Mapping the Intercellular Adhesion Molecule-1 and -2 Binding Site on the Inserted Domain of Leukocyte Function-associated Antigen-1*

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By extensive mutagenic analysis of the inserted domain (I-domain) of the α-chain (CD11a) of the leukocyte function-associated antigen-1 (LFA-1), we have defined a putative binding surface for intercellular adhesion molecules 1 and 2 (ICAM-1 and -2). This analysis showed that individually mutating Leu-205 or Glu-241 to alanine completely abolished LFA-1 binding to ICAM-1 or -2 without affecting I-domain structure, as assayed by antibody binding. Mutating Thr-243 to alanine also had a profound effect on LFA-1 binding to ICAM-1 and -2, as seen by complete loss of binding to ICAM-1 and a significant reduction (70% decrease) in binding to ICAM-2. Mutating Glu-146 to alanine reduced LFA-1 binding to ICAM-1 or -2 by 70%, and mutating His-264 or Glu-293 to alanine reduced binding to ICAM-1 or -2 by about 30–40%. Mutating Thr-175 to alanine reduced binding to ICAM-1 by about 30% and binding to ICAM-2 by about 70%. Interestingly, mutating Lys-263 to alanine preferentially abolished LFA-1 binding to ICAM-2. Using these data, we have generated a model of the interface between the LFA-1 I-domain and residues in the first domain of ICAM-1 that have been shown to be critical for this interaction. In addition, this model, together with the ICAM-2 crystal structure, has been used to map residues that are likely to mediate LFA-1 I-domain binding to ICAM-2.

Leukocyte function-associated antigen-1 (LFA-1) is a member of the leukocyte integrin family. Like other members of this family, it is expressed exclusively on leukocytes and shares a common β-chain (β2, CD18). The four members of this family that differ in their α-chains are LFA-1 (CD11a, CL), Mac-1 (CD11b, CD103), p150,95 (CD11c, CD10), and αβ1 (CD11d, CD18). LFA-1 is constitutively expressed on the cell surface and is thought to undergo a conformational change that renders it capable of high affinity ligand binding. The primary ligands for LFA-1 are intercellular adhesion molecules 1 and 2, -3 (ICAM-1, -2, and -3). The interaction of ICAM-3 with LFA-1 is of considerably lower affinity than ICAM-1 or -2 binding to LFA-1 (1, 2). LFA-1/ICAM-1 binding is the best understood β2 integrin-ligand interaction and is known to play a critical role in leukocyte function. This interaction is important for the adhesion of activated leukocytes to capillary endothelium, the activation of helper T-cells for the production of chemokines, and the activation of cytotoxic T-cells for the killing of infected or abnormal cells (3–5). Furthermore, antibodies that block the interaction of LFA-1 and ICAM-1 have been shown to be effective in prolonging the survival of allogenic grafts (6–12). Recently, two new members of the ICAM family have been identified and shown to bind to LFA-1: LW (13, 14) and telencephalin (15). The role of these two new ligands has yet to be established.

Much of our understanding of the binding of β2 integrins to ligands comes from analysis of the LFA-1/ICAM-1 interaction and the Mac-1/ICAM-1, iC3b, or fibrinogen interaction. The α-chains of all the β2 integrins contain a region of approximately 200 amino acids, called the inserted or I-domain, that is N-terminal to the divalent cation binding EF hand motifs. In addition, the α1, α2, and αE integrin subunits contain an I-domain, which is also known as the A-domain and was first identified as a sequence motif in von Willibrand factor. Both the I-domains and the EF hand motifs have been shown to bind divalent cations. A large body of evidence suggests that the I-domain plays a critical role in the binding of these integrins to their respective ligands. Numerous function-blocking antibodies to LFA-1, Mac-1, p150,95, αβ1, and αβ2 map to their respective I-domains (16–22). When expressed as an immunoglobulin (Ig) fusion protein, the CD11a I-domain binds ICAM-1 and also blocks T-cell binding to ICAM-1 (17, 23). When the CD11b I-domain is expressed as a recombinant protein, it has been shown to bind ICAM-1, fibrinogen, and IC3b (23, 24). In addition, a GST fusion protein of the α2 I-domain binds to collagen I (25) and recombinant α1 I-domain binds to collagen I, collagen IV, and laminin (26). High resolution crystal structures of the CD11a and CD11b I-domains, as well as the α2 I-domain, have been solved (27–29). The CD11a and CD11b I-domains consist of six β-strands surrounded by seven α-helices. The cation coordinating amino acids are located on one face of the I-domain. It has been hypothesized that the region of the I-domain that coordinates cation binding (Asp-137, Ser-139, Ser-141, Thr-206, and Asp-239 in CD11a) is part of the ligand binding surface (28). In addition, mutagenesis of the LFA-1 I-domain showed that amino acids outside the cation binding domain contribute to ICAM-1 binding (30). Using a two-step approach consisting of alanine scanning mutagenesis and targeted mutagenesis based on the crystal structure, we have carried out extensive mutagenesis of the LFA-1 I-domain and have identified amino acids that are critical to LFA-1 binding to ICAM-1 and ICAM-2.

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1 The abbreviations used are: LFA, leukocyte function-associated antigen; ICAM, intercellular adhesion molecule; I-domain, inserted domain; ECD, extracellular domain; PBS, phosphate-buffered saline; FACS, fluorescence-activated cell sorter.

2 C. Edwards, unpublished observations.
Using this information, we have generated a model of the binding interface between the I-domain and the first Ig-like domain of ICAM-1, which accounts for the amino acids in both molecules shown to be important for ligand binding.

**EXPERIMENTAL PROCEDURES**

**Generation of ICAM Reagents—**The production and purification of the ICAM-1-Ig fusion protein, as well as the K39A and K39E ICAM-1 mutants, has previously been described (31). We have established the convention that the ICAM amino acids will be underlined. ICAM-2-IgG fusion protein was generated in a similar fashion. Briefly, cDNA was made from U937 RNA. Using PCR and forward (5'-GTCGATATCGAT-TGGAGACTGCCAGAGATGTC-3') and reverse (5' AAAGTCGGTGAC-GTCAGTATCGAT) primers, a fragment containing the extracellular domain of ICAM-2 was generated from that cDNA. At the 5'-end of the forward primer, a ClaI site was placed and at the 5'-end of the reverse primer, a BstEII site was placed. Using the plasmid containing the human interferon-γ receptor extracellular domain (ECR) human Ig fusion protein construct (32), the interferon-γ receptor ECD was replaced with the ICAM-2 ECD at the ClaI and BstEII sites. The entire ICAM-2 ECD was sequenced to assure fidelity. The plasmid was stably transfected into human 293 cells and secreted ICAM-2-IgG was purified using protein A affinity chromatography.

**Generation of the I-domain Mutants—**The I-domain mutants were generated using a plasmid containing the sequence encoding amino acids 115-411 of human CD11a and in which the first P/FMI site 3’ of the I-domain had been abolished (pLFA.ID.Δp). A series of oligonucleotides were synthesized to incorporate the indicated base pair substitutions within the I-domain. Then, using oligonucleotide-directed mutagenesis (33), the indicated I-domain mutations were incorporated into the pLFA.ID.Δp plasmid. The entire sequence from the N9Rl site to the remaining P/FMI site for each mutant was sequenced to ensure the accuracy of each mutant. The mutant I-domains were then inserted into the human CD11a plasmid (pRKLA∞lom) at the comparable N9Rl and P/FMI sites.

**Transfections—**Human 293 cells were transfected by a standard calcium phosphate method as described previously (34). Briefly, 1 day prior to transfection, 293 cells were seeded at 30–40% confluence in 10-cm plates and incubated overnight. For each condition, 10 μg of pRKCD18 (35), 1 μg of pAdvRNA (34), and 10 μg of the plasmid containing the specified CD11a constructs were precipitated in CaCl₂ and HEPES buffer and added to the plates. Mock-transfected cells were transfected with only the pAdvRNA construct. The cells were incubated with the DNA for 3–4 h, shocked with 15% glycerol in PBS for 45 s, and then placed in fresh media. After 3 days, transfectants were tested for 1) adhesion to ICAM-1-IgG or ICAM-2-IgG that had been captured by anti-human Fc antibody (Caltag, Burlingame, CA), or 2) expression of the CD11a/CD18 complexes on the cell surface using FACS analysis.

**Adhesion Assays—**200 ng of goat anti-human Fc (Caltag) in PBS was added to each well of Nunc Maxisorb 96-well plates and incubated overnight at 4 °C. Plates were washed with PBS and blocked with 1% bovine serum albumin/PBS solution for 1 h at room temperature. The plates were washed, and 50 μl of a 1–2 μg/ml solution of ICAM-1-IgG or ICAM-2-IgG was added to the wells and incubated at 37 °C for 1–2 h. The plates were washed, and 50 μl of cells was added per well, in triplicate determinations for each condition. Each experiment testing for ICAM-1 adhesion was carried out at least three times and at least twice for ICAM-2.

**Fig. 1. Schematic map of the CD11a and I-domain mutants.** The I-domain consists of amino acids 125–311, which are indicated below the CD11a schematic. Amino acid numbers are shown above the appropriate amino acids. The clusters of charged amino acids that were mutated to alanine are designated by a box located beneath the appropriate amino acids. The I-domain antibody epitopes are denoted by shaded boxes. The N-terminal amino acids of Ide A, B, C, and D are Ile-126, Gln-143, Lys-197, and Asp-182, respectively.
TABLE I
Binding of I-domain mutants to ICAM-1 and ICAM-2

Cells expressing wild-type and mutant LFA-1 were assayed for binding to ICAM-1 and ICAM-2 coated plates. A, hImp mutants; B, point mutants. Charged amino acids that were contained within hImp mutants and were made as point mutants are boxed and shaded. The corresponding hImp designation is indicated to the left. Binding to ICAM-1/-2 by the cells expressing the LFA-1 mutants is expressed as a percentage of ICAM binding by the wild-type LFA-1 expressing cells. + + represents binding levels of between 26 and 50%, ++ represents binding levels between 51 and 75%, and +++ represents binding levels between 76 and 100%. Each condition was carried out in triplicate.

| Construct | ICAM-1 | ICAM-2 |
|-----------|--------|--------|
| hImp-1    | -      | -      |
| hImp-2    | +++    | +++    |
| hImp-3    | +++    | +++    |
| hImp-4    | +      | +      |
| hImp-5    | +++    | +++    |
| hImp-6    | +++    | +++    |
| hImp-7    | +      | +      |
| hImp-8    | +      | +      |
| hImp-9    | +      | +      |
| hImp-10   | +++    | +++    |
| hImp-11   | +++    | +++    |
| hImp-12   | +      | +      |

Transfected cells were detached with 5 mM EDTA/PBS and resuspended at a concentration of 200,000 cells/50 μl of adhesion buffer (140 mM NaCl, 20 mM HEPES, pH 7.5, 0.2% glucose) containing 1 mM each of MgCl2 and CaCl2 and 5 μg/ml of the anti-CD18 activation antibody MEM-48 (R & D Systems, Abingdon, United Kingdom). FACS analysis determined that the I-domain mutants bound MEM-48 at levels comparable to CD11a/CD18 under these conditions. The cells were added to the plates and incubated at 37 °C for 1.5 h. Nonadherent cells were removed by three washes with PBS. Cell attachment was measured using the P-nitrophenyl-N-acetyl-p-glucosaminide method of Landegren (36). The wild-type LFA-1 expressing cells bound ligand on average at levels approximately 10 times higher than mock-transfected cells. On average, 50% of the total wild-type LFA-1 expressing cells added per well bound the coated ligand.

Antibody Staining and FACS Analysis—Three days posttransfection, cells were detached, and 500,000 cells were resuspended in 100 μl of adhesion buffer containing 1 mM each of MgCl2 and CaCl2 and antibodies against either CD11a or CD18. Monoclonal antibodies against CD11a were used at 1 μg/ml for purified MHM-24 (37), The (Amac, Inc., Westbrook, ME), TS1/22 (Endogen, Boston, MA), 38 (BCA1) (R & D Systems), L16 (38), Mem-83 (1), or at 1/1000 dilution of ascites for 32E6, 3D6, 50G1 (Ref. 16 and references therein). Monoclonal antibodies against CD18 were used at 5 μg/ml for MEM-48 (R & D Systems), or 1 μg/ml for MHM-23 (37), H52 (39). Cells were incubated with antibodies on ice for 1 h and washed, and fluorescein isothiocyanate-conjugated goat anti-mouse antibodies (Cappel, Westchester, PA) were added at a dilution of 1:500. Cells were washed, fixed in 1% formaldehyde, and analyzed by FACS analysis using the CELLQuest software (Becton Dickinson). Transfection efficiencies and antibody binding levels were calculated as the percentage of the number of mutant expressing cells that bound to a given antibody compared with the number of wild-type LFA-1 expressing cells that bound to the same antibody. We have found that this provides a good estimation of transfection efficiency for our experiments, and in no instance did the relative mean fluorescence intensity differ significantly from the relative percentage of antibody binding positive cells. Typical transfection efficiencies ranged from 30 to 80%.

RESULTS

Strategy for Analysis of I-domain Mutants—A panel of 35 mutants of the LFA-1 I-domain were generated and subdivided into two classes. The first class of mutants was a series of alanine scans in which clusters of charged amino acids in the I-domain were changed to alanines. These mutants, designated hImp-1 through hImp-13, are depicted in Fig. 1. Asp-137, which was mutated in our previous work, was not changed (40). The second class of mutants was a series of 21 point mutants in which the single amino acids were mutated to alanine (Fig. 1 and Table I, B). The nomenclature for these point mutants identifies the wild-type amino acid, its position within the polypeptide, and the amino acid substitution. Twelve of these point mutants correspond to charged residues contained within hImp-2, -8, -10, and -12 sequences, and 9 were novel mutants of uncharged amino acids.

In each experiment, cells transfected with cDNAs encoding the mutants were assayed for their ability to bind to a panel of antibodies against LFA-1 using FACS (Table II). Three antibodies that recognize CD18 and nine antibodies that recognize CD11a were used. Of the anti-CD18 antibodies, two recognize the CD18 subunit alone (H52 and MEM-48) and one recognizes CD18 (MHM-23) when complexed with an α-chain (39). The anti-CD11a antibodies, seven recognize epitopes within the I-domain (25, 3, TS1/22, 32E6, 50G1, MHM-24, and MEM-83, 38), and two recognize epitopes outside the I-domain (3D6, L16). Six of the seven anti-CD11a antibodies have been mapped to four different epitopes, Ide A, B, C, and D (16, 41). The antibodies that map to Ide A, B, and C are function-blocking antibodies and block the interaction of LFA-1 with ICAM-1, -2, and -3. Antibodies that map to Ide D specifically block LFA-1 binding to ICAM-3.

Binding of the I-domain Mutants to ICAM-1—Wild-type and mutant CD11a constructs were co-transfected with the cDNA for CD18, and cells were assayed for their ability to bind to ICAM-1 (Fig. 2 and Table I) and the panel of antibodies against LFA-1 (Table II). The hImp mutants were examined first (Fig. 2). Based on antibody binding and adhesion to ICAM-1, the mutants were subdivided into three groups. The first group is represented by hImp-3, -5, -6, -10, and -11. Cells expressing these mutants bound ICAM-1 and a majority of the antibodies at levels similar to wild-type LFA-1 transfectedants. The second group of mutants is represented by hImp-1, -4, -7, and -9. These mutants appear to have major structural perturbations in LFA-1 because binding of all antibodies was abolished or reduced to less than 60% of wild-type binding. The last class of mutants is represented by hImp-2, -8, -12, and -13. Cells expressing these mutants had reduced or no binding to ICAM-1 but showed robust binding to the panel of anti-CD18 and anti-CD11a antibodies. The amino acids making up this last class of hImp mutants are likely to play a key role in ICAM-1 binding.

Our strategy in generating point mutants was 2-fold. First, the hImp mutants that were found to express structurally intact LFA-1 and also abrogate ICAM-1 binding were further dissected. Second, the region adjacent to the cation binding site of the CD11a and CD11b I-domains has been suggested as important for contacting ligand (27, 28, 30, 40, 42), and amino acids that mapped to this region were mutated. Within the hImp mutants, we mutated the following charged amino acids:

3 P. Berman and G. Nakamura, unpublished observation.
A panel of 12 anti-LFA-1 antibodies was tested by FACS for binding to cells expressing the wild-type and LFA-1 mutants. The number of positive staining cells for each mutant was calculated as a percentage of the number of positive staining cells for wild-type LFA-1. 

| Construct  | CD18 | CD11a |
|------------|------|-------|
|            | M140A | Q143A | D145A | E146A | T175A | L205A | E241A | T243A | S245A | K263A | H264A | Q266A | T267A | K287A | D290A | E293A | R294A | R295A | L205A | E241A | T243A | S245A | K263A | H264A | Q266A | T267A | K287A | D290A | E293A | R294A | R295A |
| Complex    | MHM-23 | MEM-48 | H52 | 25.3 | TS1/22 | 32E6 | 5G01 | MHM-24 | MEM-83 | 38 | Non-I-domain |
| hImp-1     | +++   | +++   | +++ | +++ | +++ | +++ | +++ | +++ | +++ | +++ | ND | +++ | +++ | +++ | +++ | +++ | +++ | +++ | +++ | +++ | +++ | +++ | +++ | +++ | +++ | +++ | +++ | +++ | +++ | +++ | +++ | +++ |
| hImp-2     | +++   | +++   | +++ | +++ | +++ | +++ | +++ | +++ | +++ | +++ | ND | +++ | +++ | +++ | +++ | +++ | +++ | +++ | +++ | +++ | +++ | +++ | +++ | +++ | +++ | +++ | +++ | +++ | +++ | +++ | +++ |
| hImp-3     | +++   | +++   | +++ | +++ | +++ | +++ | +++ | +++ | +++ | +++ | ND | +++ | +++ | +++ | +++ | +++ | +++ | +++ | +++ | +++ | +++ | +++ | +++ | +++ | +++ | +++ | +++ | +++ | +++ | +++ | +++ |
| hImp-4     | +++   |+++   |+++ |+++ |+++ |+++ |+++ |+++ |+++ |+++ |ND |+++ |+++ |+++ |+++ |+++ |+++ |+++ |+++ |+++ |+++ |+++ |+++ |+++ |+++ |+++ |+++ |+++ |+++ |+++ |+++ |
| hImp-5     | +++   |+++   |+++ |+++ |+++ |+++ |+++ |+++ |+++ |+++ |ND |+++ |+++ |+++ |+++ |+++ |+++ |+++ |+++ |+++ |+++ |+++ |+++ |+++ |+++ |+++ |+++ |+++ |+++ |+++ |+++ |
| hImp-6     | +++   |+++   |+++ |+++ |+++ |+++ |+++ |+++ |+++ |+++ |ND |+++ |+++ |+++ |+++ |+++ |+++ |+++ |+++ |+++ |+++ |+++ |+++ |+++ |+++ |+++ |+++ |+++ |+++ |+++ |+++ |
| hImp-7     | +++   |+++   |+++ |+++ |+++ |+++ |+++ |+++ |+++ |+++ |ND |+++ |+++ |+++ |+++ |+++ |+++ |+++ |+++ |+++ |+++ |+++ |+++ |+++ |+++ |+++ |+++ |+++ |+++ |+++ |+++ |
| hImp-8     | +++   |+++   |+++ |+++ |+++ |+++ |+++ |+++ |+++ |+++ |ND |+++ |+++ |+++ |+++ |+++ |+++ |+++ |+++ |+++ |+++ |+++ |+++ |+++ |+++ |+++ |+++ |+++ |+++ |+++ |+++ |
| hImp-9     | +++   |+++   |+++ |+++ |+++ |+++ |+++ |+++ |+++ |+++ |ND |+++ |+++ |+++ |+++ |+++ |+++ |+++ |+++ |+++ |+++ |+++ |+++ |+++ |+++ |+++ |+++ |+++ |+++ |+++ |+++ |
| hImp-10    | +++   |+++   |+++ |+++ |+++ |+++ |+++ |+++ |+++ |+++ |ND |+++ |+++ |+++ |+++ |+++ |+++ |+++ |+++ |+++ |+++ |+++ |+++ |+++ |+++ |+++ |+++ |+++ |+++ |+++ |+++ |
| hImp-11    | +++   |+++   |+++ |+++ |+++ |+++ |+++ |+++ |+++ |+++ |ND |+++ |+++ |+++ |+++ |+++ |+++ |+++ |+++ |+++ |+++ |+++ |+++ |+++ |+++ |+++ |+++ |+++ |+++ |+++ |+++ |
| hImp-12    | +++   |+++   |+++ |+++ |+++ |+++ |+++ |+++ |+++ |+++ |ND |+++ |+++ |+++ |+++ |+++ |+++ |+++ |+++ |+++ |+++ |+++ |+++ |+++ |+++ |+++ |+++ |+++ |+++ |+++ |+++ |
| hImp-13    | +++   |+++   |+++ |+++ |+++ |+++ |+++ |+++ |+++ |+++ |ND |+++ |+++ |+++ |+++ |+++ |+++ |+++ |+++ |+++ |+++ |+++ |+++ |+++ |+++ |+++ |+++ |+++ |+++ |+++ |+++ |

TABLE II
Anti-LFA-1 antibody binding to LFA-1 mutants

The structure of the I-domain mutants to ICAM-2 abrogated binding. As mentioned, the E293A mutant did show an approximately 40% reduction in binding. This suggests that more than one amino acid from the hImp-12 mutant must be simultaneously mutated in order to generate the hImp-12 phenotype.

Binding of the I-domain Mutants to ICAM-2—The structure of the extracellular domain of ICAM-2 has been solved (43). The three-dimensional structure of the first domain of ICAM-2 shows a high degree of similarity to the predicted three-dimensional structure of the first domain of ICAM-1 (31). Therefore, we determined whether the amino acids in the LFA-1 I-domain important for ICAM-1 binding were also important for ICAM-2 binding. All mutants that abolished or reduced binding to ICAM-1 also abolished or reduced binding to ICAM-2 (Table I).

In addition, the hImp-10 mutant preferentially abrogated ICAM-2, but not ICAM-1, binding. hImp-10 was found to bind to all the antibodies against LFA-1, except 25.3 and TS1/22 (Table II).

In order to determine the role of individual hImp-10 amino acids in ICAM-2 binding, each charged amino acid (Lys-263, His-264, Lys-265, Glu-269, and Glu-272) was individually mutated to alanine and assayed for binding to ICAM-1 and ICAM-2 (Table I). The K263A mutant abolished LFA-1 binding to ICAM-2 but had no effect on binding to ICAM-1. The other mutants did not abolish binding to either of the ICAMs. The K263A mutant bound the entire panel of anti-CD11a/CD18 antibodies, suggesting that its structure is similar to that of wild-type LFA-1 (Table II). As with the amino acids important for ICAM-1 binding, Lys-263 maps to the cation binding region of the I-domain (see Fig. 4). Thus, whereas ICAM-1 and ICAM-2 appear to bind LFA-1 with similar affinities, ICAM-2 shows a high degree of similarity to the predicted three-dimensional structure of the first domain of ICAM-1 (31). Therefore, we determined whether the amino acids in the LFA-1 I-domain important for ICAM-1 binding were also important for ICAM-2 binding. All mutants that abolished or reduced binding to ICAM-1 also abolished or reduced binding to ICAM-2 (Table I).

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binding appears to involve at least one additional amino acid compared with ICAM-1.

Refinement of Anti-CD11a Function Blocking Antibody Epitopes—In addition to elucidating amino acids in the I-domain that are important for ICAM-1 and -2 binding, these I-domain mutants further define the epitopes of a number of anti-CD11a function-blocking antibodies that have been previously mapped to this domain (16, 17, 30). Six of the anti-CD11a antibodies used in this study have been mapped to four distinct epitopes within the I-domain (IdeA, IdeB, IdeC, and IdeD) (Table II and Figs. 1 and 3). The hImp-10 mutant displayed full binding to all the antibodies against LFA-1 except 25.3 and TS1/22 (Table II). These two antibodies have previously been mapped to the IdeA epitope, which is composed of the 126IKGN motif at the N terminus of the I-domain (16). Because this motif is not contained within or near the hImp-10 mutant, these results expand the binding epitope of these two antibodies. In previous work, we showed that mutations that map within the IdeA epitope abolish LFA-1 binding to ICAM-3 but not to ICAM-1 (44). Interestingly, the hImp-10 mutant also fails to bind to ICAM-3. Each of the charged amino acids in hImp-10 (Lys-263, His-264, Lys-268, Glu-269, and Glu-272) was mutated to alanine. Surprisingly, all five of these hImp-10 point mutants bound all the anti-LFA-1 antibodies, including 25.3 and TS1/22. Thus, it is possible that multiple amino acids within the hImp-10 mutant must be simultaneously mutated in order to decrease antibody binding. Alternatively, simultaneously changing the charged amino acids within this stretch of the I-domain may induce local structural changes such that the amino acid(s) defining the binding epitope of 25.3 or TS1/22 are no longer exposed to the antibody. In support of this hypothesis, we observed that each of the uncharged amino acids Glu-266 and Thr-267, which are contained within the hImp-10 sequence, showed significant reduction in 25.3 and TS1/22 binding when mutated to alanine (Table II). Two other hImps mutants, hImp-5 and hImp-6, abolished or significantly reduced binding of antibodies that map to IdeB/C or IdeD. Both hImp-5 and -6 failed to bind to MEM-83, which maps to IdeD, and hImp-5 showed significant reduction in binding to 50G1 (IdeB/C). Additionally, hImp-5 resulted in a moderate reduction in binding in both 32E6 (IdeB/C) and MHM-24 (IdeC). In the three-dimensional structure, hImp-5 and -6 are located in close proximity to IdeD (Fig. 3). It is, therefore, conceivable that these hImp mutants define other amino acids that the MEM-83 antibody recognizes. hImp-5 also maps in close proximity to IdeC and may define additional amino acids required for 50G1, 32E6, and MHM-24 binding. Mutation of individual amino acids within these hImp mutants would be required to precisely map the antibody epitopes and will not be covered here.
DISCUSSION

In this work, we have generated a high resolution model of the interaction of LFA-1 with its ligands, ICAM-1 and ICAM-2. We constructed a series of CD11a I-domain mutants and assayed the effects of the mutations on LFA-1 binding to ICAM-1 and ICAM-2. Cells expressing each mutant were tested for binding to a panel of 12 anti-CD11a/CD18 antibodies. Mutations that caused gross structural perturbations as determined by loss of binding to multiple antibodies were not considered when developing our model. Using these criteria, seven amino acids that were defined as playing a role in LFA-1 binding to a ligand were located on the crystal structure of the LFA-1 I-domain (Fig. 3). Using the structural model of the LFA-1 binding site on ICAM-1, we have generated a model of the binding interface of the CD11a I-domain and the first Ig-like domain of ICAM-1 (Fig. 4) and ICAM-2. We identified three amino acids (Leu-205, Glu-241, and Thr-243) that are absolutely required for LFA-1 binding to ICAM-1. Two of these (Leu-205 and Glu-241) are also absolutely required for ICAM-2 binding as mutating these amino acids to alanine completely abolished ligand binding. Mutating Thr-243 to alanine reduced binding to ICAM-2 by approximately 70%. Four additional amino acids (Glu-146, Thr-175, His-264, and Glu-293), when mutated to alanine, caused a partial reduction in ICAM-1 and ICAM-2 binding. Binding of anti-LFA-1 antibodies suggests that these mutations do not cause structural perturbations; therefore, it is likely that these amino acids play a role as contact points in LFA-1 binding to its ligands. Additionally, mutating Lys-263 to alanine preferentially abolished LFA-1 binding to ICAM-2.

By examining the location of these amino acids on the crystal structure of the I-domain, Leu-205, Glu-241, and Thr-243, and the coordinated divalent cation, map to a relatively flat surface on the upper face of the I-domain (Fig. 3). This flat surface complements the flat LFA-1 binding surface that has been described on ICAM-1 (31) and is corroborated by the crystal structure of ICAM-2 (43). The size of this putative binding surface area on the I-domain, as defined by Leu-205, Glu-241, and Thr-243 and the cation, is approximately 9 × 10 Å. The amino acids in ICAM-1 known to be important for LFA-1 binding are Glu-34, Lys-39, Met-64, Tyr-66, Asn-68, and Gln-73. (ICAM amino acids are underlined.) Glu-34, Met-64, Asn-68, and Gln-73 define a surface area of approximately 8 × 13 Å. Initial modeling of the interaction of the I-domain with the first Ig-like domain of ICAM-1 allowed several juxtapositions of these two molecules, one of which involved the Glu-241 of the I-domain interacting with Lys-39 of ICAM-1. We hypothesized that these two amino acids could contribute to the binding interface via a charge/charge interaction. To test this hypothesis, we made amino acid substitutions at positions Glu-241 and Lys-39, which would either conserve or disrupt this putative interaction. We found that an E241K mutant of CD11a at equivalent, albeit low, levels to either a K39A or K39E mutant of ICAM-1 (data not shown). Thus, it is unlikely that a charge/charge interaction occurs between these two amino acids; however, the exact nature of this interaction remains to be defined.

In our current model, Glu-34 is the sixth ligand for the cation in the I-domain (Fig. 4), replacing a chloride ion found in the crystal structure of the I-domain. This is in agreement with Casasnovas et al. (43), who propose that Glu-37 of ICAM-2 coordinates the Mg$^{2+}$ in the CD11a I-domain when binding LFA-1 (43). In our model, Glu-34 also makes a hydrogen bond to the backbone of Ser-141, thereby stabilizing the Glu-34/cation coordination. The importance of stabilizing the Glu-34/cation interaction is supported by the observation that there is no tolerance for change at the Glu-34 position. Mutations that conserved charge (E34D) still abrogated LFA-1 binding (31). In addition, Tyr-66 forms a hydrogen bond with Glu-34.

By holding Glu-34 constant, allowing it to coordinate the cation in the I-domain, and assuming that Glu-241 does not interact directly with Lys-39, we rotated the I-domain and the first domain of ICAM-1 with respect to one another. This was done in an attempt to define an interface orientation that accounts for all the amino acids we had defined as important for both LFA-1 and ICAM-1 binding. In the current model, each of the oxygens in the side-chain carboxyl group of Glu-241 simultaneously interacts with the side-chain amide groups in Asn-68 and Gln-73 (Fig. 4). The other essential amino acids within the I-domain, Leu-205 and Thr-243, come together with Met-64 and Tyr-66 to make a hydrophobic pocket (Fig. 4). In addition to the Thr-243 C=O forming part of the hydrophobic pocket, Thr-243 also forms an intrachain hydrogen bond with backbone amide of Thr-206. The importance of Thr-243 is underscored by our work and the work of Huang and Springer (30). In their work, even the conserved amino acid substitution of T243S resulted in a significant decrease in LFA-1 binding to ICAM-1. Met-140 is situated near the periphery of the hydrophobic pocket (Fig. 4). Although no effect on LFA-1 binding to ICAM-1 was evident when Met-140 was mutated to alanine, Huang and Springer (30) reported that mutating Met-140 to glutamine resulted in a significant reduction in ICAM-1 binding. This could result from glutamine at position 140 being unable to fit into the hydrophobic pocket. It is also possible that a glutamine at position 140 disrupts the nearby Glu-34 interactions with CD11a by competing for hydrogen bonds.

There are several amino acids within the I-domain (Glu-146, Thr-175, His-264, and Glu-293) that when mutated to alanine resulted in a partial reduction in ICAM binding. These results were also incorporated into our working model. In the crystal structure of the CD11a I-domain, Thr-175 makes a hydrogen bond to the backbone of Leu-205. Disruption of this hydrogen bond when Thr-175 is mutated to alanine may result in movement of Leu-205 and, hence, disrupt the hydrophobic interaction in the complex. In this model, the His-264 side-chain simultaneously forms hydrogen bonds to the backbone of Asp-239 and to the backbone carbonyl of Leu-31 of ICAM-1. Glu-146 and Glu-293 are involved in 3-helix capping (45). Mutating them to alanine may affect the ability of the helices to form properly and could lead to the moderate decreases observed in these mutants binding to ICAM-1. In the crystal structure of ICAM-2, Lys-42 forms a hydrogen bond to the backbone of Leu-36. This interaction was maintained in the ICAM-1/LFA-1 model, except in this case, Lys-39 makes a hydrogen bond to the backbone of Ile-33. When ICAM-1 is complexed with LFA-1, the Lys-39 side-chain could also hydrogen bond to the Ser-141 backbone or to the Gln-143 side-chain. Finally, the ICAM-1-Leu-30 side-chain is positioned between Glu-241, His-264, and Ser-270 and interacts with their aliphatic atoms. This positioning is consistent with the reduced binding observed for the L30A mutant of ICAM-1 (31).

In the model of the LFA-1 interaction with ICAM-2, residues Glu-37, Glu-47, Gln-66, His-68, Thr-70, and Gln-75 can be docked on to the I-domain in a manner similar to ICAM-1. In this case, Glu-37 of ICAM-2 is the sixth ligand for the cation, and it receives an intramolecular bond from His-68. The oxygens of the side-chain carboxyl group of Glu-241 simultaneously hydrogen bond to the side-chain hydroxyl group of Thr-70 and the side-chain amide group of Gln-75. The hydrophobic pocket consists of Leu-205, Thr-243, Gln-66, and His-68. As with LFA-1 binding ICAM-1, the T175A mutant of the I-domain may cause reduction in binding of LFA-1 to ICAM-2 by allowing movement of Leu-205, thereby disrupting the hydrophobic pocket. This disruption may be slightly more pro-
nounced in the LFA-1/ICAM-2 interaction because of the ICAM-2 Gln-66 compared with Met-64 in ICAM-1, which probably alters the character of this hydrophobic pocket. This hydrophobic pocket in ICAM-2 is likely to be thermodynamically less favorable than the one formed between LFA-1 and ICAM-1; thereby contributing less binding energy to the LFA-1/ICAM-2 interaction. Yet ICAM-1 and ICAM-2 appear to bind LFA-1 with similar affinities.4 An additional contact point in the ICAM-2/LFA-1 interaction could be between Lys-263 of CD11a and Gln-47 of ICAM-2. ICAM-1 does not contain a Glu in the analogous position, and this could explain why the K263A mutant of CD11a preferentially abolishes LFA-1 binding to ICAM-2. Although our results support the hypothesis that the I-domain provides a complementary contact surface for the ICAMs, we have not ruled out that other regions of LFA-1 are required for contacting ICAM-1 and ICAM-2. There have been several reports suggesting a role for the CD18 (β2)-chain in ICAM binding and a role for regions of the CD11a-chain outside the I-domain (46–49). Additional mutagenic analysis on both the CD18 and CD11a-chains will be needed in order to fully characterize the ICAM binding site on LFA-1.

Very recently, after this work was completed, two groups published the crystal structure of domains 1 and 2 of ICAM-1 (50, 51). The coordinates of the two ICAM-1 crystal structures were superimposed on our ICAM-1 model using backbone atoms. The two crystal structures and the ICAM-1 model all differ in the conformation of loops between β-strands D and E and β-strands F and G, but these differences would not affect any of the proposed interactions portrayed in Fig. 4. The conformations of the side-chains in the model (e.g. Leu-30, Gln-34, and Gln-73) differ from the two crystal structures; however, these same side-chains also differ in conformation between the two crystal structures. Hence, even if the ICAM-1 coordinates from either crystal structure replaced the ICAM-1 in the model complex, one would still have to alter side-chain conformations at the ICAM-1/LFA-1 interface in order to propose the specific interaction shown in Fig. 4.

Bella et al. (51) have also proposed a docking model of the first domain of ICAM-1 to the I-domain of LFA-1. The orientation of the ICAM-1 with respect to the LFA-1 differs in the two models. For example, we propose that Gln-73 of ICAM-1 interacts with Gln-241 of LFA-1, whereas Bella et al. (51) have Gln-73 interacting with Thr-243; the specifics of the hydrophobic pocket also differ between the two models. Regardless of these differences, the overall orientation of the two models seems similar.

In addition to defining residues on the I-domain critical for ICAM binding, these studies have expanded the epitopes for several of the anti-CD11a function-blocking antibodies. Gln-266 and Thr-267, located within the hImp-10 mutant, define a region of novel residues, in addition to the previously defined IdeA, that are important for 25.3 and TS1/22 antibody binding to CD11a. This result is in agreement with the work of Huang and Springer (30), who mapped these antibodies to amino acids 250–303. The hImp-5 and hImp-6 mutants completely abolished binding of 25.3 and TS1/22 antibody binding to CD11a. This result is in agreement with the work of Huang and Springer (30), who mapped these antibodies to amino acids 250–303. The hImp-5 and hImp-6 mutants completely abolished binding of 25.3 and TS1/22 antibody binding to CD11a. This result is in agreement with the work of Huang and Springer (30), who mapped these antibodies to amino acids 250–303. The hImp-5 and hImp-6 mutants completely abolished binding of 25.3 and TS1/22 antibody binding to CD11a. This result is in agreement with the work of Huang and Springer (30), who mapped these antibodies to amino acids 250–303. The hImp-5 and hImp-6 mutants completely abolished binding of 25.3 and TS1/22 antibody binding to CD11a. This result is in agreement with the work of Huang and Springer (30), who mapped these antibodies to amino acids 250–303. The hImp-5 and hImp-6 mutants completely abolished binding of 25.3 and TS1/22 antibody binding to CD11a. This result is in agreement with the work of Huang and Springer (30), who mapped these antibodies to amino acids 250–303. The hImp-5 and hImp-6 mutants completely abolished binding of 25.3 and TS1/22 antibody binding to CD11a. This result is in agreement with the work of Huang and Springer (30), who mapped these antibodies to amino acids 250–303. The hImp-5 and hImp-6 mutants completely abolished binding of 25.3 and TS1/22 antibody binding to CD11a. This result is in agreement with the work of Huang and Springer (30), who mapped these antibodies to amino acids 250–303. The hImp-5 and hImp-6 mutants completely abolished binding of 25.3 and TS1/22 antibody binding to CD11a. This result is in agreement with the work of Huang and Springer (30), who mapped these antibodies to amino acids 250–303. The hImp-5 and hImp-6 mutants completely abolished binding of 25.3 and TS1/22 antibody binding to CD11a. This result is in agreement with the work of Huang and Springer (30), who mapped these antibodies to amino acids 250–303. The hImp-5 and hImp-6 mutants completely abolished binding of 25.3 and TS1/22 antibody binding to CD11a. This result is in agreement with the work of Huang and Springer (30), who mapped these antibodies to amino acids 250–303. The hImp-5 and hImp-6 mutants completely abolished binding of 25.3 and TS1/22 antibody binding to CD11a. This result is in agreement with the work of Huang and Springer (30), who mapped these antibodies to amino acids 250–303. The hImp-5 and hImp-6 mutants completely abolished binding of 25.3 and TS1/22 antibody binding to CD11a. This result is in agreement with the work of Huang and Springer (30), who mapped these antibodies to amino acids 250–303. The hImp-5 and hImp-6 mutants completely abolished binding of 25.3 and TS1/22 antibody binding to CD11a. This result is in agreement with the work of Huang and Springer (30), who mapped these antibodies to amino acids 250–303. The hImp-5 and hImp-6 mutants completely abolished binding of 25.3 and TS1/22 antibody binding to CD11a. This result is in agreement with the work of Huang and Springer (30), who mapped these antibodies to amino acids 250–303. The hImp-5 and hImp-6 mutants completely abolished binding of 25.3 and TS1/22 antibody binding to CD11a. This result is in agreement with the work of Huang and Springer (30), who mapped these antibodies to amino acids 250–303. The hImp-5 and hImp-6 mutants completely abolished binding of 25.3 and TS1/22 antibody binding to CD11a. This result is in agreement with the work of Huang and Springer (30), who mapped these antibodies to amino acids 250–303. The hImp-5 and hImp-6 mutants completely abolished binding of 25.3 and TS1/22 antibody binding to CD11a.
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