Small Molecule Inhibitors Induce Conformational Changes in the I Domain and the I-like Domain of Lymphocyte Function-associated Antigen-1

Molecular Insights into Integrin Inhibition*

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The β₂ integrin lymphocyte function-associated antigen-1 (LFA-1) is a conformationally flexible αβ heterodimeric receptor, which is expressed on the surface of all leukocytes. LFA-1 mediates cell adhesion crucial for normal immune and inflammatory responses. Intracellular signals or cations are required to convert LFA-1 from a nonligand binding to a ligand binding state. Here we investigated the effect of small molecule inhibitors on LFA-1 by monitoring the binding of monoclonal antibodies mapped to different receptor domains. The inhibitors were found to not only induce epitope changes in the I domain of the αL chain but also in the I-like domain of the β₂ chain depending on the individual chemical structure of the inhibitor and its binding site. For the first time, we provide strong evidence that the I-like domain represents a target for allosteric LFA-1 inhibition similar to the well established regulatory L-site on the I domain of LFA-1. Moreover, the antibody binding patterns observed in the presence of the various inhibitors establish a conformational interaction between the LFA-1 I domain and the I-like domain in the native receptor that is formed upon activation. Differentially targeting the binding sites of the inhibitors, the L-site and the I-like domain, may open new avenues for highly specific therapeutic intervention in diseases where integrins play a pathophysiological role.

Integrins are a family of αβ heterodimeric cell surface receptors that mediate cell-cell and cell-extracellular matrix interactions. They are involved in various pathophysiological processes leading to acute and chronic disease states (1). Lymphocyte function-associated antigen-1 (LFA-1, αLβ₂, CD11a/CD18) belongs to the β₂ integrin subfamily and is constitutively expressed on all leukocytes. LFA-1 is involved in leukocyte extravasation into tissue and T cell costimulation (2, 3). Several studies underscore the importance of LFA-1 in the activation process. It has been recently confirmed by the crystal structure of the extracellular segment of the integrin αLβ₂ (12). The I-like domain of the LFA-1 β₂ subunit is predicted to contact the putative β-propeller region of the αL subunit near β sheets 2 and 3 (13). A recent study suggests that the I-like domain has a regulatory role rather than a direct role in ligand binding (14).

As described for other integrins, the ligand binding activity of LFA-1 is tightly regulated (1, 15). LFA-1 is expressed on leukocytes in an inactive state and can be rapidly activated by cations or by intracellular signals (2, 15). Receptor clustering and conformational changes are proposed to be the major mechanisms by which LFA-1 is converted from a low to a high affinity form (15). Several studies provide strong evidence that in particular the C-terminal helix of the I domain plays an important role in the activation process. It has been recently demonstrated that LFA-1 can be locked in an open ligand binding and a closed nonbinding conformation by mutational introduction of disulfide bonds between the C-terminal helix and a central β sheet of the I domain (16). The positions of the disulfide bonds were modeled according to the crystal structures of Mac-1 (αMβ₂, CD11b/CD18) and VLA-2 (αLβ₁, CD49b/CD29) I domains. These I domains have been crystallized in two conformations, which differed by a major shift in the positioning of the C-terminal helix (17, 18). Moreover, transfectants expressing mutant LFA-1 with alanine or tryptophan substitutions in the C-terminal helix region of the I domain show impaired or constitutively active binding to ICAM-1 (19, 20). The importance of this region in controlling LFA-1 activity is further underlined by the fact that small molecule LFA-1 inhibitors bind to a hydrophobic pocket between the C-terminal helix and the central β-sheet of the LFA-1 I domain, termed the
The ELISA-type LFA-1/ICAM-1 binding assay (LFA-1/ICAM-1) measures the binding of purified immobilized LFA-1 to recombinant biotinylated ICAM-1. The adhesion assays (HUT78/ICAM-1, -2, -3) quantify the adhesion of the human T cell lymphoma cell line HUT78 to immobilized ICAM-1, -2, and -3. Binding of the compounds to the LFA-1 I domain was quantified by NMR. The ELISA-type Mac-1/ICAM-1 assay (Mac-1/ICAM-1) measures the binding of purified immobilized Mac-1 to recombinant biotinylated ICAM-1. Values represent independent experiments or the mean ± S.D. In parentheses is shown the number of independent experiments performed; uniformly $^{15}$N-labelled I-domain was titrated with increasing amounts of compound dissolved in Me$_2$SO. The change in the $^{15}$N resonance frequency for the peak corresponding to Thr$^{291}$ was followed and analyzed. + and − correspond to a change of $>50$ Hz and $<5$ Hz at a concentration for the I domain and compound of 100 μM, respectively.

### Table I

| Assay                        | Lovastatin | Pravastatin | LFA703 | LFA451 | XVA143 (Genentech) |
|------------------------------|------------|-------------|--------|--------|-------------------|
| **LFA-1/ICAM-1**<sup>IC50 [μM]</sup> | 2.1 ± 0.8 (n=8) | >100 (n=5) | 0.2 ± 0.1 (n=5) | 0.04 ± 0.010 (n=6) | 0.020 ± 0.008 (n=5) |
| **HUT78/ICAM-1**<sup>IC50 [μM]</sup> | 25.4 ±13.3 (n=5) | >100 (n=3) | 0.7 ± 0.5 (n=7) | 0.40 ± 0.07 (n=7) | 0.005 ± 0.004 (n=4) |
| **HUT78/ICAM-2**<sup>IC50 [μM]</sup> | 36.2 ± 22.7 (n=4) | >100 (n=2) | 1.2; 1.7 | 0.6 ± 0.251 (n=3) | 0.007, 0.007 |
| **HUT78/ICAM-3**<sup>IC50 [μM]</sup> | 16, 3.5 | >100 (n=2) | 0.4, 0.1 | < 0.1, 0.06 | 0.002, 0.001 |
| I domain binding             | +          | −           | +       | +      | −                 |
| **Mac-1/ICAM-1**<sup>IC50 [μM]</sup> | >100 (n=3) | >100 (n=3) | >100 (n=3) | >100 (n=3) | 0.002 ± 0.001 (n=3) |

lovasatin binding site (L-site) (20, 21). This pocket has been also termed the I domain allosteric site (IDAS) by others (19). The first compound described to interact with the L-site was the HMG-CoA reductase inhibitor lovastatin (20). Only recently it became evident that compounds of diverse chemical classes, including hydantoin and cinnamide derivatives, utilize the same pocket for inhibition of LFA-1 (22, 23). Since the L-site is distant from the MIDAS, it is thought that these compounds inhibit LFA-1 via an allosteric mechanism (20–23). However, molecular details of this inhibition in the context of the whole receptor are unknown. Similarly, the molecular details of the natural activation process are not well understood. Addressing both molecular mechanisms, LFA-1 activation and inhibition, is the objective of this study.

Here we investigate the effect of small molecule LFA-1 inhibitors on the putative β-propeller region, and the β2 I-like domain using monoclonal antibodies (mAbs) mapped to these different regions of the receptor. We show that inhibitors induce epitope changes in the I-domain, the putative β-propeller region, and both domains depending on their chemical structure and binding site. For the first time we establish the I-like domain on the β2 subunit as a molecular target for LFA-1 inhibition. Moreover, utilizing the native LFA-1 receptor, as compared with mutated LFA-1 studied previously (14), we provide strong evidence for a functional relationship between the I domain and the I-like domain, which is induced upon activation.

**EXPERIMENTAL PROCEDURES**

Reagents and Antibodies—LFA-1 was purified from JY cells as described earlier (20). Mac-1 was purified from peripheral blood leukocytes by as described by Diamond et al. (24). ICAM-1 mouse Cx fusion protein (ICAM-1-mCx) was produced and biotinylated as described (21). ICAM-1-human Fc, ICAM-2 human-Fc, and ICAM-3 human-Fc fragment fusion proteins were purchased from R & D Systems (Oxon, UK). 3′-O-Acetyl-2′,7′-bis(carboxyethyl)-4 or 5-carboxyfluorescein diacetoxymethyl ester was purchased from Molecular Probes (Leiden, The Netherlands). Streptavidin-peroxidase conjugate (SPOD) was purchased from Roche Molecular Biochemicals GmbH. 2,2-Azino-bis(3-ethylbenz-thiazoline-6-sulfonic acid) was obtained from Bio-Rad. All cell culture reagents were purchased from Life Technologies. Hybridoma cells producing mAbs TS2/4.1.1 (anti-CD11a) and TS1/22.1.3 (anti-CD11a) and mAbs 44aacb (anti-CD11b) and LM2/1 (anti-CD11b) were obtained from the American Type Culture Collection (ATCC). Antibodies were purified from culture supernatants using protein A-Sepharose (Amersham Biosciences) separation. Amino-directed biotinylation of these antibodies was performed using N-hydroxysuccinimide-biotin following the manufacturer’s instructions. The biotinylated and the nonbiotinylated anti-human LFA-1 mAb RT.1 (anti-CD11a) and R3.3 (anti-CD18) were purchased from BIOSOURCE (Camarillo, CA). The biotinylated and the nonbiotinylated mAb TS1/18 (anti-CD18) were from Endogen (Woburn, MA); the mAb IB4 (anti-CD18) was from Ancell Corp. (Bayport, MN); YFC118.3 (anti-CD18) was from Serotech. CLB-LFA-1/1 (anti-CD18) was from Caltag Laboratories (Burlingame, CA). Antibody 25.3.1 (anti-CD11a) was from Immunotech (Marseille, France). The biotinylated and the nonbiotinylated mAb clone 38 (anti-CD11a) and isotype controls for mouse IgG1, IgG2a, IgG2b, and rat IgG1 as well as goat anti-mouse IgG C× conjugate and Alexa Fluor®488 goat anti-mouse IgG (H + L) conjugate and Alexa Fluor®488 goat anti-rat IgG (H + L) conjugate were purchased from Molecular Probes. Goat anti-human IgG-Fc fragment was purchased from KPL (Gaithersburg, MD). All other assay reagents were bought from Sigma.

Cell Culture—Jurkat E6–1 and HUT78 cells were obtained from the ATCC and grown in RPMI 1640 medium supplemented with 10% fetal calf serum, 1% Glutamax I, and 1% nonessential amino acids.
LFA-1/ICAM-1 ELISA-type Binding Assay—LFA-1 inhibitors were tested in a LFA-1-ICAM-1 ELISA-type binding assay, which was performed as previously described (20). Briefly, 96-well microtiter plates (Nunc Maxisorb) were coated with 2–10 μg/ml purified human LFA-1 and blocked with 1% bovine serum albumin (BSA) in Tris-buffered saline (TBS) for 1 h. After a washing step, compounds dissolved in Me2SO and diluted in PBS containing 2 mM MgCl2 and 0.5% fetal calf serum, pH 7.4 (assay buffer) were added to the plates. After 15 min of incubation, ICAM-1-mC was added in assay buffer (4 μg/ml) and incubated at 37 °C for 1 h. After several washing steps, SPOD diluted 1:5000 in assay buffer was added and incubated at 37 °C for 35 min. After the removal of unbound SPOD by washing, ICAM-1-mC was quantified using 3,3,5,5-tetramethylbenzidine as substrate.

Bioinylated Antibody Binding to Purified LFA-1—The binding of biotinylated anti-CD11a and anti-CD18 mAbs to purified LFA-1 was investigated in the presence of the LFA-1 inhibitors. The compounds were dissolved in MeSO and serially diluted with PBS containing 2 mM MgCl2 and 0.5% fetal calf serum, pH 7.4 (assay buffer). After a 15-min incubation at room temperature, biotinylated anti-LFA-1 mAbs in assay buffer (0.1 μg/ml) were added and incubated at room temperature on a shaker for 30 min. Then the plates were washed, and SPOD was added, diluted 1:5000 in assay buffer. After 35 min at room temperature and a washing step, bound anti-LFA-1 mAbs were quantified using 2,2-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) as substrate. For experiments in the presence of cations, the immobilized LFA-1 was washed three times with 1 mM EDTA in PBS, and PBS containing 0.5% BSA and either 0.1 or 1 mM EDTA, pH 7.4, was used as assay buffer.

Mac-1/ICAM-1 ELISA-type Binding Assay—Purified Mac-1 was immobilized at 2–10 μg onto 96-well microtiter plates (Nunc Maxisorb) and blocked with 5% nonfat dry milk in PBS. The inhibitors were dissolved in MeSO and serially diluted in Tris-buffered saline (TBS) containing 150 mM NaCl, 2 mM MgCl2, 1 mM CaCl2, 2 mM MnCl2, and 0.2% nonfat dry milk, pH 7.4 (assay buffer). The compounds were added to the Mac-1-coated plates. After a 15-min incubation, biotinylated ICAM-1-mC in assay buffer (2 μg/ml) was added and incubated at 37 °C for 1 h. After washing steps with assay buffer, SPOD diluted 1:5000 in assay buffer was added and incubated at 37 °C for 35 min. After washing, bound ICAM-1-mC was quantified using 3,3,5,5-tetramethylbenzidine as substrate.

Bioinylated Antibody Binding to Purified Mac-1—The binding of the anti-CD18 mAb IB4 to purified Mac-1 was tested in the presence of LFA-1 inhibitors dissolved in MeSO and diluted in Tris-buffered saline containing 150 mM NaCl, 2 mM MgCl2, 1 mM CaCl2, 2 mM MnCl2, and 0.2% nonfat dry milk, pH 7.4 (assay buffer). The compounds were added to the microtiter plates coated with Mac-1 and incubated at room temperature for 15 min. Then biotinylated mAb IB4 was added (0.1 μg/ml) in assay buffer. After a 30-min incubation, bound antibodies were detected by the streptavidin-peroxidase reaction as described above.

Cell-based Adhesion Assays—LFA-1-dependent adhesion of HUT78 cells to immobilized ICAM-1-Fc, ICAM-2-Fc, and ICAM-3-Fc fusion proteins was carried out as described earlier for the HUT78/ICAM-1-mC assay (21). Briefly, 96-well microtiter plates were coated with goat anti-human IgG-Fc fragment in PBS, pH 7.8 (5 μg/ml), followed by a blocking step with 1.5% BSA in TBS. After washing with TBS, ICAM-1-Fc, ICAM-2-Fc, or ICAM-3-Fc were added at 0.1–0.3, 0.3, or 1 μg/ml,
respective, in TBS containing 150 mM NaCl, 1.5% BSA, 5 mM glucose, 2 mM MgCl₂, and 2 mM MnCl₂ (assay buffer). 3-O-Acetyl-2,7-bis(carboxyethyl)-4 or 5-carboxyfluorescein diacetoxy-methyl ester-labeled HUT78 cells (1.25 × 10⁵ cells/well) in assay buffer were transferred to the plates and incubated together with the inhibitors at 37 °C for 30 min. The plates were then gently washed with assay buffer, and bound cells were quantified by measuring fluorescence using a VIC-TORE2 microplate reader (Wallac).

**Flow Cytometry**—The effect of the LFA-1 inhibitors on the conformation of cell-expressed LFA-1 was tested by monitoring the binding of anti-LFA-1 mAbs to Jurkat E6–1 cells using flow cytometry. Jurkat E6–1 cells were harvested and washed two times with TBS containing 150 mM NaCl, 2 mM MgCl₂, 2 mM MnCl₂, 0.5% BSA, pH 7.4 (assay buffer). For experiments in the absence of cations, cells were washed three times with TBS containing 150 mM NaCl, 1 mM EDTA, and the assay buffer was replaced by TBS containing 150 mM NaCl, 0.5% BSA, 0.1 mM EDTA, pH 7.4.

3 × 10⁵ cells were preincubated with the LFA-1 inhibitors at a final concentration of 50 μM in assay buffer at room temperature for 20 min followed by the addition of anti-CD11a and anti-CD18 mAbs (1–2 μg/ml). After a 25-min incubation, cells were washed two times with assay buffer and counterstained with Alexa Fluor® 488 goat anti-mouse IgG (H + L) conjugate or Alexa Fluor® 488 goat anti-rat IgG (H + L) conjugate diluted 1:175 in assay buffer for 30 min. After a washing step, antibody binding was immediately analyzed by flow cytometry on a FACSCalibur (Becton Dickinson, San Jose, CA). Control experiments at low temperatures were performed as described above except that the antibody binding steps were carried out on ice.

Mean fluorescence intensities (geometric mean) were calculated using the CellQuest software. Mean fluorescence intensities were corrected for background staining by subtracting the mean fluorescence intensities of the appropriate isotype-matched negative control. Mean fluorescence intensities of the solvent controls were set as 100%. Inhibition of anti-LFA-1 mAb binding induced by inhibitor treatment was expressed as a percentage of these controls.

**NMR Binding Assay**—The NMR binding assay was performed as previously described (21). Briefly, 1H,15N heteronuclear single-quantum coherence spectra were recorded on a 600-MHz Bruker Avance spectrometer (Bruker AG, Karlsruhe) at 23 °C. Uniformly 15N-labeled α1 I domain was titrated with increasing amounts of ligand dissolved in MeSO. The protein concentration was 100 μM, and the maximal ligand concentration varied between 200 and 600 μM depending on the ligand. The change in the 15N resonance frequency for the cross-peak corresponding to the amide group of Thr was followed for a qualitative assessment of the binding affinity. For the assessment of compound binding to α1 I domain, one-dimensional 1H spectra of the isolated Mac-1 I domain were recorded. The α1 I domain (residues 131–321) was first cloned into a PET9a vector using standard molecular biology procedures (25). The protein was expressed as His-tagged fusion protein and purified using nitriotriacetic acid and size exclusion chromatography. The N-terminal His tag was thereafter cleaved from the purified protein using thrombin.

**RESULTS**

**Compounds of Two Different Chemical Classes Inhibit LFA-1 Function**—Table I summarizes the properties of small molecule LFA-1 inhibitors of two different chemical classes. Lovastatin and the optimized statin-derived LFA-1 inhibitors LFA703 and LFA451 blocked ICAM-1 binding to purified immobilized LFA-1 with IC₅₀ values of 2.1, 0.2, and 0.04 μM, respectively (Table I). The inhibitors not only interfered with the LFA-I/ICAM-1 but also with the LFA/I-ICAM-2 and LFA-I/ICAM-3 interaction as shown in T cell adhesion assays (Table I). ICAM-3-mediated adhesion was more potently inhibited by the compounds than ICAM-1- and ICAM-2-mediated adhesion (Table I). This result is in agreement with previous studies showing that LFA-1 binds to ICAM-1 and ICAM-2 stronger than to ICAM-3 (26). The IC₅₀ values determined for the LFA-I inhibitors in the HUT78/ICAM-1 adhesion assay were found to be similar to IC₅₀ values determined in an ICAM-1 adhesion assay utilizing the human lymphoma cell line Jurkat (data not shown). Jurkat/ICAM-2 or Jurkat/ICAM-3 adhesion assays were not performed. As shown by NMR spectroscopy, the inhibitors were able to interact with the L-site of the α1 I domain.

| Table II | Effect of LFA-1 inhibitors on the binding of different mAbs to LFA-1 expressed on Jurkat cells |
|----------------|---------------------------------------------------------------|
| mAb | IC₅₀ (μM) | % inhibition | p-value |
| Clone 38 | 23 | 102 | n.s. |
| 2.3.1 | 25.5 | 98 | n.s. |
| R3.3 | 27 | 97 | n.s. |
| 384 | 30 | 95 | n.s. |
| 14 | 31 | 94 | n.s. |
| 5 | 32 | 93 | n.s. |
| 19 | 33 | 92 | n.s. |
| 14 | 34 | 91 | n.s. |
| 13 | 35 | 90 | n.s. |
| 12 | 36 | 89 | n.s. |
| 11 | 37 | 88 | n.s. |
| 10 | 38 | 87 | n.s. |
| 9 | 39 | 86 | n.s. |
| 8 | 40 | 85 | n.s. |
| 7 | 41 | 84 | n.s. |
| 6 | 42 | 83 | n.s. |
| 5 | 43 | 82 | n.s. |
| 4 | 44 | 81 | n.s. |
| 3 | 45 | 80 | n.s. |
| 2 | 46 | 79 | n.s. |
| 1 | 47 | 78 | n.s. |
| 0 | 48 | 77 | n.s. |

**Effect of Small Molecule Inhibitors on the Integrin LFA-1**

Effect of LFA-1 inhibitors on the binding of different mAbs to LFA-1 expressed on Jurkat cells

| Inhibitor and IC₅₀ | mAb | IC₅₀ (μM) | % inhibition | p-value |
|-------------------|-----|-----------|--------------|---------|
| LFA703 (50 μM)    | Clone 38 | 23 | 102 | n.s. |
|                   | 2.3.1   | 25.5 | 98 | n.s. |
|                   | R3.3    | 27 | 97 | n.s. |
|                   | 384     | 30 | 95 | n.s. |
|                   | 14      | 31 | 94 | n.s. |
|                   | 5       | 32 | 93 | n.s. |
|                   | 19      | 33 | 92 | n.s. |
|                   | 14      | 34 | 91 | n.s. |
|                   | 13      | 35 | 90 | n.s. |
|                   | 12      | 36 | 89 | n.s. |
|                   | 11      | 37 | 88 | n.s. |
|                   | 10      | 38 | 87 | n.s. |
|                   | 9       | 39 | 86 | n.s. |
|                   | 8       | 40 | 85 | n.s. |
|                   | 7       | 41 | 84 | n.s. |
|                   | 6       | 42 | 83 | n.s. |
|                   | 5       | 43 | 82 | n.s. |
|                   | 4       | 44 | 81 | n.s. |
|                   | 3       | 45 | 80 | n.s. |
|                   | 2       | 46 | 79 | n.s. |
|                   | 1       | 47 | 78 | n.s. |
|                   | 0       | 48 | 77 | n.s. |
A close analogue of the statin-derived inhibitors, pravastatin, does not inhibit LFA-1 function and has only marginal affinity for the $\alpha_1$ I domain (Table I). This compound was included in the study as a negative control (Table I).

A unique property of the statin-derived LFA-1 inhibitors is their specificity for LFA-1. The function of other integrins including the $\beta_2$ integrin Mac-1 ($\alpha_{\beta_2}$, CD11b/CD18) was not affected by the compounds (Table I). In contrast, a small molecule LFA-1 inhibitor recently described by others was less specific (32). This inhibitor, which we term here XVA143, is a peptidomimetic with an unknown LFA-1 binding site (32).

The Statin-derived LFA-1 Inhibitors Induce Epitope Changes in the I Domain and $\beta_2$-Like Domain of LFA-1—As previously shown for many residues of the central $\beta$-sheet as well as helices $\alpha_2$ and $\alpha_4$, indicating ligand-induced structural rearrangements in solution (21). The same was observed for the statin-derived inhibitors LFA703 (Fig. 1A) and LFA451 (not shown) at equimolar concentrations of protein and ligand. In contrast, XVA143 influenced the protein spectra to a lesser extent even at high concentrations (Fig. 1B). We note that none of the residues associated with the LFA-1 MIDAS motif was affected by XVA143, ruling out the possibility that inhibition of LFA-1 is mediated by competitive binding of the compound to the MIDAS region implicated in ICAM-1 binding (Fig. 1B). Likewise, protein spectra of the $\alpha_3$ I domain acquired in the presence and absence of XVA143 did not show changes in the chemical shift of resonances affected by magnesium (i.e. residues located in the vicinity of the MIDAS pocket (data not shown)). These results indicate that LFA-1 and Mac-1 inhibition by XVA143 is not mediated by the engagement of the $\alpha$ chain MIDAS pocket. The data further suggest that the molecular target for XVA143-driven inhibition of LFA-1 and Mac-1 is different from the L-site utilized by the statin-derived LFA-1 inhibitors.

Selection of mAbs Specific for Different LFA-1 Domains—To investigate the effect of the inhibitors described above on the different domains of LFA-1, the binding of mAbs to LFA-1 expressed on Jurkat cells was determined in the presence and absence of compounds. The selected mAbs are reported to map to different regions of the $\alpha_1$ chain and the $\beta_2$ chain of LFA-1. mAbs TS1/22, 25.3.1, R7.1, and clone 38 bind to the $\alpha_1$ I domain (8, 10, 28). The epitopes of mAbs R7.1 and mAb clone 38 on the I domain are unknown, whereas mAbs TS1/22 and 25.3.1 map to a region in the close vicinity of the L-site (10) (Fig. 2). mAb TS2/4 maps outside the I domain recognizing the putative $\beta$-propeller of the $\alpha_1$ chain (10). mAbs TS1/18, YFC118.3, IB4, and R3.3 are reactive with the putative I-like domain of the $\beta_2$ chain (11, 29). According to the recognized epitopes, consisting of at least two noncontiguous sequences the anti-I-like domain, mAbs can be divided into two groups; the first group includes TS1/18 (Leu$^{154}$-Glu$^{159}$ and Glu$^{154}$-Asp$^{168}$) and YFC118.3 (Leu$^{154}$-Glu$^{159}$, Glu$^{154}$-Asp$^{168}$, and His$^{168}$-Asn$^{173}$), and the second group includes IB4 and R3.3 (Arg$^{144}$-Lys$^{148}$ and Pro$^{158}$-Glu$^{178}$) (11). All mAbs that map to the $\alpha_1$ I domain or the putative $\beta_2$ I-like domain of LFA-1 are reported to block LFA-1 binding to ICAM-1 (8, 11, 28–30). In contrast, TS2/4 only marginally inhibits LFA-1 function (30).
antibody binding (Table II, Fig. 3A). The epitopes recognized by mAbs TS1/22 and 25.3.1 were only partially or not affected by the compounds (Table II). Interestingly, L-site engagement by lovastatin also influenced the presentation of epitopes displayed on the I-like domain of the β2 chain. This phenomenon became even more pronounced in the presence of the statin-derived LFA-1 inhibitor LFA703 (Table II). The compound potently blocked the binding of mAbs IB4, TS1/18, and YFC118.3, shown to recognize two distinct regions of the I-like domain (11). This finding indicates that LFA703 is able to alter epitopes in the I-like domain of the β2 chain by binding to the L-site of the α4 chain (Table II). In contrast, the effect of the inhibitor LFA451 on the I-like domain was much less evident. Only the epitope recognized by mAb YFC118.3 was marginally affected by the inhibitor (Table II). The fact that LFA451 and LFA703 show different effects on mAb binding to the I-like domain was not entirely unexpected. The compounds differ in their pattern of chemical modifications on the parent lactone ring of lovastatin. While LFA703 is an N-substituted lactam with a modification in position 2 of the lactam ring, LFA451 is modified in position 3 of the corresponding carbamate ring (Table I). These chemical modifications, in conjunction with the accompanying changes in the stereochemistry of the lactam/carbamate ring, were expected to result in a different orientation of the newly introduced groups with respect to the native LFA-1 receptor. This assumption was confirmed by the crystal structure determination of the LFA451 and LFA703 I domain complexes (not shown). Our data suggest that the naphthyl part of LFA703 interacts with pockets of the L-sites that are not reached by the vanillyl group of LFA451. Likewise, the recently described small molecule LFA-1 inhibitor BIRT 377 does not extend into pockets of the L-site used by LFA703, as indicated in the modeled structure published recently (22). Consistent with this observation, BIRT 377 was found to inhibit the binding of mAbs to the I domain but not the I-like domain (28). Taken together, these results provide evidence that conformational changes of the I-like domain are induced by the unique interaction of the naphthyl part of LFA703 with the L-site. In contrast to the I domain and I-like domain, the putative β-propeller region of LFA-1 was not affected by the binding of the statin-derived LFA-1 inhibitors (Table II). As expected, pravastatin did not inhibit antibody binding to LFA-1 (Table II). To exclude the possibility that internalization of antibodies

![Effect of Small Molecule Inhibitors on the Integrin LFA-1](image)

**Fig. 4.** Effect of LFA-1 inhibitors on the binding of mAbs to cation-activated purified LFA-1. Purified LFA-1 was immobilized onto 96-well plates and incubated with biotinylated mAb R7.1 (anti-α4 I domain) (A) or biotinylated mAb IB4 (anti-β2 I-like domain) (B) in the presence and absence of increasing concentrations of indicated LFA-1 inhibitors. The experiment was performed in the presence of 2 mM MgCl2. Bound antibody was detected via streptavidin-peroxidase as described under “Experimental Procedures.” Compound-induced inhibition of mAb binding to LFA-1 is expressed as the percentage of solvent control. Each value represents the mean ± S.D. of triplicates. A representative experiment out of two is shown.

![Effect of LFA-1 inhibitors on the binding of mAb IB4 to cation-activated purified Mac-1](image)

**Fig. 5.** Effect of LFA-1 inhibitors on the binding of mAb IB4 to cation-activated purified Mac-1. Purified Mac-1 was immobilized onto 96-well plates and incubated with biotinylated mAb IB4 (anti-β2 I-like domain) in the presence of different LFA-1 inhibitors (50 μM). Binding of the antibody in the absence of compounds is indicated as + Mac-1, and background binding in the absence of immobilized Mac-1 is indicated as – Mac-1. The experiment was performed in the presence of cations. Bound antibody was quantified via streptavidin-peroxidase. Each bar represents the mean ± S.D. of triplicates. A representative experiment out of two independent experiments is shown.

in the presence of compounds led to the effects described above, all experiments were also performed at 4°C. The results at low temperatures were comparable with those generated at room temperature (data not shown).

**Statin-derived LFA-1 Inhibitors Induce Epitope Changes in the I Domain and I-like Domain of Purified LFA-1**—To further substantiate our findings, we investigated the interaction of mAb R7.1 and mAb IB4 to immobilized purified LFA-1 in the presence of the LFA-1 inhibitors. The analysis was performed with the magnesium-activated form of LFA-1. Consistent with the flow cytometry experiments described above, lovastatin, LFA703, and LFA451 inhibited the binding of the mAb R7.1 in a dose-dependent manner, whereas pravastatin had no effect (Fig. 4A). Interestingly, the IC50 values determined for the compounds in the LFA-1/mAb R7.1 binding assay (Fig. 4A; lovastatin IC50 = 2.9 μM, LFA703 IC50 = 0.14 μM, LFA451 IC50 = 0.03 μM) correlated well with those generated in the cell-free LFA-1/ICAM-1 binding assay (Table I). Moreover, LFA703
blocked the binding of the mAb IB4 to purified LFA-1 in a dose-dependent manner, confirming the effect of LFA703 on the I-like domain of the \( \beta_2 \) chain (Fig. 4B). In contrast, lovastatin and LFA451 did not inhibit the interaction of mAb IB4 with purified LFA-1, corroborating that their binding to the L-site does not affect the I-like domain to the same extent as observed for LFA703 (Fig. 4B). As expected, pravastatin showed no effect on antibody binding (Fig. 4, A and B).

The LFA-1 Inhibitor LFA703 Does Not Interact with the I-like Domain—To exclude the possibility that LFA703 directly binds to the I-like domain of the \( \beta_2 \) chain and by this inhibits the interaction of the mAbs with the I-like domain, we tested the effect of LFA703 on Mac-1, which shares the \( \beta_2 \) chain with LFA-1 (2). Purified Mac-1 was immobilized on microtiter plates, and the binding of the mAb IB4 was measured in the presence of LFA703 (Fig. 5). The compound did not inhibit the binding of mAb IB4 to purified Mac-1 at a concentrations of 50 \( \mu \)M (Fig. 5) or 200 \( \mu \)M (not shown), suggesting that LFA703 does not bind to the I-like domain and indeed has to bind to the L-site on the \( \alpha_4 \) chain to influence epitopes on the \( \beta_2 \) chain. Likewise, lovastatin and LFA451 were inactive in the Mac-1/mAb IB4 binding assay, indicating that the effect of XVA143 on the I-like domain is independent from the type of \( \alpha_4 \) chain associated with the \( \beta_2 \) chain (Fig. 5). In contrast, the binding of the mAb TS2/4 to LFA-1 was not impaired in the presence of XVA143 (Table II). These findings provide strong evidence that the binding site of XVA143 is located on the I-like domain of the \( \beta_2 \) chain shared by LFA-1 and Mac-1. Moreover, the IC\(_{50}\) values determined in the LFA-1/ICAM-1 and the Mac-1/ICAM-1 assays suggest that the engagement of this binding site allows very efficient \( \beta_2 \) integrin inhibition (Table I).

Effect of the LFA-1 Inhibitors on Inactive LFA-1 on Jurkat Cells—We also investigated the effect of the inhibitors on the inactive form of LFA-1. To keep LFA-1 in its inactive state, Jurkat cells were washed several times with 1 mM EDTA before compounds and antibodies were added. The experiments were performed in the presence of 0.1 mM EDTA. Under these conditions, LFA-1 did not interact with ICAM-1 (data not shown). The antibodies utilized for the study bound the active and inactive form of LFA-1 equally well (data not shown). As observed for active LFA-1, lovastatin, LFA703, and LFA451 most prominently inhibited the binding of the anti-I domain mAb R7.1 to inactive LFA-1 on Jurkat cells (Table II). This suggests that the L-site ligands interact with the L-site irrespective whether the receptor resides in its ligand binding or nonbinding state. However, the effect of LFA703 on the reactivity of anti-I-like domain mAbs with the inactive receptor was less pronounced than observed for the active receptor (Table II). This result indicates that the conformational linkage between
the I domain and the I-like domain observed in the active receptor is modified upon inactivation of LFA-1 by cation removal. In contrast to the statin-derived LFA-1 inhibitors, XVA143 had almost no effect on the epitopes of inactive LFA-1 expressed on Jurkat cells (Table II). As observed for activated LFA-1, the inhibitors did not affect or only marginally affected the β-propeller region of inactive LFA-1 recognized by mAb TS2/4 (Table II).

Effect of the LFA-1 Inhibitors on Inactive Purified LFA-1—LFA-1 immobilized on microtiter plates was washed several times with 1 mM EDTA, and antibody binding experiments were performed in the presence of 0.1 mM EDTA or 1 mM EDTA to further examine the effect of the compounds on the inactive integrin. At both EDTA concentrations, lovastatin, LFA703, and LFA451 inhibited the interaction of mAb R7.1 with the I domain in a dose-dependent manner, whereas XVA143 and pravastatin had no effect on mAb R7.1 binding (Fig. 6, A and B). This result is in agreement with the data described above for inactive LFA-1 expressed on Jurkat cells. In the mAb IB4/LFA-1 assay at 0.1 mM EDTA, lovastatin and LFA451 were inactive, whereas XVA143 and AAY703 significantly influenced the interaction of the antibody with the inactive receptor (Fig. 6C). At 1 mM EDTA, however, the statin-derived inhibitors and XVA143 had no effect or only a marginal effect on the IB4 epitope (Fig. 6D). The latter data at 1 mM EDTA are consistent with the flow cytometry experiment involving LFA-1 on Jurkat cells in 0.1 mM EDTA. This indicates that EDTA more effectively removes cations from the membrane-bound receptor than from the purified receptor. The results show that the binding of XVA143 to LFA-1 is highly cation-dependent. This property is not shared by the statin-derived LFA-1 inhibitors. We note that the binding of the mAbs directed against the I-like domain of LFA-1 was not altered by the removal of cations (data not shown). This clearly indicates that the effects of XVA143 on the antibody/LFA-1 interaction are not due to competitive inhibition but a consequence of epitope alterations.

DISCUSSION

The data reported here demonstrate that small molecule LFA-1 inhibitors can induce conformational changes in both the αI chain and β2 chain of the LFA-1 receptor. The nature of these changes is dependent on the binding site of the compound and its chemical structure. This is schematically illustrated in Fig. 7 for two of the inhibitors analyzed. All statin-derived LFA-1 inhibitors shown to bind to the L-site and to be highly LFA-1-specific lead to the partial or total loss of epitopes on the αI I domain as monitored by impaired binding of several αI domain-specific antibodies. This finding suggests that L-site ligands induce conformational changes in the αI I domain, resulting in LFA-1 inhibition, and is consistent with earlier data showing that the I domain is directly involved in ICAM-1 binding (8).

Dependent on the binding mode, some inhibitors targeting the L-site were found to induce conformational changes in both the α chain I domain as well as the β2 chain I-like domain. This result is in agreement with a recent study showing that mutational activation or inactivation of LFA-1 at the IDAS/L-site results in enhanced or reduced binding of a novel mAb recognizing an activation epitope on the β2 chain (31). Further, Lu et al. (14) demonstrated that LFA-1 locked in an active form by introducing a disulfide bond within the L-site region is resistant to inhibition by blocking mAbs to the I-like domain and conclude that a functional relationship between the I domain and I-like domain exists (14). The data reported here using L-site ligands demonstrate that indeed an interaction between the I domains exists in the native LFA-1 receptor.

Interestingly, the additional involvement of the I-like do-
above) and a recent study investigating the role of the I-like domain of LFA-1 (14) provide evidence in favor of an indirect mechanism. Thus, we hypothesize that the binding of XVA143 to the I-like domain induces conformational changes that disturb the interaction between I domain and I-like domain necessary for ICAM-1 binding. This hypothesis is supported by a study analyzing the inhibition of the \( \alpha_{IIb}\beta_3 \) integrin interaction by RGD-containing peptides. This study indicates that inhibitor binding to the \( \beta_3 \) I-like domain induces the dissociation of fibrinogen from its binding site located on the \( \alpha \) chain of \( \alpha_{IIb}\beta_3 \) via an allosteric mechanism (27). Taken together, these data provide strong evidence that I-like domains on integrin \( \beta \) subunits represent targets for allosteric integrin inhibition, similar to the well-established allosteric L-site on the \( \alpha \) chain (20–23).

We also examined the effect of the different LFA-1 inhibitors on inactive LFA-1 in the absence of cations. The interaction of XVA143 with the inactive receptor was found to be very weak as indicated by marginal changes of a few mAb epitopes. This observation is in agreement with the assumption that XVA143 interacts with a cation binding site on the I-like domain. In contrast, the statin-derived LFA-1 inhibitors mediated epitope changes in the I\( \alpha \) I domain of LFA-1 in absence of cations, indicating that the L-site is accessible in the inactive and active receptor and that inhibitor binding to the L-site is not dependent on cations. However, L-site engagement in the inactive receptor no longer resulted in conformational alterations in the I-like domain of the \( \beta \) chain. This finding indicates that the conformational interaction between the I domain and I-like domain observed in the active receptor is cation-dependent and does not take place in the inactive receptor. Similarly, a conformational interaction between the I domain of the LFA-1 \( \alpha \) chain and the stalk region of the LFA-1 \( \beta \) chain was abolished in the inactive receptor in the absence of cations.\(^2\) These results suggest that during the LFA-1 activation process, cation-dependent domain linkages are built up, which allow inter-subunit communication. Our data imply that the putative \( \beta \) propeller region of LFA-1 is less affected by the activation process, although this region is thought to be in close contact with both I domains.

In conclusion, using mAbs directed against different domains of LFA-1, we were able to analyze how inhibitor binding to the L-site or the I-like domain triggers epitope changes within and across LFA-1 domains. For the first time, we can demonstrate these processes in the native receptor without introducing mutations. Our study demonstrates that both the L-site and the I-like domain constitute suitable targets for the design of integrin inhibitors that could be used to prevent or treat a wide range of diseases.

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\(^2\) K. Welzenbach and G. Weitz-Schmidt, unpublished data.