The second domain of intercellular adhesion molecule-1 (ICAM-1) maintains the structural integrity of the leucocyte function-associated antigen-1 (LFA-1) ligand-binding site in the first domain

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The first domain of intercellular adhesion molecule-1 (ICAM-1) binds to the leucocyte function-associated antigen-1 (LFA-1) I domain, which contains the principal ligand-binding site of this leucocyte integrin. Whether the function of the second domain is also to directly bind LFA-1 has been unclear. Our data show that mutation in the hydrophilic EF loop of ICAM-1 domain 2 resulted in impaired binding of the isolated I domain when compared with wild-type ICAM-1. LFA-1 on T-cells also binds with reduced affinity to this ICAM-1 mutant. A hybrid construct containing the first domain of vascular cell-adhesion molecule-1 joined to domains 2–5 of ICAM-1 was unable to bind to the I domain, showing that there is no direct interaction between the second domain of ICAM-1 and the I domain. This construct was also not bound by LFA-1 expressed in T-cells. Function-blocking monoclonal antibodies that map to domain 2 of ICAM-1, implicating this domain in ligand binding, were found to act indirectly. In summary our data suggest that the second domain of ICAM-1 has a role in maintaining the structure of the LFA-1 ligand-binding site in the first domain of ICAM-1 but does not appear to have a direct role in ligand binding.

Key words: adhesion, I domain, integrin, lymphocyte, structure.

INTRODUCTION

Intercellular adhesion molecule-1 (ICAM-1, CD54) is a cell-surface glycoprotein that mediates leucocyte adhesion during immune responses [1–3]. It is expressed on leucocytes, endothelium, epithelium and fibroblasts and is upregulated in response to cytokines and bacterial antigens [4,5]. ICAM-1 is a heavily glycosylated polypeptide chain of ≈90 kDa with an extracellular region consisting of five tandemly repeated immunoglobulin-like domains [6,7]. It exists on the membrane as a non-covalently linked dimer [8,9]. Electron microscopy shows soluble ICAM-1 to be a rod-shaped structure ≈19 nm in length with a bend ≈7.6 nm from one end between domains 3 and 4 [7,10]. Recently, the crystal structure of the first two domains of ICAM-1 has been solved [11,12].

The ICAM family consists of four other members, ICAM-2, -3, -4 and -5 (reviewed in [3]). All are members of the immunoglobulin superfamily (IgSF) and have been reported to bind to the β2 integrin leucocyte function-associated antigen-1 (LFA-1), but ICAM-1 is the principal ligand for LFA-1 in many interactions [13,14]. LFA-1 is expressed on the surface of leucocytes and consists of two non-covalently associated subunits, αL (CD11a) and β2 (CD18) [15]. In the α subunit a major binding site for ICAM-1 has been located in the inserted (I) domain [16], with other sites in the cation-binding domains V and VI [17] as well as in the β subunit [18] also implicated in ligand binding. In addition to LFA-1, ICAM-1 is a receptor for Mac-1 [19], fibrinogen [20], the major group of human rhinoviruses [21] and Plasmodium falciparum-infected erythrocytes [22].

The ICAM-1 binding sites for LFA-1 and all of its other binding partners, with the exception of Mac-1, have been located in the first domain and have been found to be overlapping [7,19,23–25]. Mutagenesis studies have shown that residues E34 and Q73 in domain 1 of ICAM-1 (and corresponding residues E37 and Q75 in ICAM-2 and -3) are essential for binding to LFA-1 [7,26,27]. The critical residue E34 in ICAM-1 has recently been shown to interact with the isolated LFA-1 I domain [28].

The second domain of ICAM-1 has also been implicated in ligand binding by mutagenesis and mapping of function-blocking monoclonal antibody (mAb) epitopes to the top of the domain [7,23], but the function of this domain has not been established. In this study we have investigated the role of the second domain of ICAM-1 in binding to the β2 integrin LFA-1.

MATERIALS AND METHODS

mAbs and cell lines

The following mAbs were used in this study: anti-LFA-1 mAbs mapping to the I domain [29], mAb 38 (this laboratory) and MEM 83 (a gift from Dr V. Horejsi, Institute of Molecular Genetics, Prague, Czech Republic); anti-ICAM-1 mAbs specific for domain 1, mAb 15.2 (this laboratory) [23], RR1 (a gift from Dr R. Rothlein, Boehringer Ingelheim, Ridgefield, CT, U.S.A.) and WEHI-CAM-1 (a gift from Dr A. Boyd, Walter and Eliza Hall Institute, Royal Melbourne Hospital, Melbourne, Australia); anti-ICAM-1 mAbs specific for domain 2, mAb R6.5 (a gift from Dr R. Rothlein) and mAb 8.4A6 (a gift from Dr D. Haskard, Imperial College School of Medicine, Hammersmith Hospital, London, U.K.); mAbs specific for ICAM-1 domains 4 and 5, P3.58a, P3.58BA.11 and P3.58BA.14 (a gift from Dr J. Johnson, Institute for Immunology, Munich, Germany); CA7 (a gift from Dr K. Kishimoto and Dr J. Woros, Boehringer Ingelheim); anti-α4 mAb HP1/2 (a gift from Dr R. Lobb, Biogen, Germany) and mAb HP1/2 (a gift from Dr R. Lobb, Biogen, Germany).

Abbreviations used: ICAM-1, intercellular adhesion molecule-1; LFA-1, leucocyte function-associated antigen-1; VCAM, vascular cell-adhesion molecule; MAAdCAM-1, mucosal addressin cell-adhesion molecule-1; GST, glutathione S-transferase; IgSF, immunoglobulin superfamily; mAb, monoclonal antibody.

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protocols. All were fully sequenced using dideoxy sequencing. COS-1 cells were transfected using DEAE-dextran and the secreted proteins were collected in serum-free medium for 4 days. The proteins were purified on a Protein A-Sepharose affinity column using standard methods. To ensure that equivalent amounts of wild-type and mutant ICAMs were used in the assays, protein estimations were carried out using the BCA protein assay kit (Pierce) and confirmed on SDS/polyacrylamide gels.

A panel of 10 mAbs against distinct epitopes in domains 1 and 2 of ICAM-1 [23] were tested in an ELISA and bound to the domain 2 mutant proteins in a comparable manner to wild-type ICAM-1Fc, suggesting that the proteins were folded into native conformation.

**Chimaeric proteins V1/I2-5Fc and I1/V2-3/I4-5Fc**

The vascular cell-adhesion molecule (VCAM)/ICAM hybrid (V1/I2-5Fc) was made by joining the first domain of VCAM-1 to domains 2–5 of ICAM-1Fc. This was done by PCR using site-directed mutagenesis with oligonucleotides P2 and P6 [31]. The ICAM/VCAM hybrid (I1/V2-3/I4-5Fc) was made by adding a *Bsu*36I site to the end of domain 1 of ICAM-1 using oligonucleotide P4 [31] and a *Bam*HI site to the start of ICAM-1 domain 4 using oligonucleotide 5′-CCCAGGATCCCTCTGACGAGCCAGCAGAG-3′ (the *Bam*HI site is underlined). VCAM-1 domains 2 and 3 were taken from plasmid VCAM-1/pCDM8 using a *Bsu*36I/ *Bgl*II digest and ligated to the ICAM-1 PCR fragments.

Both chimaeric proteins were fully sequenced and made by transfecting COS-1 cells as described above. ELISAs showed that V1/I2-5Fc expressed ICAM-1 domain 2–5 mAb epitopes and therefore had ICAM-1 domains with wild-type conformation (results not shown). The I1/V2-3/I4-5Fc protein was tested by ELISA using ICAM-1 mAbs with epitopes specific for domains 1, 2, 4 and 5 but only domains 4 and 5 were folded correctly (see Results).

**Synthesis of LFA-1 I domain-glutathione S-transferase (GST) fusion protein**

As described previously [28], the LFA-1 I domain (Leu-111–Ser-327) including the predicted I domain sequence and a 17-amino acid N-terminal extension was synthesized by PCR and ligated into pGEX2T (Pharmacia) to make the I-GST construct. Protein expression was induced in *Escherichia coli* Topp 2 (Stratagene) using isopropyl-β-D-thiogalactoside and the I-GST extracted from bacterial pellets using the GST purification kit (Pharmacia). In some experiments the I domain was cleaved from the GST tag with thrombin.

**Solid-phase ELISA**

The ELISAs were carried out as described previously [28]. The wells of Maxisorp 96-well plates (Nunc) were coated with 500–750 ng/well ICAM-1Fc in PBS for 16 h at 4 °C, then blocked with 3.5% BSA (Calbiochem) in TBS (20 mM Tris/HCl, pH 7.5/150 mM NaCl) at room temperature for 1 h. Wells were washed three times between each step with TBS. I-GST and GST control protein or I domain cleaved from the GST tag were washed three times between each step with TBS. I-GST and GST control protein or I domain cleaved from the GST tag were diluted in TBS/1 mM MgCl₂/1 mg/ml BSA and incubated for 3 h at 37 °C. Bound I-GST and GST were detected using 5 µg/ml rabbit anti-GST mAb (Sigma) followed by peroxidase-conjugated goat anti-rabbit IgG (Dako) diluted 1:1000. Bound cleaved I domain was detected using mAb MEM 83 at 5 µg/ml.
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Figure 2  Binding of LFA-1 I domain to wild-type (wt) ICAM-1 and ICAM-1 domain 2 mutants

Equivalent amounts of wild-type and mutant ICAM-1Fc proteins were plated on 96-well plates. Binding of equimolar quantities of I-GST and control protein GST to wild-type ICAM-1Fc is shown compared with I-GST binding to ICAM-1 domain 2 mutant proteins. (A) H152HG/AAA, (B) R125GE/AAA, (C) N156/A and (D) R149RD/AAA. Data are means ± S.D. from one representative experiment of triplicates (n = 3).

followed by peroxidase-conjugated goat anti-mouse IgG (Dako) at 1:1000. All antibodies were diluted in TBS/1 mM MgCl2/0.5 mg/ml BSA and incubated for 30 min at room temperature. Bound proteins were detected using o-phenylenediamine dihydrochloride (Sigma) and plates were read in an ELISA plate reader at 492 nm.

T-cell and Jurkat-β7 cell-adhesion assay

Immulon 1 96-well plates (Dynatech) were coated overnight at 4 °C with 50 μl/well of wild-type or mutant ICAM-1Fc diluted in PBS. Plates were blocked with 2.5% BSA in PBS for 1 h at room temperature, then washed 4× in Hepes buffer (20 mM Hepes/140 mM NaCl/2 mg/ml glucose, pH 7.4). T-cells, cultured as described previously [32], or J-β7 cells were suspended at 4×10⁶ cells/ml in Hepes buffer and labelled with 2.5 μM BCECF/AM [2′,7′-bis-(carboxyethyl)-5(6)-carboxyfluorescein/acetoxyethyl ester; Calbiochem] for 30 min at 37 °C. The labelled cells were washed and 2×10⁵ T-cells or 1.5×10⁵ J-β7 cells were added per well in Hepes buffer containing MgCl2 and EGTA. mAbs were added to a final concentration of 5 μg/ml. The plates were centrifuged for 1 min at 30 g, incubated at 37 °C for 30 min, then washed twice with warm Hepes buffer containing 0.4 mM MgCl2 and 0.4 mM CaCl2 to remove unbound cells. Bound cells were quantified using a CytoFluor multi-well plate reader series 4000 (PerSeptive Biosystems).

RESULTS

Analysis of hydrophilic loops in ICAM-1 domain 2

It was reasoned that if ICAM-1 domain 2 has a role in ligand binding, loops which are hydrophilic and therefore exposed might be involved. To determine likely sequences, domains 1 and 2 of ICAM-1 were analysed using a Kyte and Doolittle hydrophobicity plot [33] (results not shown) together with the recently published ICAM-1 crystal structure [11,12] (Figure 1). The two most hydrophilic loops in these domains were found to be the CC’ and EF loops in domain 2. Mutations were made in these loops at the most exposed residues. Therefore R<sup>135</sup>GE, which are the only residues in the CC’ loop, and H<sup>152</sup>HG, which are the most hydrophilic residues in the EF loop, were mutated to alanine residues in an ICAM-1Fc construct.

Isolated LFA-1 I domain binding to ICAM-1Fc is inhibited by domain 2 mutation H<sup>152</sup>HG/AAA

As the LFA-1 I domain contains the major binding site for ICAM-1, we investigated the effect of the domain 2 mutants on binding of the isolated I domain in an ELISA. The I domain made as a GST fusion protein (I-GST) showed impaired binding to H<sup>152</sup>HG/AAA mutant ICAM-1Fc compared with wild-type ICAM-1Fc (Figure 2A). However, the R<sup>135</sup>GE/AAA mutation in the CC’ loop had no effect (Figure 2B).

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Further mutations were made in the EF loop to investigate whether the region involved in reduction of I domain binding included residues in addition to H$_{152}$HG. Residues R$_{125}$RD and N$_{156}$A, which lie on either side of H$_{152}$HG, were mutated to alanine residues. Unlike H$_{152}$HG ICAM-1Fc, the R$_{125}$RD/AAA and N$_{156}$A ICAM-1Fc mutant proteins showed no reduction in I domain binding (Figures 2C and 2D). Similar data were also obtained using I domain cleaved from the GST tag (results not shown).

To test whether mutation of residues H$_{152}$HG had disrupted the structure of ICAM-1, ELISAs were carried out using a panel of 33 mAbs mapped to specific domains of ICAM-1. No significant differences in epitope expression were found between wild-type ICAM-1 and H$_{152}$HG AAA ICAM-1 (results not shown). The presence of all the epitopes tested shows that the inhibitory effects of the mutation are not simply due to disruption of ICAM-1 structure.

These results show that mutation of residues H$_{152}$HG affects the I domain-binding site; however, as the LFA-1 I domain has been shown to bind to domain 1 of ICAM-1 [28] there are two possible explanations for this. The mutation might either affect a ligand-binding site in ICAM-1 domain 2 or indirectly affect the I domain-binding site in ICAM-1 domain 1.

Isolated LFA-1 I domain does not bind to a hybrid construct containing domain 1 of VCAM-1 and domains 2–5 of ICAM-1

To directly investigate whether ICAM-1 domain 2 contains a ligand-binding site for the LFA-1 I domain, we made a hybrid protein replacing ICAM-1 domain 1 with VCAM-1 domain 1. This construct, V1/I2-5Fc, was used in an ELISA to test for ligand binding. As shown in Figure 3, the I domain did not bind to V1/I2-5Fc. Therefore these data indicate that there is no direct interaction between domain 2 of ICAM-1 and the LFA-1 I domain.

The LFA-1 receptor on T-cells has reduced affinity for domain 2 mutant H$_{152}$HG/AAA ICAM-1

We next tested the ability of T-cell LFA-1 to bind to the ICAM-1 domain 2 mutants. The greatest reduction in binding affinity was seen with mutation H$_{152}$HG/AAA. Although H$_{152}$HG/AAA ICAM-1Fc and wild-type ICAM-1Fc could support similar maximal levels of T-cell binding, a higher concentration of H$_{152}$HG/AAA ICAM-1Fc than wild-type ICAM-1Fc was required to achieve this. This reduction in binding affinity was seen whether T-cell LFA-1 was activated directly by Mg$^{2+}$ and EGTA (Figure 4A), Mn$^{2+}$ or activating mAbs, or indirectly via intracellular signalling pathways using phorbol ester, thapsigargin or ionomycin (results not shown). The effect of mutation...
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Figure 5 Adhesion of T-cells and J-β2.7 cells to V1/I2-5Fc compared with ICAM-1Fc and VCAM-1Fc

ICAM-1Fc, VCAM-1Fc and V1/I2-5Fc proteins were plated on 96-well plates at 10 μg/ml and tested for adhesion of (A) T-cells stimulated with 2 mM MgCl2 and 1 mM EGTA and (B) J-β2.7 cells stimulated with 1 mM MgCl2 and 1 mM EGTA. mAbs were used at 5 μg/ml. Data are means ± S.D. from one representative experiment of triplicates (n = 4).

H134HG/AAA was therefore independent of the route of activation. The protein concentrations required for saturated T-cell binding to the R149RD/AAA, N156/A and R158GE/AAA mutants was comparable with wild-type ICAM-1Fc (Figure 4B). The reduced binding of T-cell LFA-1 to ICAM-1 domain 2 mutant H134HG/AAA reflects the result observed with the isolated I domain.

T-cells and Jurkat-β2.7 cells bind V1/I2-5Fc via VCAM-1 domain 1 and not via ICAM-1 domain 2

Although the LFA-1 I domain did not bind to ICAM-1 domain 2, it was possible that another region of LFA-1 might bind to this domain. Therefore V1/I2-5Fc was tested for binding to T-cell LFA-1 and compared with wild-type ICAM-1Fc and VCAM-1Fc. All three proteins were bound equally by LFA-1. Blocking by anti-LFA-1 mAb 38 and anti-ICAM-1 mAb 15.2 shows that ICAM-1Fc binds to T-cells via LFA-1, whereas blocking by anti-α1 mAb HP1/2 and anti-β1 mAb P5D2 shows that VCAM-1Fc and V1/I2-5Fc bind T-cells via integrin α1β1 (Figure 5A).

Unexpectedly, the anti-ICAM-1 mAbs R6.5 and 8.4A6, which have epitopes at the top of domain 2 and block T-cell binding to ICAM-1Fc [7,23], also blocked adhesion to V1/I2-5Fc (Figure 5A). Since the anti-LFA-1 I domain mAb 38 does not block the interaction between T-cells and V1/I2-5Fc, this result suggested that LFA-1 might bind to ICAM-1 domain 2 via a domain other than the I domain or, alternatively, that the mAbs might indirectly block binding of α1β1 to VCAM-1 domain 1.
To test whether the anti-ICAM-1 domain 2 mAbs blocked indirectly, we used the Jurkat cell line J-b#7, which expresses $\alpha_b$ but not $\beta_2$ integrins on the cell surface [30]. It can be seen that, like binding of T-cells, J-b#7-cell binding to V1/I2-5Fc was blocked by anti-\(\alpha_b\) and anti-\(\beta_2\) mAbs and also by anti-ICAM-1 domain 2 mAbs (Figure 5B). Since there was no binding to ICAM-1 by this cell line, the mAbs to ICAM-1 domain 2 must be indirectly blocking $\alpha_b/\beta_2$ binding to VCAM-1 domain 1 rather than directly inhibiting an LFA-1 ligand-binding site in domain 2.

**Domain 1 of ICAM-1 does not fold correctly in the ICAM/VCAM hybrid protein I1/V2-3/I4-5Fc**

To further investigate the apparently exclusive role of ICAM-1 domain 1 in LFA-1 recognition, we made a second chimaeric protein in which ICAM-1 domains 2 and 3 were replaced by the homologous domains 2 and 3 of VCAM-1. This protein could not be used for ligand-binding studies as the epitopes of mAbs specific for ICAM-1 domain 1 were absent, indicating the complete loss of ICAM-1 domain 1 structure (Figure 6). In contrast, all the ICAM-1 domain 4 and 5 mAb epitopes tested were expressed at similar levels to wild-type ICAM-1. This shows that the correct conformation of ICAM-1 domain 1 is particularly dependent upon ICAM-1 domain 2, whereas folding of the membrane-proximal domains 4 and 5 is more independent.

**DISCUSSION**

It has been suggested that the second domain of ICAM-1 might contain a ligand-binding site for LFA-1 [7,23], but the precise role of this domain has not been established. We investigated a
possible role in ligand binding for the two most hydrophilic regions in the first two domains of ICAM-1, the CC’ and EF loops in domain 2. Our results show that binding of the I domain, which contains the major ligand-binding site in LFA-1, was impaired by mutation of residues H\textsuperscript{126HG} in the EF loop of domain 2 compared with wild-type ICAM-1. This same sensitivity was reflected by reduced binding affinity of LFA-1 on T-cells. A hybrid construct with VCAM-1 domain 1 fused to ICAM-1 domains 2–5 (V1/I2-5Fc) was not recognized by isolated I domain or LFA-1 on T-cells. Further, mAbs specific for ICAM-1 domain 2 inhibited binding of LFA-1 to ICAM-1 and also \( \alpha_\beta \beta_1 \) to domain 1 of VCAM-1 in the hybrid protein, V1/I2-5Fc, suggesting they have an indirect effect. Together these results suggest that the second domain of ICAM-1 does not bind LFA-1 directly but has an essential indirect role in ligand binding.

The mAbs R6.5 and 8.4A6, which have epitopes localized to the top of ICAM-1 domain 2 on the BC and FG loops respectively, inhibit binding to LFA-1 and thus implicate domain 2 in ligand binding [7,23]. Our data show that these mAbs are also able to block \( \alpha_\beta \beta_1 \) binding to VCAM-1 domain 1 in the V1/I2-5Fc construct. Since \( \alpha_\beta \beta_1 \) does not bind to ICAM-1, this result argues against the idea that the mAbs inhibit a domain 2 ligand-binding site. Instead it suggests that these mAbs act indirectly, either sterically inhibiting ligand binding to the first domain, or blocking interdomain movements essential for ligand recognition. The crystal structures of the first two domains of IgSF proteins ICAM-1, ICAM-2, VCAM-1 and mucosal addressin cell-adhesion molecule-1 (MAdCAM-1) have been solved and show that the junction between these domains is conserved (reviewed in [34]). The structures show that there are two hydrophobic patches at the interface of the two domains created by hydrogen bonding and non-polar interactions. These patches are consistent with interdomain flexibility around a pivot point between the last residue of domain 1 and the first residue of domain 2. One of these hydrophobic patches includes the BC and FG loops to which the ICAM-1 domain 2 function-blocking mAbs R6.5 and 8.4A6 have been mapped. Therefore these mAbs may inhibit ligand binding to ICAM-1 and V1/I2-5Fc by preventing interdomain movement in this region, which is essential for ligand binding to domain 1. In support of this, the BC and FG loops at the top of domain 2 in CD4 have been shown to be important for interdomain movements required during ligand binding [35].

Ligand binding to ICAM-1 domain 1 seems to be sensitive not only to structural features at the top of domain 2, but also to the integrity of the bottom of the domain, as we have shown through mutation of the EF loop of domain 2. The ICAM-1 binding site for LFA-1 has been located in the C, D, F and G strands in the top half of domain 1 (Figure 1) [7,36]. The LFA-1 I domain binds to domain 1 and includes residue E\textsuperscript{24} on the C strand in its recognition sequence [28]. Since the I domain binding face is \( \approx 25 \AA \) in diameter and the first domain of ICAM-1 is \( \approx 38 \AA \) in length, a single I domain would be unable to be in contact with residues in the EF loop at the bottom of domain 2 as well as the C strand in domain 1. In addition, the ICAM-1 crystal structure shows that residues H\textsuperscript{126HG} are located on the back of the molecule relative to the domain 1-binding site, indicating further that LFA-1 would be unlikely to make contact with both regions. Finally the lack of binding of LFA-1 or its I domain to the V1/I2-5Fc construct suggests that the EF loop mutation H\textsuperscript{126HG} affects the ligand-binding site in domain 1. In spite of extensive analysis of mAb epitopes throughout domains 1–5 of ICAM-1 there were no apparent structural differences between H\textsuperscript{126HG}/AAA ICAM-1 and wild-type ICAM-1. However, the hydrogen-bonding network at the bottom of domain 2 would be perturbed by mutation of residues H\textsuperscript{126HG} and this could have a ‘knock-on’ effect, transmitting through domain 2 to the domain 1-binding site. It is possible that docking of the E34 region of ICAM-1 to the LFA-1 I domain may have very precise requirements. These conclusions are supported by a study showing that the affinity of rhinovirus binding to ICAM-1 domain 1 is affected by folding at the bottom of domain 2 [37].

In an attempt to show that ligand binding is confined to the first domain of ICAM-1 we made a hybrid construct in which ICAM-1 domains 2 and 3 were replaced by VCAM-1 domains 2 and 3. In this construct the first domain of ICAM-1 failed to fold correctly but domains 4 and 5 expressed all mAb epitopes tested. This demonstrates further the dependence of the first domain of ICAM-1 on domain 2. The second domains of ICAM-1 and VCAM-1 have similar structure, both belonging to the I2 subset [34]; therefore it was expected that domain 2 of VCAM-1 could be substituted for domain 2 of ICAM-1. However, in spite of the structural similarities between ICAM-1 and VCAM-1, there is obviously less dependency of the first domain of VCAM-1 on its second domain, since domain 2 of ICAM-1 could be substituted in hybrid protein V1/I2-5Fc, resulting in ligand binding equivalent to wild-type VCAM-1.

A previous study of the IgSF molecules VCAM-1 and MAdCAM-1 reports a requirement for domains 1 and 2 for ligand binding [38]. The ligand-binding site in domain 1 of ICAM-1 is located on a flat surface [12], whereas the binding sites in domain 1 of VCAM-1 and MAdCAM-1 protrude from the domain on a loop [39]. Therefore these ligands may require domain 2 for integrin binding whereas ICAM-1 does not. However, it is possible that the mutations made in domain 2 of VCAM-1 and MAdCAM-1 indirectly affect the domain 1-binding site in a similar manner to the H\textsuperscript{126HG} mutation in ICAM-1. For ICAM-3, which is expected to have a similar domain 1-binding surface to ICAM-1, chimaeric constructs and mutations in domains 1 and 2 indicate that, as our data suggests for ICAM-1, the first domain of ICAM-3 contains the complete LFA-1-binding site [26,40,41]. A recent study of ICAM-5 using domain deletion constructs and mapped mAbs indicates that, for ICAM-5 also, only the first domain is required for binding LFA-1 [42]. Therefore location of the LFA-1-binding site solely in domain 1 seems to be a common feature for the ICAMs. However, unlike the other ICAMs, the first domain of ICAM-1 is dependent on the second domain for maintaining its structure, although the reason for this difference is unclear.

In summary, we have shown that domain 2 of ICAM-1 indirectly affects binding of the LFA-1 I domain to domain 1 of ICAM-1 although it does not seem to directly bind to LFA-1. Our data give further insight into the interaction between LFA-1 and ICAM-1 and demonstrates that immunoglobulin-like domains which do not bind ligand can have an essential role in maintaining the structure of the ligand-binding site.

We thank Dr L. Osborn and Dr R. Lobb (Biogen, Cambridge, MA, U.S.A.) for VCAM-1 cDNA and VCAM-1Fc protein; and Dr D. Altiere, Dr A. Boyd, Dr D. Haskard, Dr V. Horesji, Dr J. Johnson, Dr K. Kishimoto, Dr R. Rothlein, Dr J. Woska and Leukocyte Typing workshops IV and V for mAbs. We thank our colleagues Birgit Leitinger and Matthew Robinson for their helpful comments on this manuscript.

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