

Directed evolution to probe protein allostery and integrin I domains of 200,000-fold higher affinity

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Understanding allostery may serve to both elucidate mechanisms of protein regulation and provide a basis for engineering active mutants. Herein we describe directed evolution applied to the integrin α_L inserted domain for studying allostery by using a yeast surface display system. Many hot spots for activation are identified, and some single mutants exhibit remarkable increases of 10,000-fold in affinity for a physiological ligand, intercellular adhesion molecule-1. The location of activating mutations traces out an allosteric interface in the interior of the inserted domain that connects the ligand binding site to the $\alpha 7$ -helix, which communicates allostery to neighboring domains in intact integrins. The combination of two activating mutations (F265S/F292G) leads to an increase of 200,000-fold in affinity to intercellular adhesion molecule-1. The F265S/F292G mutant is potent in antagonizing lymphocyte function-associated antigen 1-dependent lymphocyte adhesion, aggregation, and transmigration.

Allostery is important in many signaling proteins (1). In the inserted domain (I domain), present in some integrin α subunits, allosteric activation pathways have been studied structurally and functionally (2–8). The ligand-binding site of the I domain, a metal ion-dependent adhesion site (MIDAS), exists as two distinct conformations allosterically regulated by the C-terminal $\alpha 7$ -helix (Fig. 1). Intercellular adhesion molecule-1 (ICAM-1) is the ligand for $\alpha_L \beta_2$ integrin. The N-terminal domain (D1) of ICAM-1 binds to the α_L I domain through an interface that buries 1,250 Å²; at the center of this interface ICAM-1 residue Glu-34 coordinates to the MIDAS metal (Fig. 1A).

Allosteric activation in I domains was first suggested from structural studies of I domains that were crystallized in two conformations termed closed and open; subsequent mutational studies and cocrystallization with ligands demonstrated that closed and open conformations have low affinity and high affinity (HA), respectively, for ligand (5, 8, 9). The two conformations differ in the MIDAS (Fig. 1B) and the position of the $\alpha 7$ -helix (Fig. 1C), which exhibits axial C-terminal or “downward” displacement of 10 Å in the open conformation. In an intact integrin, interaction of the linker region after the $\alpha 7$ -helix with the neighboring I-like domain of the β subunit exerts the force that pulls the $\alpha 7$ -helix downward (3, 10).

Experts using structural insight have designed mutations for studying allostery in α I domains. In the α_M I domain, two buried hydrophobic residues (Phe-275 and Phe-302) become more exposed to solvent in the open conformation. The mutations F275R and F302W led to a 2-fold increase in cell adhesion (2). The allosteric role of the $\alpha 7$ -helix was more directly demonstrated in the α_L I domain. Pairs of cysteines introduced to stabilize the $\alpha 7$ -helix in intermediate or open positions led, respectively, to 500- and 10,000-fold higher affinity to ICAM-1 (4, 5).

Here, we have asked whether directed evolution, an approach based on random mutagenesis coupled with functional screening, and requiring no *a priori* knowledge of protein structure, could be used to identify key residues in the transmission of allostery within the I domain. In this study, we have chosen the α_L I domain as a model system and demonstrate that directed evolution using a yeast

display system (11) can be used to probe protein allostery with great efficiency. Furthermore, we have constructed an allosteric mutant with a 200,000-fold increase in affinity.

Results

Expression and Affinity Selection of I Domain Libraries. As positive controls, wild-type I domain, and disulfide-bonded, intermediate affinity (IA) and HA mutant I domains were displayed on yeast. Good display on the yeast cell wall was shown by the binding of the anti-hemagglutinin (12CA5) and anti-c-myc (9E10) antibodies and I domain-specific antibodies TS1/22 and MEM83 (Fig. 1D and data not shown). Binding was also measured for ICAM-1-Fc chimera and a mAb selective for the open conformation, AL-57 (Fig. 1D). Binding was undetectable to the wild-type I domain, and ICAM-1 and AL-57 bound $56 \pm 19\%$ and $65 \pm 12\%$, respectively, as well to the IA as to the HA I domain as measured by adjusted specific fluorescence intensity (ASFI).

Next, the yeast display library was tested for the binding of antibodies and ICAM-1. Binding of 12CA5, 9E10, TS1/22, and MEM83 mAbs was reduced significantly after error-prone mutagenesis (Fig. 1D and data not shown). Only 30% of cells in the mutant library bound to the 9E10 mAb, compared with 80% for the wild-type I domain before mutagenesis. The I-domain mutant library was then selected by magnetic cell sorting for binding to AL-57 or ICAM-1. After sorting once with AL-57, the majority of the cells in the enriched library bound to 9E10, MEM83, and AL-57 mAbs and ICAM-1 (Fig. 1D), suggesting that the vast majority of selected clones contained mutations that stabilized the HA conformation of the α_L I domain. When the same mutant library was selected with ICAM-1-Fc, multiple cycles were needed to enrich for positive cells. After three cycles, the percentage of cells that bound to 9E10, MEM83, and AL-57 mAbs and ICAM-1 was comparable (Fig. 1D).

Activating Mutations in the Error-Prone PCR Library. A total of 25 yeast colonies selected with AL-57 and ICAM-1-Fc were tested for binding of ICAM-1-Fc and sequenced (Table 1). Of these, 15 had unique sequences and were designated as m1–m15, and all bound ICAM-1 better than wild type (Table 1). Five clones contained single mutations, and the remainder contained two to five mutations each (Table 1). From the clones containing multiple mutations, one to three residues were selected and independently tested as a single substitution (designated by a, b, or c suffixes in Table 1) or independently isolated from the ratchet residue library (see below).

Some clones containing single mutations (F265S, L274I, and

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Abbreviations: I domain, inserted domain; MIDAS, metal ion-dependent adhesion site; ICAM-1, intercellular adhesion molecule-1; IA, intermediate affinity; HA, high affinity; SFI, specific fluorescence intensity; ASFI, adjusted SFI; LFA-1, lymphocyte function-associated antigen 1; PMA, phorbol ester 12-tetradecanoylphorbol-13 acetate; MFI, mean fluorescence intensity.

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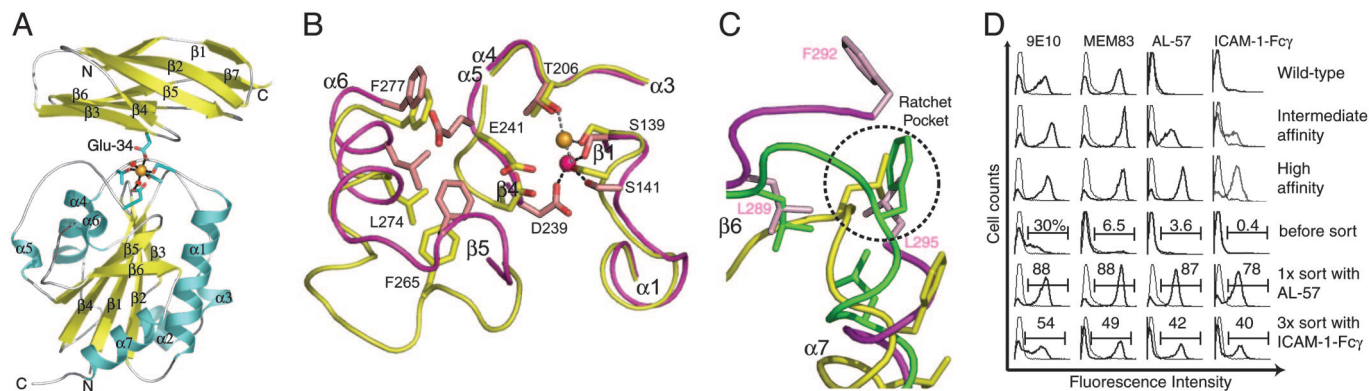


Fig. 1. α_L I domain structure and detection by flow cytometry. (A) Ribbon diagram of the α_L I domain in an open conformation in complex with ICAM-1 domain. The β -strands (yellow), α -helices (cyan), and N and C termini are labeled. I-domain MIDAS and ICAM-1 Glu-34 side chains are shown as stick models with oxygen atoms in red. The MIDAS metal ion and the oxygen atoms of two water molecules are shown as gold and red spheres, respectively. All structure diagrams are drawn in PYMOL (Delano Scientific, San Carlos, CA). (B) Top view of the α_L I domain. The $C\alpha$ traces, key side chains as sticks, and MIDAS metal ions as large spheres are in magenta (closed) and yellow (open); oxygens are in red. Primary coordinations are shown as dashed lines. (C) The $C\alpha$ trace and ratchet side chains (Leu-289, Phe-292, Leu-295 shown in stick models) for the closed (magenta), intermediate (green), and open (yellow) conformations of the α_7 -helix are depicted. The structure of the α_7 -helix of the closed α_L I domain was modeled based on the crystal structure of Protein Data Bank ID codes 1LFA and 1ZON; the structure of the α_7 -helix of the open α_L I domain (Protein Data Bank ID code 1MQ9) was modeled as described (7). (D) Detection by immunofluorescence before (thin lines) and after (thick lines) induction of anti-c-myc antibody 9E10, I domain-specific antibody MEM83, I domain activation-dependent antibody AL-57, and ICAM-1-Fc γ . The percentages of the cells within the gated regions are shown.

Table 1. ICAM-1 binding to I domains

Clones	Mutations	Expression, %SFI of HA	ICAM-1, %ASFI of HA
WT	Wild type	102	0
m1	I258T	197	7
m2	I258T, T267A, F277S	76	10
m3	G262E	110	4
m4	F134L, K252E, F265S	24	31
m5	L274I, S279T	138	51
m5a	L274I	81	91
m6	F209Y, L289P	133	33
m7	F292S	146	47
m8	F292S, K280E	131	54
m9	F292S, K178R, L204Q	155	28
m10	F265L, E293G, K294E, K296E, V308I	86	16
m10a	F265L	58	1
m10b	E293G	97	4
m11	L295P, Q303R	77	107
m11a	Q303R	80	7
m12	I150T, G246V, F299L, I309T	113	27
m12a	I150T	80	0
m12b	F299L	89	0
m12c	I309T	91	17
m13	F265S	57	152
m14	I288N	132	14
m15	F277L, I288T	143	30
m15a	F277L	94	135
f1	L289A	131	1
f2	L289G	136	59
f3	L289P	82	24
f4	L289W	93	51
f5	F292A	88	29
f6	F292G	126	63
f7	F292P	58	5
f8	L295A	222	25
f9	L295P	86	6
f10	L295Q	129	4
f11	L295W	99	65

Clones isolated from the error-prone PCR library are designated as m1 to m15. Clones identified by focused mutagenesis are designated as f1 to f11. Clones tested as single substitutions are designated by a, b, and c after the names of the parental clones. Expression level was quantified from anti-c-myc mAb 9E10 binding.

F277L) showed higher binding of ICAM-1 than clones containing these and additional mutations. In contrast, some single mutants that were made showed lower binding to ICAM-1 than their parental multiply mutant clones, suggesting either that the wrong residues were selected for single substitutions or the activating effect of the mutations were interdependent (e.g., L295P and Q303R in m11). The significance of the location of these mutations for understanding I domain allostery is highlighted in *Discussion*.

Activating Mutations in the Ratchet-Residue Library. Leu-289, Phe-292, and Leu-295 in the β_6 - α_7 loop of the I domain alternatively occupy the same hydrophobic pocket in the closed, intermediate, and open conformation (5) and are termed “ratchet” residues (Fig. 1C). Mutations in each of these ratchet residues were selected from the error-prone PCR library that increased affinity for ICAM-1 (m6, m7, m8, m9, and m11 in Table 1). To obtain higher saturation of mutagenesis of ratchet residues, oligonucleotides randomized in the first two bases of each ratchet residue were used to construct a focused library, and 11 unique clones designated as f1–f11 were selected with AL-57 (Table 1). The particular residues that were selected suggest that the small amino acids Gly and Ala, Pro, and the large amino acid Trp are particularly activating at the ratchet positions (Table 1).

Kinetics of Binding of Soluble I Domains to ICAM-1. Several of the most activating mutants were expressed in *Escherichia coli* and refolded to measure the kinetics of binding to ICAM-1 by surface plasmon resonance (Fig. 2). All of the single mutants, F265S, F292A, and F292G, exhibited an association rate to ICAM-1 in a similar range from 9,500 to 16,000 $M^{-1}s^{-1}$ (Table 2). The double-mutant F265S/F292G showed a 2-fold higher k_{on} of 25,000 $M^{-1}s^{-1}$. These association rates are lower than observed for the IA and HA mutants of 105,000–133,000 $M^{-1}s^{-1}$. In contrast to the association rates, the mutants isolated in this study differ very markedly from one another in their dissociation rates. The F265S and F292G mutants showed a 100-fold slower dissociation rate than the F292A mutant. The double-mutant F265S/F292G exhibited a further 10-fold decrease in dissociation rate compared with the F265S and F292G single mutants. The dissociation rate for the double mutant was slower than the HA I domain by close to 100-fold.

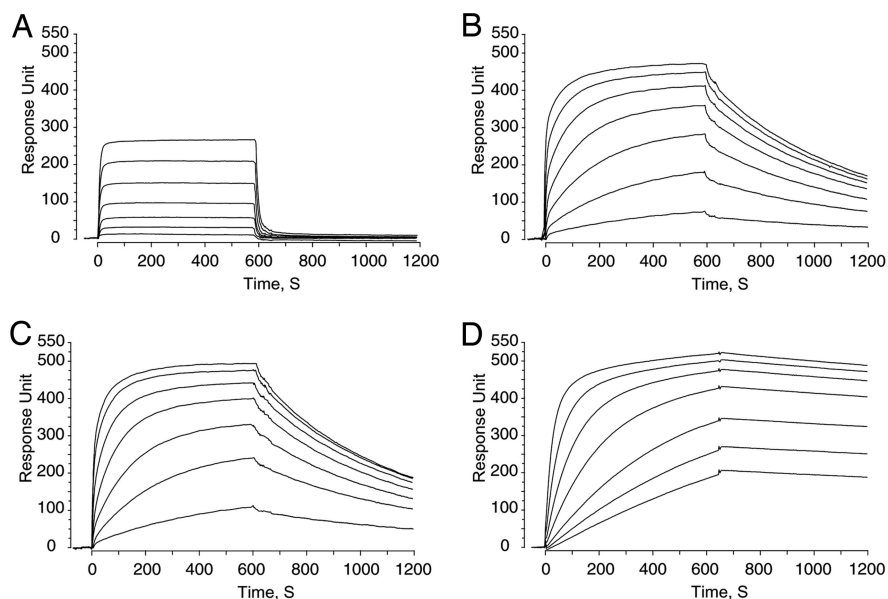


Fig. 2. Binding of α_L I-domain mutants to ICAM-1-Fc γ -coated sensor chip measured with surface plasmon resonance (Biacore). In all sensograms, the signals from the control surface, as described in *Materials and Methods*, were subtracted. The I-domain mutants were injected in a series of 2-fold dilutions starting from 100 μ M for F292A (A) and 1 μ M for F265S (B), F292G (C), and F265S/F292G (D).

The K_D of the single mutants F265S and F292G of ≈ 100 nM is similar to that of the HA I domain. Remarkably, the K_D of the double-mutant F265S/F292G is 6 nM, which corresponds to an increase of 200,000-fold in affinity compared with the wild-type I domain. All of the I-domain mutants were monomeric in solution as assessed by size-exclusion chromatography (data not shown), and therefore the slower dissociation rate was not affected by avidity modulation.

Soluble I Domains as Inhibitors of Adhesion, Homotypic Aggregation, and Transmigration. The potential to antagonize lymphocyte function-associated antigen 1 (LFA-1)-dependent lymphocyte adhesion to ICAM-1 with soluble I domains was examined *in vitro*. Consistent with the differences in affinity measured by surface plasmon resonance, the F265S/F292G mutant ($IC_{50} = 0.35$ μ M) was much more potent than the disulfide-bonded HA mutant ($IC_{50} = 5.3$ μ M) in blocking the adhesion of phorbol ester 12-tetradecanoylphorbol-13 acetate (PMA)-activated lymphocytes to ICAM-1-coated surfaces (Fig. 3A). The wild-type I domain gave no inhibition in the same assay.

Homotypic aggregation of lymphocytes stimulated by PMA occurs through the interaction between LFA-1 and ICAMs (12). When the soluble I domains were tested as inhibitors for blocking the aggregation of JY lymphoblastoid cells, the wild-type I domain was slightly effective only at the highest concentration (Fig. 3B).

Table 2. Kinetics of the binding of the soluble I domains to ICAM-1 measured by Biacore

I domain	k_{on} , $M^{-1}s^{-1} \times 10^{-3}$	k_{off} , s^{-1}	K_D , μM
Wild type*	3.1 ± 0.1	4.6 ± 0.36	$1,500 \pm 200$
IA*	133 ± 10	0.43 ± 0.07	3 ± 0.44
HA*	115 ± 7	0.014 ± 0.001	0.15 ± 0.016
HA	105 ± 49	0.011 ± 0.0003	0.105 ± 0.05
F265S	11.8 ± 0.9	0.0017 ± 0.0003	0.145 ± 0.031
F292A	9.5 ± 1.2	0.19 ± 0.005	20.0 ± 0.8
F292G	16.0 ± 0.8	0.0019 ± 0.0004	0.119 ± 0.017
F265S/F292G	25.0 ± 0.76	0.00015 ± 0.000003	0.006 ± 0.0002

The values shown are mean \pm SD from three independent measurements. The parameters of k_{on} , k_{off} , and K_D were calculated by fitting the 1:1 Langmuir binding model to the Biacore sensogram.

*The values were from ref. 5.

The HA I-domain mutant at as low as 5 μ M completely inhibited the aggregation. In contrast, the F265S/F292G mutant at 1 μ M completely blocked aggregation, and at 0.5 μ M <10% of cells were in aggregates. Even at 0.01 μ M, the F265S/F292G was effective in inhibiting the formation of large and compact clusters, which were already observed at 0.1 μ M of the HA I domain.

Migration of leukocytes across vascular endothelium is required for emigration from the bloodstream. The interaction between LFA-1 on leukocytes and ICAM-1 and ICAM-2 on endothelium has been identified as important for this process (13). When the I domains were tested at 1 μ M, the F265S/F292G mutant was found to be much more efficient than the HA mutant I domain at blocking transmigration (Fig. 3C). The amount of inhibition by the F265S/F292G mutant at 1 μ M was comparable to that of the anti-ICAM-1 antibody RR1/1 and the anti-LFA-1 I domain antibody MHM24, which were used at 0.33 μ M and are bivalent.

Discussion

In this study, we tested whether directed evolution could be used (i) to engineer HA in a protein in which affinity is allosterically regulated and (ii) to structurally probe allosteric pathways within a protein. Our results show that directed evolution is an efficient method for identifying key residues in the transmission of allostery within the I domain. From 25 clones that were isolated with AL-57 and ICAM-1, all previously speculated or tested hot spots based on expert inspection were found [Phe-265 (2), Leu-289 (4), Phe-292 (2, 4), and Leu-295 (4)], together with many new positions (Ile-258, Gly-262, Leu-274, Phe-277, Ile-288, Glu-293, and Ile-309). Furthermore, using focused mutagenesis of the ratchet positions, we found that the identity of the substituting amino acid was markedly more important than previously suspected. None of the activating mutations were found in the ICAM-1-binding interface, supporting our hypothesis that they are allosteric mutations that shift the conformational equilibrium toward the open, HA conformation.

The mutations found here identify residues that are key in the shape-shifting pathway that connects the ligand-binding site to the α 7-helix that communicates allostery to neighboring domains in intact integrins. In the transition of the MIDAS from closed to open conformation, the MIDAS metal moves ≈ 2 \AA toward Thr-206 and away from Asp-239 (Fig. 1B). The α 1-helix, particularly near its N-terminal end, and the β 1- α 1 loop bearing Ser-139 and Ser-141, move inward along with the Mg^{2+} ion. Furthermore, there is a

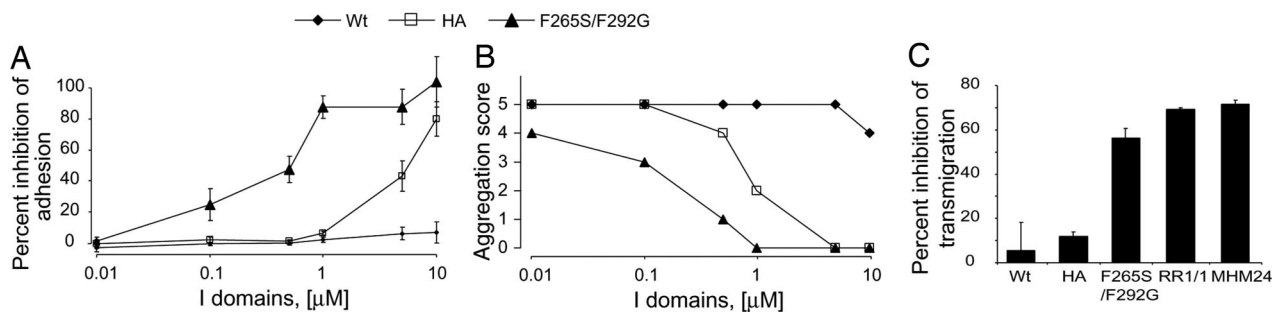


Fig. 3. Soluble I domains as inhibitors of LFA-1-ICAM-1 interaction. (A) Inhibition of PMA-stimulated lymphocyte adhesion to ICAM-1-coated surface. Data are mean \pm SEM from two experiments, each in triplicate. (B) Inhibition of PMA-stimulated JY lymphoblastoid homotypic aggregation. Scoring scheme for the aggregation assay is as follows (30): 0, no cells are in clusters; 1+, <10% of cells are in aggregates; 2+, <50% of cells are in aggregates; 3+, up to 100% of cells are in small and loose clusters; 4+, up to 100% of cells are aggregated in larger clusters; and 5+, up to 100% of cells are in large, very compact clusters. Data are from two independent experiments, which showed identical results. (C) Lymphocyte migration through endothelial monolayer. I domains were used at 1 μ M, and anti-ICAM-1 antibody RR1/1 and anti-LFA-1 antibody MHM24 were at 0.33 μ M. Data are mean \pm SEM from three experiments.

backbone flip at Gly-240 of the β 4- α 5 loop, enabling its Asp-239 side chain to alter metal ion coordination and its Glu-241 side chain to form a crucial salt bridge to ICAM-1 (Fig. 1B). These changes all involve MIDAS loops and are directly involved in ligand binding; we will refer to the region containing them as the “active site” region (Fig. 4A). Remarkably, none of the allosteric activating mutations we have selected map to the active site; instead they define another region that we term the region of “switch allostery.” The switch-allostery region corresponds to a single segment of primary structure containing secondary structure elements β 5, α 6, β 6, and α 7 (Fig. 4A).

Our findings provide insights into how the switch-allostery and active-site regions are coupled. The switch-allostery region undergoes a shearing motion with respect to the remainder of the I domain, including the active-site region (Fig. 4B). Previously, the importance of movements of key residues and isolated segments of the switch-allostery regions have been discussed (8, 14), but not their shearing motion as a whole. In allostery, the twist of the central β -sheet increases, with a significant increase in twist between β -strands 4 and 5, and a marked increase between β -strands 5 and 6 (Fig. 4C and D). This twisting/shearing motion normal to the

plane of the central β -sheets is accompanied by movements across the entire interface, as shown in the *en face* view in Fig. 4C and D. Indeed, the allosteric mutations we have identified are distributed from one side of this interface to the other, demonstrating that residues throughout this extensive interface participate in balancing the energy between the open and closed conformational states.

In the closed conformation, the residues identified by allosteric mutations all are structurally contiguous and linked by hydrophobic packing, backbone hydrogen bonds, and peptide bonds (Fig. 4C). The hydrophobic side chains of Phe-277 and Leu-274 in the α 6-helix and Ile-258 in the β 5-strand pack against one another on one side of the switch-allostery-active-site interface. This hydrophobic interface extends by contacts with Leu-274 to the side chains of Phe-265 and Ile-288 (Fig. 4C). Furthermore, backbone hydrogen bonds connect Phe-265 to Gly-262, and in turn Gly-262 to Leu-289 (Fig. 4C). Leu-289 is also covalently linked to Ile-288 in the above hydrophobic cluster. In the closed conformation, Leu-289 packs closely with ratchet-residues Phe-292 and Leu-295, and Leu-295 is in the hydrophobic ratchet pocket, which is formed in part by Phe-292 and Leu-289. These residues outline one rim of the interface

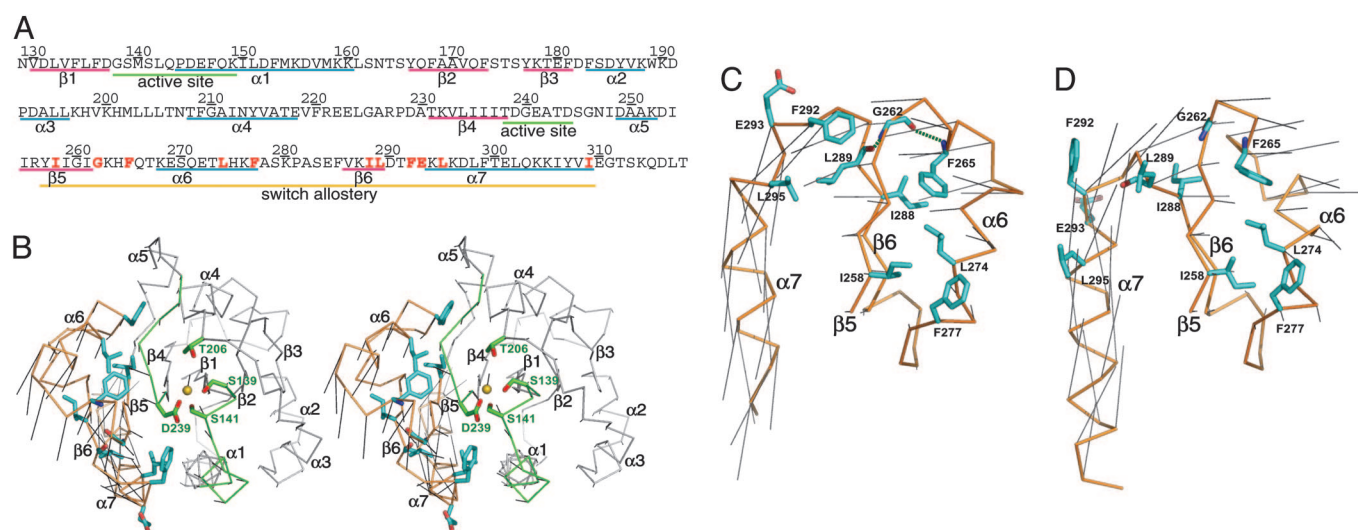


Fig. 4. Active-site and switch-allostery regions and hot spots for activation in α I domain. (A) Wild-type I-domain sequence is shown with the secondary-structure assignment. Hot-spot residues with activating mutations are in red. Active-site and switch-allostery regions are underlined in green and orange, respectively. (B) Stereoview of the I domain in the closed conformation. $C\alpha$ trace is green for the active site, yellow for switch allostery, and gray for the rest of the I domain. MIDAS side chains are green, and hot-spot side chains are cyan. (C and D) *En face* view of the switch-allostery region $C\alpha$ trace and the hot-spot side chains in the closed (C) and open (D) structures. Black lines in B–D are drawn by connecting the same $C\alpha$ atoms in the closed and open structures.

Immunofluorescence Flow Cytometry. Induced cells were harvested, washed with 100 μ l of labeling buffer (PBS/0.5% BSA/10 mM MgCl₂), and then incubated with ligands at 10 μ g/ml in 50 μ l of the labeling buffer for 20 min with shaking at 30°C. Ligands used in this study were anti-c-myc antibody 9E10 (Santa Cruz Biotechnology) and anti-hemagglutinin antibody 12CA5 (Roche Applied Science), α _L I domain-specific antibodies TS1/22 and MEM83 (18), and ICAM-1-Fc γ (R & D Systems). AL-57, which was engineered from a phage display system (unpublished data), was used as an activation-dependent I domain-specific antibody. After incubation, cells were washed in 100 μ l of the labeling buffer and incubated with secondary antibodies at 5 μ g/ml in 50 μ l of the labeling buffer for 20 min at 4°C. Finally, cells were washed once in 100 μ l and suspended in 100 μ l of the labeling buffer for flow cytometry (FACScan, BD Biosciences).

To calculate SFI, the mean fluorescence intensity (MFI) of uninduced clones was subtracted from the MFI of induced clones. Because the SFI of AL-57 and ICAM-1 is affected both by the affinity to the I domains and the difference in the level of I domain expression, the SFI of AL-57 and ICAM-1 was corrected by dividing them by the SFI of 9E10 mAb to c-myc tag, and these values were defined as ASFI. For example, the ASFI of ICAM-1 is computed as: [MFI (ICAM-1) of induced - MFI (ICAM-1) of uninduced]/[MFI (9E10) of induced-MFI (9E10) of uninduced]. Then the ASFI of AL-57 and ICAM-1 of clones were compared with that of the clone expressing the HA I-domain mutant, e.g., the percentage HA-ASFI of clone m1 = 100 \times ASFI (clone m1)/ASFI (HA).

Magnetic Cell Sorting. Sorting of the I domain library was performed with a magnetic cell sorter (MACS LS Column, Miltenyi Biotec). The I-domain library ($\approx 10^8$ cells) induced in 1 ml of selective galactose media was spun down, washed with 1 ml of the labeling buffer, and incubated with 5 μ g/ml of AL-57 or ICAM-1-Fc γ in 200 μ l of the labeling buffer for 20 min with shaking at 30°C. After incubation with primary ligands, cells were washed once with 1 ml of the labeling buffer, incubated in 80 μ l of the labeling buffer and 20 μ l of mouse anti-human IgG microbeads (Miltenyi Biotec) for 20 min at 4°C, and sorted.

Expression of Soluble I Domains and Surface Plasmon Resonance Measurements. Mutant I domains (F265S, F292A, F292G, F265S/F292G, and HA) with residues of Asn-129 to Tyr-307 were expressed in *E. coli* BL21 (DE3) (Novagen), refolded, and purified as described (5). ICAM-1-Fc γ -coupled or mock-coupled CM5 sensor chip as control was prepared with the amine coupling kit (Biacore) as described (4). Surface plasmon resonance was measured by using a Biacore 3000 optical biosensor.

I domains were injected over the chip in 20 mM Tris-HCl (pH 8.0), 150 mM NaCl, and 10 mM MgCl₂, at a flow rate of 10 μ l/min at room temperature.

Cell Adhesion Assays. V-bottom 96-well plates (Corning) were coated with ICAM-1-Fc γ (10 μ g/ml in PBS, pH 7.4) or 2% BSA as a control at 4°C overnight, and then blocked with 2% BSA for 1 h at 37°C. I domains were added in 50 μ l of L15 medium, 2.5% FBS. Lymphocytes were isolated and cultured as described (13). PMA at 100 ng/ml and 2',7'-bis-(2-carboxyethyl)-5-(and-6)- carboxyfluorescein acetoxyethyl ester at 2 μ g/ml were added to 10⁶ cells/ml in L15 and 2.5% FBS. After 30 min at 37°C, cells were washed and added to the wells in 50 μ l of L15 and 2.5% FBS. The plates were immediately centrifuged at 200 \times g for 15 min at room temperature (19). Nonadherent cells that accumulated at the center of the V-bottom were quantified by a fluorescence plate reader (Spectra MAX Gemini XS, Molecular Devices). Percent inhibition was calculated from the fluorescence intensity of experimental measurements (*F*), the positive control coated with ICAM-1-Fc γ without added I domains (*F*_{ICAM-1}), and the negative control coated with BSA alone (*F*_{BSA}): 100 \times (*F* - *F*_{ICAM-1})/(*F*_{BSA} - *F*_{ICAM-1}).

Homotypic Aggregation. The effect of the soluble I domains on blocking homotypic aggregation of JY PMA-stimulated B lymphoblastoid cells was evaluated by a qualitative aggregation assay (score 0–5) after 1-h incubation as described (12).

Transmigration Assay. Primary human dermal microvascular endothelial cells (HDMVECs) in 24-well plates were activated for 12 h with TNF- α (100 ng/ml), washed three times in buffer A (Hanks' balanced salt solution supplemented with 20 mM Hepes, pH 7.2 and 1% human serum albumin) and preincubated for 5 min at 37°C in buffer A alone (control) or buffer A containing the wild-type, HA, and F265S/F292I domains (1 μ M) or RR1/1 (20) or MHM24 (21) mAbs (0.33 μ M) (13). IL-2-cultured primary human lymphocytes (10⁵ cells/ml) in 500 μ l of buffer A containing I domains or mAbs were added to HDMVECs and incubated at 37°C for 10 min. For each condition complete Z-stacks were obtained in each of 10 randomly selected fields by using the Bio-Rad Radiance 2000 laser-scanning confocal microscope system and then analyzed to determine the number of cells in the process of, or having completed, diapedesis (13). Percent inhibition of transmigration was calculated as: 100 \times (1 - fraction of TEM in the presence of I domains or mAbs/fraction of TEM in control).

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