, H201	98 $\pm$ 1	98 $\pm$ 0	97 $\pm$ 1
MHM24	α_L I domain	K197	96 $\pm$ 2	97 $\pm$ 1	96 $\pm$ 0
TS1/22	α_L I domain	Q266, S270	96 $\pm$ 1	97 $\pm$ 2	92 $\pm$ 1
TS2/14	α_L I domain	S270, E272	99 $\pm$ 0	99 $\pm$ 0	14 $\pm$ 2
CBR LFA-1/1*	α_L I domain	301–338	97 $\pm$ 2	2 $\pm$ 0	2 $\pm$ 1
May.017	β_2 I-like domain	E175, ?	98 $\pm$ 0	70 $\pm$ 8	3 $\pm$ 2
MHM23	β_2 I-like domain	E175	97 $\pm$ 2	40 $\pm$ 6	2 $\pm$ 2
TS1/18	β_2 I-like domain	R133, H332	98 $\pm$ 1	4 $\pm$ 3	0 $\pm$ 2
YFC51	β_2 I-like domain	R133, H332	98 $\pm$ 0	2 $\pm$ 2	0 $\pm$ 1
CLB LFA-1/1	β_2 I-like domain	H332, N339	97 $\pm$ 1	2 $\pm$ 2	0 $\pm$ 0

Wild-type $\alpha_L\beta_2$ in K562 transfectants was activated by preincubation with mAb CBR LFA-1/2. Binding to soluble, multimeric ICAM-1 in medium containing 1 mM CaCl_2 and 1 mM MgCl_2 was in the presence of the indicated mAb. Results are means $\pm$ SD of three experiments. HA, high-affinity I domain mutant (8).

*The epitope spans the linker including α_L -E310C. Binding of CBR LFA-1/1 to the α_L -E310C/ β_2 -A210C and HA mutants was $\approx$ 50% of binding to wild-type $\alpha_L\beta_2$ (8). All other mAbs bound to α_L -E310C/ β_2 -A210C, HA $\alpha_L\beta_2$, and wild-type $\alpha_L\beta_2$ equally well (data not shown).

wild-type $\alpha_L\beta_2$ was inactive under basal conditions in Ca^{2+} / Mg^{2+} , whereas the α_L -E310C/ β_2 -A210C double mutant was maximally active in Ca^{2+} / Mg^{2+} both in adhesion assays and in binding of soluble multimeric ICAM-1 (Fig. 2 C and D). In contrast to wild-type $\alpha_L\beta_2$, α_L -E310C/ β_2 -A210C was active

independent of whether Ca^{2+} plus Mg^{2+} , Mg^{2+} , Mn^{2+} , or activating mAb CBR LFA-1/2 was present (Fig. 2F).

DTT was used to reduce the disulfide bond. Although reduction with 10 mM DTT at 37°C can activate β_2 integrins (8, 30), 2 mM DTT at room temperature did not affect binding of wild-type $\alpha_L\beta_2$ to immobilized or soluble ICAM-1 (Fig. 2 C, D, and F). However, 2 mM DTT abolished the binding of α_L -E310C/ β_2 -A210C to ICAM-1 (Fig. 2 C, D, and F). Ligand binding by wild-type $\alpha_L\beta_2$, but not DTT-treated α_L -E310C/ β_2 -A210C, was activated by CBR LFA-1/2 mAb and Mn^{2+} (Fig. 2 C, D, and F). The inability of DTT-treated α_L -E310C/ β_2 -A210C to bind ligand agrees with the finding above that both α_L -E310C/ β_2 and α_L / β_2 -A210C heterodimers failed to bind ligand. We conclude that (i) in the active conformation of $\alpha_L\beta_2$, residues α_L -310 and β_2 -210 are in sufficiently close proximity to form a disulfide bond when mutated to cysteine; (ii) disulfide bond formation is required for second-site reversion between the α_L -E310C and β_2 -A210C mutations; and (iii) formation of a disulfide bond between these residues constitutively activates the α_L I domain.

Susceptibility to Small Molecule Antagonists and Inhibitory Antibodies

mAbs inhibit $\alpha_L\beta_2$ function by different mechanisms. mAbs that directly, i.e., competitively block binding to ICAM-1, inhibit binding to activated wild-type $\alpha_L\beta_2$ as well as $\alpha_L\beta_2$ containing an I domain locked in the high-affinity, ligand-binding configuration with a disulfide bond (HA $\alpha_L\beta_2$) (11). By contrast, mAbs that indirectly, i.e., allosterically block binding to ICAM-1, inhibit wild-type $\alpha_L\beta_2$ but not HA $\alpha_L\beta_2$. Competitive inhibitor mAbs to the α_L I domain, i.e., TS2/6, May.035, MHM24, and TS1/22, equivalently blocked wild-type $\alpha_L\beta_2$, α_L -E310C/ β_2 -A210C, and HA $\alpha_L\beta_2$ (Table 1) (8). By contrast, TS2/14 mAb, which noncompetitively inhibits ICAM-1 binding to LFA-1,

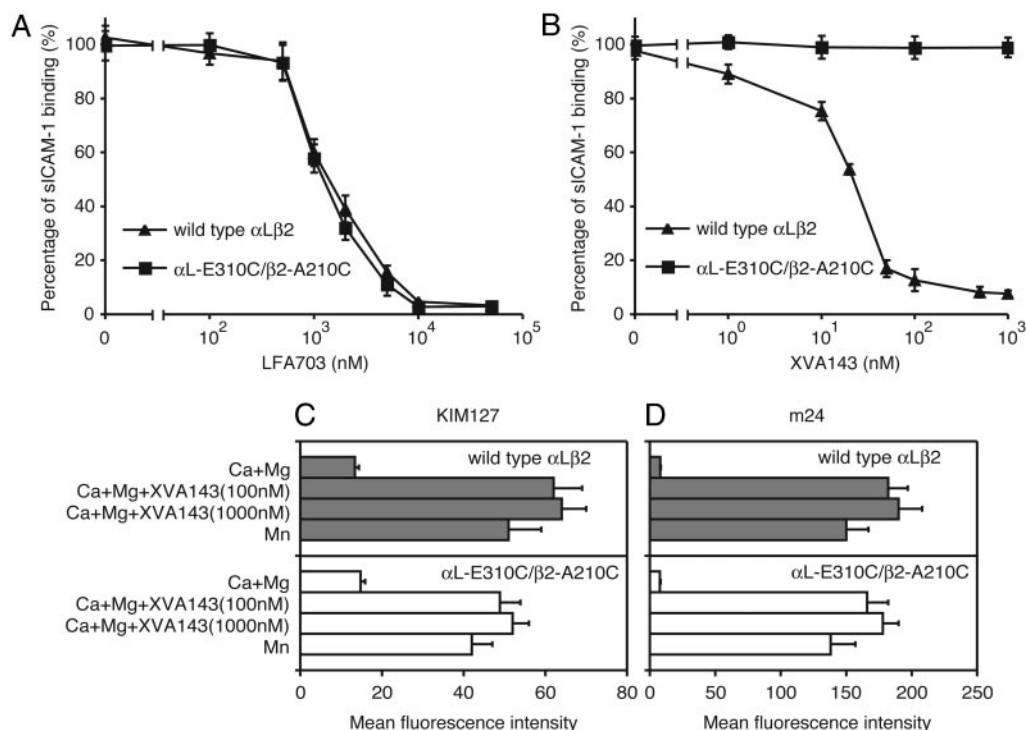


Fig. 3. Inhibition by small molecule antagonists of binding to ICAM-1 and induction of activation epitopes. (A and B) Inhibition of binding of soluble, multimeric ICAM-1 by LFA703 (A) or XVA143 (B). Binding of wild-type $\alpha_L\beta_2$ K562 transfectants activated by preincubation with mAb CBR LFA-1/2 for 30 min or α_L -E310C/ β_2 -A210C transfectants was measured in medium containing 1 mM CaCl_2 and 1 mM MgCl_2 . (C and D) Induction by XVA143 or Mn^{2+} of KIM127 (C) and m24 (D) epitopes. Transfectants in medium containing 1 mM CaCl_2 /1 mM MgCl_2 , 2 mM MnCl_2 , and XVA143 as indicated were stained with KIM127 or m24 mAbs and subjected to immunofluorescence flow cytometry. Expression of activation-insensitive mAb TS2/4 was not affected by XVA143 or Mn^{2+} (data not shown).

blocked binding of the α_L -E310C/ β_2 -A210C mutant but not the HA $\alpha_L\beta_2$ mutant to ICAM-1, suggesting that the α_L -E310C/ β_2 -A210C mutation does not irreversibly activate the I domain. mAbs to the β_2 I-like domain inhibit ICAM-1 binding allosterically, as shown by inhibition of wild-type but not HA $\alpha_L\beta_2$ (Table 1) (11). Interestingly, mAbs May.017 and MHM23, which bind to the specificity-determining loop of the I-like domain, which locates in or very near to the α I/ β I-like interface, partially inhibited ligand binding by α_L -E310C/ β_2 -A210C (Table 1). By contrast, mAbs that bind distal to this interface, to the β_2 I-like domain α 1- and α 7-helices, did not inhibit α_L -E310C/ β_2 -A210C (Table 1).

The mechanism of activation by the disulfide between the α_L I linker and the β_2 I-like MIDAS was investigated further with representatives of two distinct classes of small molecule antagonists, LFA703 and XVA143. Both are allosteric antagonists, as shown by lack of inhibition of HA $\alpha_L\beta_2$ (29). LFA703 binds to the hydrophobic pocket underneath the α 7-helix of the α_L I domain and stabilizes the low-affinity, closed conformation of the I domain (25, 26). α_L -E310C/ β_2 -A210C was as sensitive to inhibition by LFA703 as wild-type $\alpha_L\beta_2$ (Fig. 3A). XVA143 binds to the MIDAS of the β_2 I-like domain and blocks its ability to communicate activation to the α I domain (27, 29). The α_L -E310C/ β_2 -A210C mutant was totally resistant to inhibition by XVA143 (Fig. 3B).

The global conformation of the α_L -E310C/ β_2 -A210C mutant was examined with the m24 mAb to an activation epitope on the β_2 I-like domain (11, 23, 31) and the KIM127 mAb to an epitope on the β_2 I-EGF2 domain that is buried in the bent integrin conformation and exposed in the extended conformation (32, 33). The disulfide connecting the α_L I domain linker to the β_2 I-like MIDAS did not induce exposure of either epitope, suggesting that the extended conformation is not induced and that the I-like domain remains in an inactive conformation (Fig. 3C and D). This finding is as expected, because unlike coordination of α_L -Glu-310 with the β_2 MIDAS metal, the disulfide bond is not expected to alter MIDAS coordination and β_2 I-like domain conformation. Similarly, mutationally stabilizing the α_L I domain in the high-affinity conformation with the HA $\alpha_L\beta_2$ mutant does not lead to β_2 I-like domain activation or global conformational change (11). Nonetheless, the active conformation of the β_2 I-like domain detected by m24 mAb and the extended integrin conformation detected by KIM127 mAb were induced in the α_L -E310C/ β_2 -A210C mutant by Mn^{2+} and by XVA143 (Fig. 3C and D). This finding shows that the α_L -E310C/ β_2 -A210C mutant is capable of undergoing global conformational change and binds to XVA143 despite lack of inhibition of ligand binding by XVA143.

Another Second-Site Reversion $\alpha_L\beta_2$ Mutant That also Constitutively Binds ICAM-1. To obtain further evidence for an interaction of α_L -Glu-310 with the β_2 MIDAS, we mutated residue β_2 -Tyr-115 (Fig. 1B). Tyr-115 is located between the two Ser residues in the MIDAS DXSYS sequence motif. Ala-210 and Tyr-115 locate opposite one another on either side of the MIDAS metal ion (Fig. 1B). ICAM-1 binding by $\alpha_L\beta_2$ was reduced by the β_2 -Y115A mutation and totally abolished by the β_2 -Y115C mutation (Fig. 4A). The double mutant α_L -E310C/ β_2 -Y115C constitutively bound ICAM-1, exactly as observed for the α_L -E310C/ β_2 -A210C mutant (Fig. 4A). Moreover, immunoprecipitation from 293T transfectants and SDS/PAGE demonstrated that the α_L -E310C and β_2 -Y115C subunits were covalently linked together with a disulfide bond, with an efficiency of formation of 63% (Fig. 4B). Ligand binding by α_L -E310C/ β_2 -Y115C was abolished by DTT reduction, demonstrating that the intersubunit disulfide bond was indispensable for ligand-binding activity (Fig. 4A).

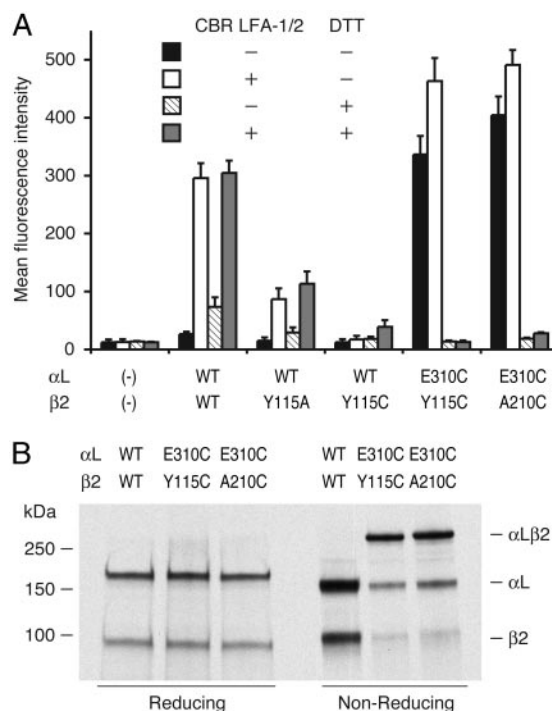


Fig. 4. Constitutive activation of α_L -E310A/ β_2 -Y115C with an intersubunit disulfide bond. (A) Binding of 293T cell transfectants to soluble ICAM-1 complexes. Binding was assayed in 2.5% FBS/L15 medium supplemented with 10 μ g/ml CBR LFA-1/2 or 2 mM DTT as indicated at room temperature. All mutants were as well expressed as wild-type $\alpha_L\beta_2$ in 293T cells. (B) Immunoprecipitation. Lysates from ^{35}S -labeled 293T transfectants were immunoprecipitated with TS2/4 mAb and subjected to SDS/7.5% PAGE and fluorography.

Discussion

To the classical technique of second-site reversion mutations (21), we have added the twists of disulfide bond formation and mutations that activate function as well as suppress loss of function. The α_L mutation E310C and the β_2 mutations A210C and Y115C individually abolish activation of ligand binding by $\alpha_L\beta_2$, but use of the α_L mutation in combination with either of the β_2 mutations constitutively induces $\alpha_L\beta_2$ activation. The formation of the intersubunit disulfide bonds and their requirement for activation of ligand binding directly demonstrate interaction between these regions of α_L and β_2 in the active integrin conformation. The β I-like MIDAS metal ion is centered immediately between residues Ala-210 and Tyr-115, only 5 Å from their C α atoms. The activating crosslinks to these residues strongly support the hypothesis that interaction between α_L residue Glu-310 and the metal of the β_2 I-like domain MIDAS induces the high-affinity conformation of the α_L I domain. Glu-310 is the only acidic residue in the α_L I domain α 7-helix or its linker to the β -propeller domain that is conserved in all integrin I domains, and mutation of the other α_L acidic residues in the same polypeptide segment, Glu-301 (16), Asp-316, and Glu-323 (data not shown) does not abolish $\alpha_L\beta_2$ activation. In integrins that lack I domains, the β I-like domain MIDAS metal ion when activated directly coordinates an acidic residue in the ligand, e.g., the Asp of the Arg-Gly-Asp (RGD) motif common to many integrin ligands (7). Our data strongly support the concept that integrins that contain I domains are activated by a similar interaction in which an intrinsic ligand-like residue, α_L -Glu-310, rather than an extrinsic ligand, binds to a metal ion at the β -subunit I-like MIDAS.

Receptor-ligand-like interaction between neighboring domains/subunits is a previously uncharacterized mechanism for

signal transmission in the extracellular environment and in adhesion molecules and may turn out to have parallels in the diverse range of pro- and eukaryotic intracellular enzymes and extracellular recognition molecules in which von Willebrand factor type A, i.e., I domains, are present (34). α I domains appear in integrins late in metazoan evolution, whereas β I-like domains are present in all integrins, including those in *Porifera*, *Drosophila*, and *Caenorhabditis elegans*. Binding of I-like domains to an intrinsic ligand in the linker following the I domain must have evolved when I domains became inserted in integrin α -subunits, to enable regulated binding to an extrinsic ligand to be transferred from the β I-like domain to the α I domain.

Our study provides insight into the mechanics of the linkage between β I-like and α I domains. Movement of residue α_L -310 to a position near the β_2 I-like MIDAS is sufficient to pull down the α 7-helix, reshape the β 6- α 7 loop, and activate the α_L I

domain. The conformation of the β 6- α 7 loop, but not that of the α 7-helix, is important for transducing conformational change to the α I MIDAS (9). Allosteric inhibition of α_L -E310C/ β_2 -A210C by certain mAbs to the α_L and β_2 I-like domains and LFA703 shows that the segment connecting the β 6- α 7 loop (α_L residue $\approx$ 293) to α_L residue 310 remains elastic. The connecting segment thus should be viewed as a pull spring rather than a bell rope or connecting rod. A pull spring, but not the other two types of mechanical connections, has the important feature that it would enable the β 6- α 7 loop to assume three ratchet positions, as recently observed for the closed, intermediate, and open conformations of the α_L I domain, which have low, intermediate, and high affinity for ICAM-1, respectively (9).

We thank Uli von Andrian for reviewing the manuscript. This work was supported by National Institutes of Health Grant CA31798.

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