Molecular Biology of the Cell
Vol. 8, 501–515, March 1997

Identification of the Binding Site in Intercellular Adhesion Molecule 1 for Its Receptor, Leukocyte Function-associated Antigen 1

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Submitted October 3, 1996; Accepted December 27, 1996
Monitoring Editor: Masatoshi Takeichi

Intercellular adhesion molecule 1 (ICAM-1, CD54) is a member of the Ig superfamily and is a counterreceptor for the β2 integrins: lymphocyte function-associated antigen 1 (LFA-1, CD11a/CD18), complement receptor 1 (MAC-1, CD11b/CD18), and p150,95 (CD11c/CD18). Binding of ICAM-1 to these receptors mediates leukocyte-adhesive functions in immune and inflammatory responses. In this report, we describe a cell-free assay using purified recombinant extracellular domains of LFA-1 and a dimeric immunoadhesin of ICAM-1. The binding of recombinant secreted LFA-1 to ICAM-1 is divalent cation dependent (Mg^{2+} and Mn^{2+} promote binding) and sensitive to inhibition by antibodies that block LFA-1-mediated cell adhesion, indicating that its conformation mimics that of LFA-1 on activated lymphocytes. We describe six novel anti-ICAM-1 monoclonal antibodies, two of which are function blocking. Thirty-five point mutants of the ICAM-1 immunoadhesin were generated and residues important for binding of monoclonal antibodies and purified LFA-1 were identified. Nineteen of these mutants bind recombinant LFA-1 equivalently to wild type. Sixteen mutants show a 66–2500-fold decrease in LFA-1 binding yet, with few exceptions, retain binding to the monoclonal antibodies. These mutants, along with modeling studies, define the LFA-1 binding site on ICAM-1 as residues E34, K39, M64, Y66, N68, and Q73, that are predicted to lie on the CDFG β-sheet of the Ig fold. The mutant G32A also abrogates binding to LFA-1 while retaining binding to all of the antibodies, possibly indicating a direct interaction of this residue with LFA-1. These data have allowed the generation of a highly refined model of the LFA-1 binding site of ICAM-1.

INTRODUCTION

Leukocyte function-associated antigen 1 (LFA-1)[1] is a member of the integrin family of cell adhesion receptors that is expressed exclusively on leukocytes. LFA-1 is a noncovalent heterodimer of an αL (CD11a) subunit of relative mass (M_r) ~180,000 and a β2 (CD18) subunit of M_r ~95,000. A common β2 subunit is shared by LFA-1, MAC-1, p150,95, and αdβ2 which are expressed on lymphoid and myeloid leukocytes (Kishimoto *et al.*, 1987; Arnaout *et al.*, 1988; Corbi *et al.*, 1987, 1988; Larson *et al.*, 1989; Van der Vieren *et al.*, 1995). The extracellular domains of LFA-1 play a role in ligand binding, whereas the cytoplasmic domains are thought to mediate transmembrane signaling and modulation of LFA-1 affinity for ligands (Hynes, 1992; Diamond and Springer, 1994). Binding of LFA-1 to its counterreceptors mediates adhesive interactions at multiple stages in the immune response, including

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[1] Abbreviations used: ELISA, enzyme-linked immunosorbent assay; HRP, horseradish peroxidase; ICAM-1, intercellular adhesion molecule 1; LFA-1, leukocyte function-associated antigen 1; mAb, monoclonal antibody; OPD, *o*-phenylenediamine dihydrochloride; sLFA-1, secreted leukocyte function-associated antigen-1; VCAM-1, vascular cell adhesion molecule 1.

leukocyte adhesion to activated endothelium and antigen-dependent T and B cell responses (Kishimoto *et al.*, 1989; Dustin and Springer, 1991).

Thus far, three counterreceptors for LFA-1 have been identified: ICAM-1, ICAM-2, and ICAM-3. ICAM-1 is widely expressed on leukocytes, endothelium, epithelium, and fibroblasts and is up-regulated at sites of inflammation in response to bacterial antigens and cytokines (Dustin *et al.*, 1986; Kishimoto *et al.*, 1989; Dustin and Springer, 1991). In contrast, ICAM-2 and ICAM-3 are constitutively expressed on a subset of these same cell types. Antibodies to ICAM-1 and LFA-1 have been shown to inhibit numerous T cell functions in vitro. In vivo, these antibodies have been shown to modulate the inflammatory response by efficiently prolonging tumor and allograft survival (Heagy *et al.*, 1984; Isobe *et al.*, 1992), inhibiting acute inflammatory damage of the liver and kidney (Kawasaki *et al.*, 1993; Tanaka *et al.*, 1993), or inhibiting in a model of autoimmune disease (Connolly *et al.*, 1994). In addition, circulating forms of shed ICAMs have been detected in a number of inflammatory disease states (Simmons, 1995).

ICAM-1 is a heavily glycosylated cell surface protein of M_r ~90,000 with a large extracellular domain containing five tandem Ig-like domains, a transmembrane domain, and a cytoplasmic tail (Dustin *et al.*, 1986; Simmons *et al.*, 1988; Staunton *et al.*, 1988). As such, ICAM-1 is a member of the Ig superfamily, which also contains the integrin counterreceptors ICAM-2, -3, VCAM-1, platelet endothelial adhesion molecule 1, and mucosal addressin cell adhesion molecule 1 (Osborn *et al.*, 1989; Staunton *et al.*, 1989; Newman *et al.*, 1990; Simmons *et al.*, 1990; Stockinger *et al.*, 1990; Fawcett *et al.*, 1992; Vazeux *et al.*, 1992; Briskin *et al.*, 1993). Interactions of both ICAM-1 and -3 with LFA-1 have been shown to occur primarily through the amino-terminal (N-terminal) domain. Site-directed mutagenesis has defined amino acids in these domains that are critical to LFA-1 binding. β-strands A, B, E, F, and G of ICAM-1 domain 1 have been extensively mutagenized (Staunton *et al.*, 1990) and only two individual residues, E34 and Q73 located respectively in β-strands C and G, were found to have a major effect on LFA-1 binding. These have been aligned with residues in VCAM-1 that play a role in receptor binding to define a conserved integrin binding pentapeptide motif (Sadhu *et al.*, 1994). Although several models of the N-terminal domains of ICAM-1 and -3 (Giranda *et al.*, 1990; Staunton *et al.*, 1990; Berendt *et al.*, 1992; Sadhu *et al.*, 1994) have been proposed and the crystal structure of the N-terminal domain of human VCAM-1 has been solved (Jones *et al.*, 1995), a well-defined surface which comprises the LFA-1 binding site in ICAM-1 is lacking.

In this report, we describe structural and functional analysis of the N-terminal domain of ICAM-1. A protein assay, using the purified extracellular domains of human LFA-1 (sLFA-1) and a human ICAM-1 immunoadhesin (5dICAMIg), was established that permits analysis of binding in the absence of cellular modulation of receptor/counterreceptor affinity. We localized residues in ICAM-1 that interact with LFA-1 and defined the epitopes for a panel of novel monoclonal antibodies (mAbs) to ICAM-1. Together these data have been used to generate a high-resolution model of the ICAM-1-binding surface that may be useful in the design of ICAM mimetics as anti-inflammatory therapeutics.

MATERIALS AND METHODS

ICAM-1 Expression Constructions

A plasmid for the expression of a human ICAM-1 (Simmons *et al.*, 1988; Staunton *et al.*, 1988) immunoadhesin called pRK.5dICAMGaIg was constructed. It contains the five Ig-like domains of ICAM-1, a six-amino acid cleavage site recognized by an H64A variant of subtilisin BPN', Genenase I (Carter *et al.*, 1989), and the Fc region from human IgG1 (Ellison *et al.*, 1982) in the pRK5 vector (Eaton *et al.*, 1986).

The following primers were used to generate a fragment containing the five Ig-like domains of ICAM-1 from the full-length ICAM-1 cDNA by 20 cycles of polymerase chain reaction (PCR) amplification: 1) a 17-b forward primer that matched a portion of the vector sequence 5′ of the ICAM-1 coding sequence 5′-TGCCTTTCTCTCCACAG-3′; and 2) a 48-b reverse primer that matched seven amino acids at the 3′ end of Ig-like domain 5 and contained sequence coding for the Genenase I cleavage site and a *Dra*III restriction site 5′-GGTGGGCACAGAGTGTAGTGCGCAGCCTCATACCGGGGGGAGAGCACA-3′. This amplification produced a fragment of expected size that was digested with restriction enzymes *Cla*I and *Dra*III (New England Biolabs, Beverly, MA). The resulting 1515-bp fragment was gel isolated, purified, and ligated using *Cla*I and *Dra*III restriction sites (New England Biolabs) into a 5378-bp pRK5 vector fragment containing the human IgG1 Fc. DNA was transformed into MM294-competent cells.

An expression vector containing only the first three domains of human ICAM-1 was also constructed as an immunoadhesin called pRK.3dICAMIg. In this vector, a five-amino acid (DKTHT) linker from the C-terminus of the IgG1 CH1 domain is present between the C-terminal residue of ICAM-1 (N291) and the N-terminal Cys of the human Fc hinge, and there is no Genenase I cleavage site.

A chimera was constructed containing the two N-terminal Ig-like domains from ICAM-1 and domain 3 from murine VCAM-1 (Hession *et al.*, 1992). This plasmid, pRK.I12V3GaIg, was constructed using the same vector as pRK.5dICAMGaIg. The forward PCR primer used to generate the ICAM-1 fragment was the same as that used to make pRK.5dICAMGaIg. The 34-bp reverse primer contained ICAM-1 domain 2 and VCAM-1 domain 3 sequences and introduced a *Sst*II restriction site: 5′-TGTATTCCGCGGCAGGAC AAAGGTCTGGAGCTGG-3′. The primers used to generate VCAM-1 domain 3 are 5′-ACATCTCTCCGCGGAATACAACG A-3′ (forward) and 5′-GGTGGGCACAGAGTGTAGTGCGC AGCTTTCTCTTGAACAACTAATTCCAC-3′ (reverse) and introduced a Genenase I cleavage site and *Sst*II and *Dra*III restriction sites. Each of the final constructions were completely sequenced using dideoxy chain termination methods (Sequenase 2.0 kit, United States Biochemical, Cleveland, OH).

Expression and Purification of Recombinant Five-Domain ICAMIgG

Human embryonic kidney 293 cells (Graham *et al.*, 1977) were stably transfected with pRK.5dICAMGaIg and the RSV-neo plasmid (Gor-

man *et al.*, 1983) to generate a cell line expressing the five-domain ICAMIg (5dICAMIg). A clone that expressed 20 μg/ml of secreted 5dICAMIg was selected by the enzyme-linked immunosorbent assay (ELISA) using antibodies to human IgG Fc (Caltag, Burlingame, CA) and ICAM-1 (BBIG-I1, R & D Systems, Minneapolis, MN). Cell culture supernatant from this cell line was loaded onto a protein A column (ProsepA, Bioprocessing, Ltd., Durham, United Kingdom) equilibrated in 0.01 M *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) buffer (pH 7.0) and 0.15 M NaCl (HBS), and the column was washed with HBS followed by 0.01 M HEPES buffer (pH 7.0), 0.5 M NaCl, and 0.5 M tetramethylammonium chloride to remove nonspecifically bound material. The tetramethylammonium chloride buffer was thoroughly washed from the column with HBS, and the 5dICAMIg was eluted with 0.01 M HEPES buffer (pH 7.0), 3.5 M $MgCl_2$, and 10% (wt/vol) glycerol. The protein A pool was dialyzed extensively against HBS and concentrated. Protein concentration was determined by quantitative amino acid analysis. A similar method was used for the purification of 3dICAMIg. Purified human and murine VCAM-1 immunoadhesins were provided by S. Jones (Genentech, Inc.; Renz *et al.*, 1994).

Expression and Purification of Recombinant sLFA-1

cDNAs of the α (CD11a) and β (CD18) subunits of human LFA-1 (Kishimoto *et al.*, 1987; Larson *et al.*, 1989) were subcloned into an expression vector under the control of separate SV40 early promoters. A similar plasmid, containing the simian virus 40 (SV40) early promoter in place of the human cytomegalovirus promoter, was described previously (Eaton *et al.*, 1986). cDNAs encoding CD11a and CD18 were truncated at the amino acid residue immediately preceding the transmembrane domain, Q1063 in CD11a and N678 in CD18. This plasmid, pSVI6BLFAα+β_s, was cotransfected with a plasmid containing the dihydrofolate reductase (DHFR) cDNA, into DHFR-Chinese hamster ovary (CHO) cells, using Ca^{2+} phosphate precipitation (Gorman *et al.*, 1983). Transfected cells were grown under increasing concentrations of methotrexate, up to 1 μM, to establish a stable cell line (Wurm *et al.*, 1986). A clone that expressed 34 μg/ml sLFA-1 was selected using the ELISA. For the ELISA, microtiter plates were coated with the anti-CD11a monoclonal antibody (mAb) MHM24 (Hildreth *et al.*, 1983) and blocked in phosphate-buffered saline (PBS) containing 0.05% bovine serum albumin (BSA), 0.05% Tween 20, and 0.01% thimerosal. Samples were diluted in the same buffer and incubated for 2 h followed by a 1-h incubation with a horseradish peroxidase- (HRP) conjugated secondary mAb, MHM23 (which binds to CD18 in complex with an α subunit; Hildreth *et al.*, 1983). Plates were developed with *o*-phenylenediamine dihydrochloride (OPD; Sigma, St. Louis, MO) and absorbance was read at 492 nm.

sLFA-1-expressing cell culture supernatants were concentrated approximately 20-fold and then diafiltered into 0.01 M HEPES buffer containing 0.05 M NaCl, 1 mM $MgCl_2$, and 1 mM $CaCl_2$ at pH 7.0 (buffer A). This material was applied to Q-Sepharose fast-flow anion exchange resin (Pharmacia, Piscataway, NJ) equilibrated in buffer A, washed extensively, and eluted using a 0.05–0.25 M NaCl gradient in buffer A. Fractions were collected and the sLFA-1 was pooled based on ELISA data (see above), concentrated, clarified by centrifugation at 10,000 × *g* for 20 min, and loaded onto a size exclusion column (S300HR; Pharmacia) equilibrated in buffer A. Fractions were collected and pooled based on ELISA data. A DEAE-Sepharose fast-flow column (high capacity; Pharmacia) was equilibrated in buffer A, and the S300HR pool was applied and washed with buffer A. The column was eluted using a gradient of 0.05 M to 0.15 M NaCl in buffer A. Isocratic washing of the column was continued using 0.15 M NaCl in 0.01 M HEPES buffer (pH 7.0), 1 mM $MgCl_2$, and 1 mM $CaCl_2$ (buffer B) until the absorbance at 280 nm had returned to baseline. Fractions were collected throughout the elution, and material was pooled based on analysis by ELISA and SDS-PAGE. The DEAE pool was adjusted to a concentration of 1 M NaCl and was passed through a phenyl-Sepharose fast-flow column (low substitution; Pharmacia) that had been equilibrated in 0.01 M HEPES, 1 M NaCl, 1 mM $MgCl_2$, and 1 mM $CaCl_2$ at pH 7.0 (buffer C). The sLFA-1 does not bind under these conditions and was collected as a single flow-through fraction, concentrated, and dialyzed into buffer B.

sLFA-1/ICAM-1 Binding Assay

Nunc MaxiSorp plates (VWR Scientific Products, Brisbane, CA) were coated with goat anti-human IgG Fc antibody (Caltag, Burlingame, CA). 5dICAMIg and other reagents were diluted in divalent cation buffer containing 0.3% BSA, 1 mM $MnCl_2$, 0.05% Tween 20, 20 mM HEPES (pH 7.2), and 140 mM NaCl. Plates were blocked in 1% BSA, washed, and 5dICAMIg was bound for 2 h. Plates were washed and 2 μg/ml sLFA-1 were added, in the presence or absence of inhibitors, and bound for 2 h. Plates were washed and 2.5 μg/ml of the anti-CD18 mAb PLM-2 (Hildreth *et al.*, 1989) was bound for 1 h. A 1:3000 dilution of a HRP-conjugated rabbit anti-murine Fc (Jackson Laboratories, Bar Harbor, ME) was bound for 1 h, plates were developed with OPD, and absorbance was read at 490–405 nm.

Generation, Epitope Mapping, and Functional Characterization of Antibodies to ICAM-1

Mice were immunized in the foot pads with 5 μg/mouse of recombinant full-length ICAM-1 (Edwards *et al.*, 1995) in monophosphoryl lipid A/trehalose dicorynomycolate (Ribi Immunochem, Hamilton, MT) six times at 7-day intervals. Three days after the last immunization, popliteal lymph node cells from immunized mice were fused with P3X63Ag 8U.1 myeloma cells (Chuntharapai and Kim, 1996). Hybridoma cell lines were initially selected for the presence of anti-ICAM-1 activity using the ELISA followed by the flow cytometric analysis of 293 cells transfected with human ICAM-1. Positive hybridomas were selected, cloned twice, and retested with the ELISA and flow cytometric analysis. The isotypes of the mAbs were determined with the ELISA using isotype-specific alkaline phosphatase-conjugated goat anti-mouse Ig (Meloy, Springfield, VA).

The domains of ICAM-1 recognized by mAbs were determined in a capture ELISA using 1–3 domain ICAMIg, 1–5 domain ICAMIg, and 1–2 domain ICAM/3 domain VCAMIg. Nunc 96-well plates were coated with 5 μg/ml goat anti-human IgG Fc antibody (Organon Teknika Corp., West Chester, PA) and blocked with PBS containing 2% BSA. Plates were incubated with 2 μg/ml of purified 3dICAMIg or culture supernatant of 293 cells expressing 5dICAMIg or the ICAM/VCAM chimera for 1 h. mAb at 2 μg/ml was bound to the wells for 1 h followed by incubation with HRP-conjugated goat anti-mouse Ig for 1 h. 3,3',5,5'-tetramethylbenzidine (Microwell peroxidase substrate, Kirkegaard and Perry Labs, Gaithersburg, MD) was used for the color detection and absorbance was read at 450 nm. Between each step the plates were washed with PBS buffer containing 0.01% Tween 20. To determine whether the anti-ICAM-1 antibodies recognize the same or different epitopes, a competitive binding ELISA was performed in which the binding of biotinylated mAb was competed with excess unlabeled mAb as described previously (Kim *et al.*, 1992).

To assess the ability of mAbs to block LFA-1-mediated cell adhesion, Nunc 96-well plates were coated with 5 μg/ml full-length recombinant ICAM-1 protein (Edwards *et al.*, 1995). Nonspecific binding sites were blocked with RPMI 1640 containing 5 mg/ml BSA (RPMI-BSA) for 1 h. 293 cells transfected with human LFA-1 (Edwards *et al.*, 1995) were labeled with 10 μM calcein (Sigma) for 30 min at 37°C and 2×10^5 cells were added to ICAM-1-coated wells in the presence of 5 μg/ml anti-ICAM antibodies. Cell attachment was carried out for 15 min at 37°C, cells were washed once with 150 μl RPMI-BSA and unbound cells were removed by centrifuging the inverted plate at 200 × *g* for 5 min. Bound cells were lysed in 0.1% SDS/50 mM Tris, and the fluorescence was measured at 515 nm

(Fluostar, SLT-Labinstruments, Salzburg, Austria). Commercial antibodies to human ICAM-1 used in these studies were BBIG-I1 (R & D Systems, Minneapolis, MN) and 84H10 (AMAC, Westbrook, ME).

Mutagenesis and Functional Analysis of 5dICAMIg

Site-directed mutations were made in pRK.5dICAMGaIg by either the method of Kunkel (1985) or by using the Chameleon mutagenesis kit (Stratagene, La Jolla, CA). To reduce the amount of sequencing required on each of the mutants, an 871-bp *Eco*RI/*Stu*I fragment of pRK.5dICAMGaIg containing the 5′ untranslated sequence, the signal sequence, and 252 amino acids of mature ICAM-1 was subcloned into LITMUS28 (New England Biolabs, Beverly, MA). Site-directed mutations were made in the subclone and the *Eco*RI/*Stu*I insert cloned back into pRK.5dICAMGaIg for expression. All mutants were fully sequenced using the dideoxy chain termination method (Sequenase 2.0 kit, United States Biochemical, Cleveland, OH) or automated sequencing methods (Perkin Elmer-Cetus, Applied Biosystems Division, Foster City, CA). A list of mutagenic primers is available upon request.

Three assays were performed on cell supernatants from ICAM-1 wild-type, mutant, and mock-transfected 293 cells: a quantitative ELISA, antibody binding to a panel of anti-ICAM-1 antibodies, and a functional sLFA-1-binding assay. All assays were carried out on Nunc MaxiSorp plates coated with goat anti-human Fc antibody (Caltag). Wash buffer was 150 mM NaCl, 20 mM HEPES, and 0.05% Tween 20. After coating, plates were blocked with 1% BSA in PBS. For the ELISA, cell supernatants were serially diluted 1:5 and bound for 2 h. Purified or wild-type 5dICAMGaIg of known concentration was diluted fivefold serially from 500 ng/ml for use as a standard. Plates were washed and a 1:1000 dilution of a HRP-conjugated goat anti-human Fc antibody (Caltag) was bound. Plates were washed and developed with OPD before reading absorbance at 490–405 nm.

For antibody binding, samples and standard were diluted to 50 or 100 ng/ml and bound to coated plates for 2 h. Biotinylated anti-ICAM antibodies BBIG-I1, 1B9, 1E8, 4B2, 4D3, 4G3, and 6A12 or unconjugated 84H10 were bound at 0.4 or 1 μg/ml for 1 h. Streptavidin-HRP (1:5000 dilution, Zymed, South San Francisco, CA) was used to detect biotinylated antibodies and a 1:5000 dilution of a HRP-conjugated goat anti-mouse (gamma and light) antibody (Tago, Burlingame, CA) was used to detect 84H10. The plates were washed, developed with OPD, and absorbance was read at 490–405 nm.

To test mutants for sLFA-1 binding (see sLFA-1/ICAM-1 Binding Assay), cell supernatants were initially diluted threefold serially from 300 to 0.1 ng/ml based on ELISA quantitation of expression levels. When no or reduced binding was observed, samples were assayed undiluted and diluted twofold serially in divalent cation buffer. For each mutant, binding data from the titration were plotted and subjected to a four-parameter fit analysis to determine the EC_{50}. The average EC_{50} for wild-type 5dICAMIg was 35 ± 18 ng/ml (SEM). Mutant values are reported as the EC_{50} relative to the wild-type value for that experiment.

Modeling of ICAM-1

The model of domains 1 and 2 of human ICAM-1 was based on the crystal structure of human VCAM-1 (Jones *et al.*, 1995). The ICAM-1 sequence was aligned with human VCAM-1; all insertions and deletions in ICAM-1 relative to VCAM-1 mapped to surface loops in the VCAM-1 structure. Two *cis*-Pro residues, one in the VCAM-1 domain 1 β-strand A and the other in domain 2 loop BC (the loop connecting β-strands B and C), were retained in the ICAM-1 model. One major difference between VCAM-1 and the ICAM-1 model involves β-strand D; in VCAM-1 this β-strand interacts with β-strand E, whereas in the ICAM-1 model (Figure 5), the β-strand D interacts with β-strand C [similar to the interaction of β-strands D and C in CD4 domain 2 (Wang *et al.*, 1990) and CD2 domain 2 (Jones *et al.*, 1992)]. In a previous model (Giranda *et al.*, 1990), the β-strand D interacts with β-strand E.

VCAM-1 was transformed into ICAM-1 in two steps. First, all residues except those involving insertions/deletions were changed to the ICAM-1 sequence using the INSIGHT-II program (Biosym Technologies, San Diego, CA). If possible, conformations of ICAM-1 side chains were kept similar to those of VCAM-1, otherwise they were based on rotamer libraries (Ponder *et al.*, 1987), packing and hydrogen-bonding considerations. Second, possible loop structures for the ICAM-1 insertions/deletions were gleaned from a search of crystal structures in the Protein Data Bank (Bernstein *et al.*, 1977) using the INSIGHT-II program.

Hydrogen atoms were added to the structure using INSIGHT-II, and positions of hydrogens on Ser, Thr, and Tyr side chains were checked visually for proper alignment in hydrogen bonds, if present. The ICAM-1 model was then subjected to 7500 cycles of energy minimization using the DISCOVER program (Biosym Technologies); 500 cycles of steepest descent minimization was followed by 7000 cycles of conjugate-gradient minimization. The all-atom AMBER force field (Weiner *et al.*, 1984, 1986) was used for all calculations, employing a 15 Å cutoff for nonbonded interactions, a distance-dependent dielectric (E = 4.0*r), and scaling of 1–4 interactions by 0.5; Coulombic forces were not included.

RESULTS

Expression and Purification of ICAM-1 and sLFA-1

The extracellular domain of human ICAM-1 was expressed as an immunoadhesin to facilitate protein purification and use in structural and functional assays of wild-type and mutant proteins. Immunoadhesins containing the first three (3dICAMIg) or all five Ig-like domains (5dICAMIg) bound ICAM-1 antibodies and sLFA-1 (our unpublished results and Figure 2). The expression level of the 5dICAMIg was higher than that of the 3dICAMIg, and the five domain form was chosen for mutagenesis. Two chimeras, one containing the first two domains of ICAM-1 and the third domain of murine VCAM-1, and the other containing the first domain of ICAM-1 and the second and third domains of murine VCAM-1, were also made as immunoadhesins. The latter proved to be nonfunctional and incapable of binding to a panel of anti-ICAM-1 antibodies while the former bound sLFA-1 and was used to define the domain specificity of anti-ICAM-1 antibodies.

The expression plasmid containing 5dICAMIg was transfected into human embryonic kidney 293 cells along with the neomycin resistance marker (see MATERIALS AND METHODS) to establish a stable clonal cell line. 5dICAMIg was purified from culture supernatants over a protein A column and characterized by SDS-PAGE (Figure 1A). Purified 5dICAMIg, estimated to be >95% pure, migrated as two bands of M_r ~100,000 and ~200,000 under nonreducing conditions and M_r ~80,000 under reducing conditions. The molecular weight of the larger band corresponds to the expected size for a dimeric immunoadhesin containing the five Ig-like domains of ICAM-1. The molecular weight of the lower band, along with behavior on gel filtration chromotography (our unpublished results),

is consistent with this being a monomeric form of the immunoadhesin in which disulfides have been cleaved or failed to form. Sequence analysis of the purified 5dICAMIg revealed that the NH_2 terminus of >95% of the protein was blocked, as predicted by the ICAM-1 cDNA sequence (Simmons *et al.*, 1988; Staunton *et al.*, 1988). Final protein concentration was determined by quantitative amino acid analysis.

To analyze direct binding of purified 5dICAMIg in a cell-free assay system, a secreted form of human LFA-1 (sLFA-1) was expressed and purified. The cDNAs for both the α and β subunits were subcloned into an SV40 expression vector, cotransfected into CHO cells with a DHFR-containing plasmid, and a stable clonal cell line was established (see MATERIALS AND METHODS). Culture supernatants were concentrated and subjected to chromatography (see MATERIALS AND METHODS) to obtain purified sLFA-1 protein. Material from each step of the purification was analyzed by SDS-PAGE and final purified protein was estimated to be >95% pure (Figure 1B). Purified sLFA-1 was comprised of two protein bands which migrated at M_r ~150,000 and ~90,000 under nonreducing conditions, the expected positions for α (CD11a) and β (CD18) subunits, respectively. The identity of these bands was confirmed by immunoblotting with antibodies to the α and β subunits of LFA-1 (our unpublished results). In addition, N-terminal sequence analysis of sLFA-1 protein bands (Figure 1B, final) was carried out. The NH_2 terminus of the β subunit was blocked, as expected, and that of the α subunit was found to agree with the published cDNA sequence (Kishimoto *et al.*, 1987; Larson *et al.*, 1989). Final protein concentration was determined by quantitative amino acid analysis and was in agreement with values determined using the ELISA (see MATERIALS AND METHODS) that detects heterodimeric sLFA-1. Thus, it appears that the purified sLFA-1 is present as a highly purified heterodimeric form of LFA-1 that should be useful for functional studies of LFA-1/ICAM-1 interactions.

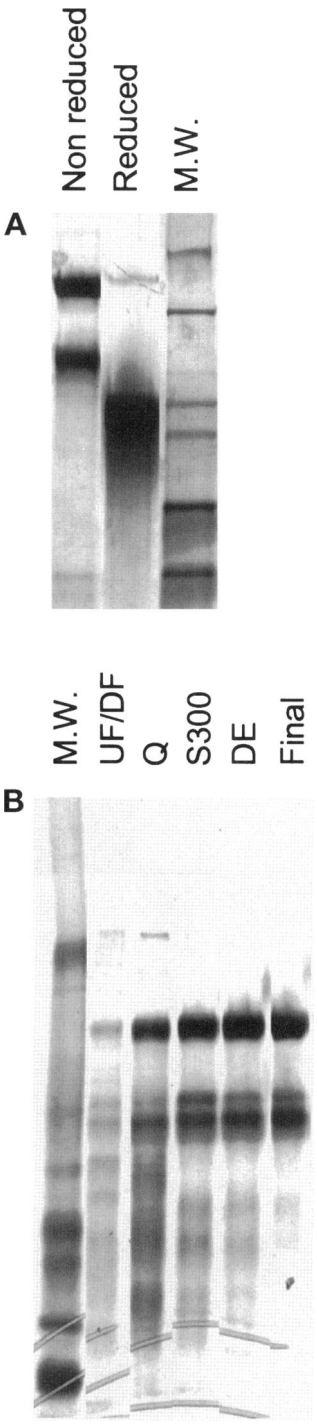

Figure 1. SDS-PAGE of purified proteins. (A) Purified recombinant human 5 domain ICAM-1 IgG was electrophoresed under reducing (R) and nonreducing (NR) conditions on an 8% SDS-polyacrylamide gel and silver stained. Molecular weight markers (M.W.) are 200,000, 116,000, 97,000, 66,000, 55,000, and 36,000. (B) Samples from each step of the purification of recombinant human soluble LFA-1 were run on 8–16% SDS-polyacrylamide gels and silver stained. Ultrafiltration/diafiltration of culture supernatant (UF/DF), Q-Sepharose eluate (Q), Pharmacia S300HR eluate (S300), and DEAE-Sepharose eluate (DE); final purified material (Final). Molecular weight markers (M.W.) are the same as those in A.

Binding of sLFA-1 to 5dICAMIg

To assess the functional state of sLFA-1 and 5dICAMIg, a cell-free binding assay was developed. An anti-Fc antibody was used to capture purified 5dICAMIg and sLFA-1 binding was detected using an anti-LFA-1 antibody (see MATERIALS AND METHODS). Purified sLFA-1 bound to 5dICAMIg in a dose-dependent manner over a range of concentrations (0.3–5 μg/ml; Figure 2A). Based on these results, a concentration of 2 μg/ml sLFA-1 was chosen for functional binding assays. The LFA-1-mediated adhesion of lymphocytes to ICAM-1 is known to be dependent on the presence of Mg^{2+} or Mn^{2+} (Rothlein and Springer, 1986; Dransfield and Hogg, 1989). Binding of the purified sLFA-1 to 5dICAMIg was facilitated by $MgCl_2$ or $MnCl_2$, whereas $CaCl_2$ did not promote binding (Figure 2B). Even at a concentration of 1.0 mM $CaCl_2$, sLFA-1 binding did not increase above background levels. Removal of divalent cations from the assay (Figure 2B, EDTA) abrogated binding, indicating that purified sLFA-1 binds 5dICAMIg in a divalent

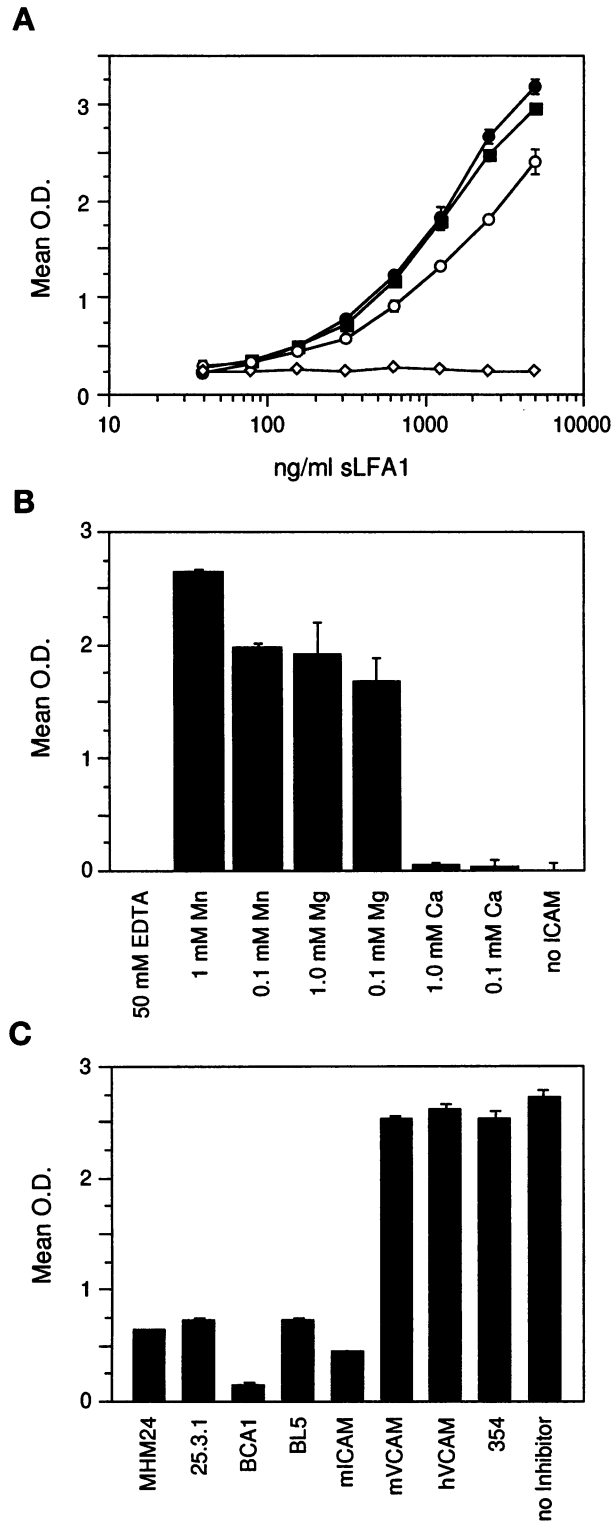

Figure 2. Binding of sLFA-1 to 5dICAMIg. (A) Purified sLFA-1 (0.04–5 μg/ml) was bound to three different coat concentrations of 5dICAMIg, 100 ng/ml ICAM-1 (●); 60 ng/ml ICAM-1 (■); 30 ng/ml ICAM-1 (○), and no ICAM-1 (◇). (B) sLFA-1 binding to anti-Fc-captured 5dICAMIg was assayed in the presence of 50 mM EDTA, 1 mM or 0.1 mM of $MnCl_2$, $MgCl_2$, or $CaCl_2$ or no ICAM-1 coated (no ICAM). (C) Binding of sLFA-1 to anti-Fc-captured 5dICAMIg was assayed in the presence of 50 μg/ml of anti-CD11a antibodies (MHM24, 25.3.1, TS1/22, BCA1), anti-CD18 antibody (BL5), full-length human ICAM (mICAM), mVCAM (murine) and hVCAM (human), isotype control antibody (354), no inhibitor.

cation-dependent manner. To test the specificity of these binding interactions, a panel of function-blocking mAbs to LFA-1 were tested for their ability to inhibit binding. Antibodies to both the α (Figure 2C, MHM24, 25.3.1, and BCA1) and β (BL5) subunits of LFA-1 inhibited sLFA-1 binding to 5dICAMIg. An isotype control antibody (Figure 2C, 354) did not affect binding. Purified full-length human ICAM-1 (mICAM; Edwards *et al.*, 1995) inhibited sLFA-1 binding while human or murine VCAM-1 did not (Figure 2C, hVCAM and mVCAM). This specific binding of a truncated form of recombinant LFA-1 to 5dICAMIg resembles that observed for full-length LFA-1 purified from the JY B-cell line (Dustin and Springer, 1989). Indeed, the divalent cation-potentiated binding of recombinant sLFA-1 to ICAM-1 appears to mimic LFA-1-mediated adhesion of HuT78 cells to ICAM-1 (our unpublished results). Thus, analysis of ICAM-1 mutants in this cell-free system should be predictive of the binding of this integrin ligand to cellular LFA-1.

Anti-ICAM-1 Antibody Characterization

A panel of six mAbs was generated (see MATERIALS AND METHODS) that, along with 84H10 (AMAC) and BBIG-I1 (R & D Systems), were used to characterize the wild-type and mutant ICAM-1 proteins. All antibodies were tested for their ability to bind to ICAM-1 immunoadhesins containing the five Ig-like domains (5dICAMIg), the three N-terminal Ig-like domains (3dCIAMIg), and a chimera of the two N-terminal Ig-like domains and the third domain of VCAM-1 (I12V3Ig). Six antibodies mapped to the two N-terminal Ig-like domains of ICAM-1; one of these, 1E8, consistently gave only low level binding to I12V3Ig but robust binding to 3dICAMIg. Two antibodies, 4G3 and 6A12, map to Ig-like domains 4 and 5 (Table 1). Of these eight antibodies, four (1B9, 4D3, BBIG-I1, and 84H10) inhibit the binding of biotinylated forms of these antibodies to 5dICAMIg-coated plates, indicating that they share a common epitope (Table 1, A). Antibodies 1E8, 4B2, 4G3, and 6A12 do not interfere with binding of any of the other biotinylated antibodies to ICAM-1 and as such bind to four distinct epitopes (Table 1, B–E). Four of the antibodies [84H10, 1B9, 4D3 (Figure 3), and BBIG-I1 (our unpublished results)] inhibit the adhesion of LFA-1-transfected 293 cells to 5dICAMIg-coated plates. All of the antibodies bound to ICAM-1-expressing 293 cells when subjected to fluorescence-activated cell sorting

Table 1. Summary of anti-ICAM-1 mAbs

mAbs	OD in ELISA[a]				ICAM-1 domains bound	Epitope[b]	Function[c] blocking	Isotype
	5dICAMIg	3dICAMIg	I12V3Ig	VCAM				
84H10	**	**	**	—	1–2	A	+	G1
BBIG-I1	***	***	**	—	1–2	A	+	G1
1B9	****	****	**	—	1–2	A	+	G1
1E8	***	***	*	—	1–3	B	−	G2a
4B2	****	****	****	—	1–2	D	−	G2a
4D3	****	****	***	—	1–2	A	+	G2a
4G3	***	—	—	—	4–5	C	−	G2b
6A12	***	—	ND[d]	ND	4–5	E	−	G1

[a] mAb binding to ICAM-1 immunoadhesins in ELISA ****, OD > 1.2; ***, OD 0.9–1.2; **, OD 0.6–0.9; *, OD 0.3–0.6.

[b] Epitope assignment was based on mAb cross-blocking data.

[c] Based on inhibition of LFA-1 293 cell adhesion to 5dICAMIg (see also Figure 3).

[d] ND, not determined.

analysis (our unpublished results). Thus, these antibodies define a panel of reagents that are useful for the characterization of the conformation and functional state of ICAM-1 mutants.

Effects of ICAM-1 Amino Acid Substitutions on LFA-1 Binding

The N-terminal Ig-like domains of both ICAM-1 and ICAM-3 have been found to play a critical role in binding to LFA-1 (Staunton *et al.*, 1990; Sadhu *et al.*, 1994; Holness *et al.*, 1995; Klickstein *et al.*, 1996). To understand further the role of individual residues in binding to LFA-1 and to elucidate more clearly the structure of the ICAM-1-binding site, 23 amino acids in the N-terminal domain of ICAM-1 were mutated to alanine. In addition, several of these residues were changed to amino acids other than alanine to investigate the effects of alternate substitutions. In all, a total of 34 single and one double mutations were made in domain 1 of the expression plasmid encoding 5dICAMIg.

Each mutant was transfected into 293 cells on two separate occasions and assayed for expression level, binding to sLFA-1, and binding to the panel of anti-ICAM-1 antibodies. Representative data are shown in Table 2. In addition, all mutants were tested for their ability to support LFA-1-mediated adhesion of HuT78 cells (ATCC TIB 161) and to stimulate proliferation of human $CD4^+$ T cells in the presence of an antibody to CD3 (our unpublished results). Results of cell-based analysis were in agreement with those obtained using purified 5dICAMIg and sLFA-1. This suggests that under conditions used in these assays, the sLFA-1 is in a conformation that is similar to the activated state found on T cells. Expression levels of ICAM-1 mutants, measured in an anti-Fc ELISA, ranged from 1 to 15.5 μg/ml compared with wild-type expression levels of 2.7–20.4 μg/ml. The functional binding to sLFA-1 is presented as the EC_{50} of a mutant relative to that of the purified wild-type 5dICAMIg. Mutants either bound to sLFA-1 with EC_{50}s within twofold of the wild type (average EC_{50} = 35 ng/ml) or they exhibited extremely low or no binding. This second group of mutants was tested for their ability to bind sLFA-1 at much higher concentrations (from 2.3 to

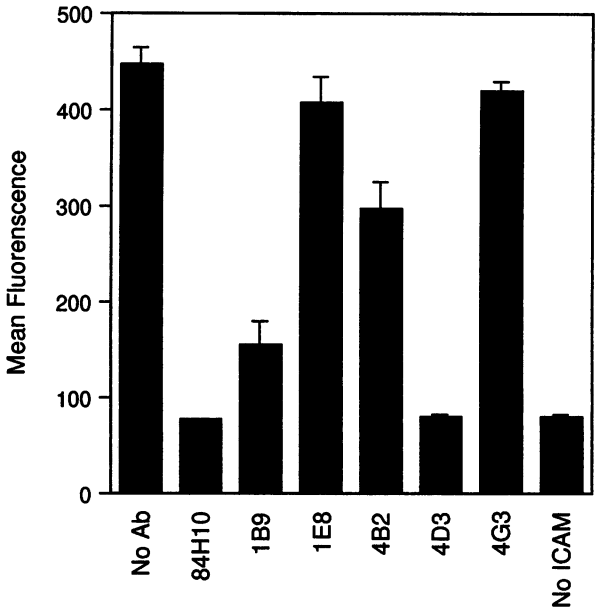

Figure 3. Antibody inhibition of adhesion of LFA-1 293 cells to 5dICAMIg. Calcein-labeled LFA-1-expressing 293 cells were bound to 5dICAMGaIg in the presence or absence of six different anti-ICAM-1 antibodies. Maximal binding was determined in the absence of antibody. Background binding is adhesion to BSA-blocked wells containing no 5dICAMIg. Each condition was carried out in triplicate.

Table 2. Expression and binding of ICAM-1 mutants to sLFA-1 and antibodies

Mutation	Expression levels (μg/ml)	EC_{50}[a]	EC_{50}^{hi}[b]	Antibody binding (% wild type)							
				84H10	BBIG-I1	1B9	1E8	4B2	4D3	4G3	6A12
S3A	2.2	1		101	98	103	156	101	101	100	98
S22A	2.4	1.5		101	95	100	172	98	99	99	98
D26A	4.7	0.9		99	100	98	—	96	94	97	98
Q27A	7	0.8		98	100	106	**29**[c]	101	108	101	101
K29A	5.1	0.7		101	—	—	—	102	—	101	98
K29M	6.6	0.8		98	—	—	**31**	101	—	101	102
K29Q	6.1	0.8		96	—	—	**26**	100	—	97	97
L30A	5.7	1.2	1.8	100	84	—	94	98	—	98	100
L31A	1.2	→	>124.0	70	**26**	—	**36**	98	—	101	100
G32A	7.1	→	>97.7	99	95	85	101	100	97	99	100
E34A	5.6	NB	>1271	97	95	100	80	96	92	96	97
E34D	7.3	NB	>2877	98	101	**39**	125	99	76	103	100
E34Q	2.9	NB	>247.6	101	94	106	107	99	103	101	101
K39A	5.4	→	>97.7	96	96	—	63	95	—	95	95
K39E	1.1	NB	>535	65	—	—	**20**	92	—	99	98
K39M	6.9	→	>122.5	96	96	—	101	99	—	100	107
K40A	8.1	1.0		**44**	93	93	78	96	97	97	98
E41A	5.1	0.8		96	100	—	71	96	**22**	97	98
L42A	6.8	1.1		93	94	94	88	95	92	99	97
L43A	8.5	0.6		53	101	96	86	97	98	104	101
L44A	6.1	0.9		92	99	85	166	99	92	103	96
R49W	2.5	1.9		98	89	100	109	100	100	98	97
E53A	2.5	1.2		99	101	98	90	101	98	98	99
D60A	1.8	1.5	1.3 & 1.6	96	97	88	49	96	87	99	98
M64A	1.7	→	>177.5	115	98	102	91	100	100	100	96
Y66S	1.1	NB	>66.9	95	—	—	**21**	100	—	96	93
N68A	7.5	→	>122.5	93	49	98	109	95	97	97	96
D71A	7.6	0.8		99	**26**	87	79	95	92	98	97
Q73A	5.1	NB	>1157	100	100	102	129	97	100	103	103
Q73E	4.1	NB	>941	97	86	95	51	95	94	95	96
Q73N	1.7	NB	>383	101	—	52	81	96	83	99	98
Q73T	7.1	NB	>2645	98	**38**	105	62	102	116	102	97
T75A	2.1	0.93		107	95	97	120	101	100	97	95
K77A	2.8	0.91		107	102	100	94	99	103	101	99
G32A, Y66S	1.0	NB	>68.1	95	**20**	—	**24**	99	—	95	96

[a] EC_{50} determined from a 4P fit of ICAM-1 titrations over a concentration range of 0.1–300 ng/ml and expressed relative to wild type; EC_{50} values <1 bind better than wild type; values >1 bind less well; mutants that displayed no binding (NB) or very low binding (→) were retested at higher concentrations.

[b] EC_{50}^{hi} values determined relative to wild type EC_{50} for NB and (→) mutants from footnote a.

[c] mAbs which bound <20% of wild type are represented as (—); those which bound 20–44% of wild type are shown in bold.

12.6 μg/ml of 5dICAMIg). Mutants, such as E34A (Figure 4), that exhibited no binding at up to 300 ng/ml are designated NB (no binding; Table 2, column 3); these mutants gave background levels of binding even when tested at concentrations from 67- to 2877-fold of the EC_{50} for wild-type 5dICAMIg (Table 2, column 4). For those mutants that exhibited low binding at higher concentrations (L31A, G32A, K39A, K39M, M64A, and N68A), the EC_{50} was at least 97-fold less than the wild-type EC_{50} (Table 2, column 4). This group of mutants is represented by mutant G32A (Figure 4), for which the binding never increased above a very low level. Of the 19 mutants that bound similarly to wild type, two mutants, D60A and L30A (Figure 4), displayed a less than twofold reduction in binding.

Several of the 35 mutations were identical to those made by Staunton *et al.* (1990) or behaved similarly in functional assays. Mutations K40A and T75A exhibited wild-type activity, whereas E34A failed to bind to sLFA-1 in the protein assay (Table 2, Figure 4) or in cell attachment and costimulation assays (our unpublished results). In addition, mutations unique to this study, E34D and E34Q, both failed to bind to sLFA-1 at concentrations 2877-fold and 248-fold above the EC_{50} of wild-type 5dICAMIg. In a number of instances, we changed single amino acids to Ala that had been mutated by Staunton *et al.* (1990) to either

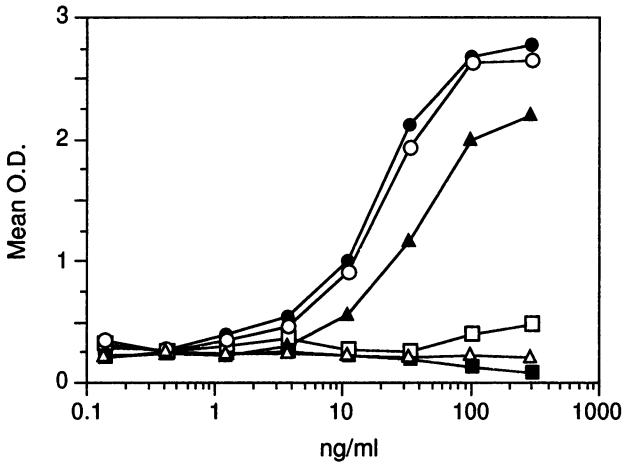

Figure 4. Binding of sLFA-1 to ICAM-1 mutants. Titration of ICAMs over a concentration range of 0.1–300 ng/ml. Wild-type 5dICAMGaIg (○) and mutants: D26A (wild-type like; ●), L30A (intermediate binding; ▲), G32A (low binding; □), E34A (no binding; ■), no ICAM-1 (△).

another residue or as one of multiple amino acid changes. These changes, S3A, S22A, E53A, D60A, D71A, and K77A, resulted in wild-type binding or less than a twofold decrease in binding to sLFA-1. These authors observed that Q73T bound only twofold less than wild-type and Q73H showed a 10-fold decrease in binding in their cell attachment assay. We observed no binding of Q73T to sLFA-1 at concentrations up to 2645-fold above the wild-type EC_{50}. Mutants Q73A, Q73E, and Q73N also failed to bind to sLFA-1 in the protein assay (Table 2) and in cell-based assays (our unpublished results). Thus, these results are consistent with those published previously (Staunton *et al.*, 1990).

At several positions our mutants behaved differently from those of Staunton *et al.* (1990). The mutation D26QPK/ALPE made by Staunton bound at 30% wild-type levels in a cell attachment assay. We made several individual mutations at these positions, D26A, Q27A, K29A, K29 M and K29Q, all of which bound at least as well as wild-type in a protein assay (Table 2) and were qualitatively similar in cell-based assays. It is possible that the charge reversal of K29 in D26QPK/ALPE caused the decrease in activity observed by Staunton *et al.* (1990). Another mutation, K39KE/ERQ, showed no loss in activity for these investigators. When we mutated K39 to Ala, Glu, or Met, we consistently observed a significant loss in activity. When tested at high concentrations, these mutants demonstrated a 100-fold or greater decrease in EC_{50}. Two mutations reported by Staunton *et al.* (1990), Q62PM/API and M64I, had near wild-type activity. In contrast, a point mutation we made, M64A, showed a greater than 100-fold loss in binding. The mutation N68K was also reported to have full activity by Staunton *et al.* (1990) while N68A showed a greater than 100-fold loss in binding in our hands. The most striking difference in our results, however, involved Y66. Staunton observed wild-type binding with mutant Y66T while we saw no evidence of sLFA-1 binding to Y66S. Even when tested at higher concentrations, we saw at least a 66-fold decrease in EC_{50} compared with wild type.

In addition to the mutations described above, two novel mutations, L31A and G32A, resulted in a large decrease in binding to sLFA-1 (Figure 4 and Table 2). Because L31A did not bind to four of the eight antibodies tested and showed a 30% decrease in binding to the 84H10 antibody, the loss of activity of this mutant is likely due to a structural perturbation. In contrast, the mutation G32A retained binding to all of the antibodies in the panel and yet there was nearly a 100-fold loss in sLFA-1 binding. This result could indicate a direct interaction between G32 and LFA-1 or, alternatively, the Ala side chain could be affecting the side chain conformation of Y66. Alignment of the amino acid sequences of ICAM-1 and ICAM-3 revealed that the analogous residues in ICAM-3 are A35 (aligns with G32 in ICAM-1) and S68 (aligns with Y66 in ICAM-1). To investigate whether changing the ICAM-1 sequence to match that of ICAM-3 at these positions influenced ICAM-1 binding, the double mutant G32A/Y66S was made. The double mutant retained the same sLFA-1 binding phenotype as Y66S, which leads us to conclude that Y66 interacts directly with LFA-1.

Previously described mutants are all thought to lie on one of the two β-sheets that comprise the Ig-fold of domain 1 of ICAM-1. We also generated mutants of conserved residues thought to lie on the opposite β-sheet. Residues S3, S22, and E53 are conserved in human, chimp, and murine ICAM-1. In addition, S22 is conserved in ICAM-2 and ICAM-3. In all cases, mutants of these residues to Ala displayed binding to antibodies and sLFA-1 at wild-type levels. Residue R49 of human ICAM-1 is highly conserved as a Trp in chimp, mouse, and rat as well as in ICAM-2 and ICAM-3. Mutant R49W also displayed a wild-type binding pattern to antibodies and sLFA-1.

Effects of ICAM-1 Amino Acid Substitutions on Antibody Binding

A panel of six novel monoclonal antibodies to ICAM-1 and two commercially available mAbs were used to determine whether the ICAM-1 mutants maintained an overall structure similar to that of the wild-type molecule. Two antibodies in the panel, 4G3 and 6A12, mapped to domains 4 and 5 and bound to all of the ICAM-1 mutants at levels comparable to wild type (Table 2). Most mutants bound the other antibodies in the panel at 80–100% of the wild-type levels. Two antibodies which mapped to domains 1 and 2, 84H10

(function-blocking antibody) and 4B2 (a nonblocking antibody) bound to most mutants equivalently to wild type. These represent different epitopes based on antibody cross-blocking data (Table 1) and their binding probably reflects proper protein folding over a substantial portion of the protein. Three mutants (K39E, K40A, and L43A) showed a loss of binding to the 84H10 antibody. K40A and L43A showed the greatest loss in binding (44 and 53% of wild type, respectively) but bound all of the other antibodies in the panel. K39E bound 84H10 at 65% wild-type levels and showed a complete loss in binding to the other function-blocking antibodies as well as the nonblocking antibody 1E8. Two other mutations (K39A and K39M) showed a loss in binding to blocking antibodies 1B9 and 4D3, but no decrease in binding to the blocking antibodies 84H10 and BBIG-I1. These data suggest that K39 and K40 are part of the epitopes for the function-blocking antibodies that, along with sLFA binding data, support K39 as part of the ICAM-1 binding site for LFA-1.

Five mutants showed a loss in binding to four antibodies (1B9, 1E8, 4D3, and BBIG-I1), which bind to domains 1 and 2, but retained binding to 84H10 and 4B2. Three of the mutations were at K29 (K29A, K29M, and K29Q) and all bound to sLFA-1 at least as well as the wild type. We conclude that K29 represents part of the epitope for 1B9, 1E8, 4D3, and BBIG-I1. Y66S and the double mutant G32A/Y66S showed a decrease in binding to sLFA-1 as well as to three of the four function-blocking antibodies (Table 2). The epitopes for antibodies 84H10 and 4B2 (domains 1 and 2) as well as 4G3 and 6A12 (domains 4 and 5) were undisturbed. This suggests that these mutants were properly folded and is consistent with the critical role of Y66 and G32 in LFA-1 binding.

Model of ICAM-1 Domains 1 and 2

To map the location of mutated residues, a model of the two N-terminal domains of ICAM-1 was generated. Residues that most profoundly affected binding of 5dICAMIg to LFA-1 and that we conclude form the LFA-1 binding site are highlighted in yellow (Figure 5). Residues that when mutated retained wild-type properties are shown in green. Mutations at E34, K39, M64, Y66, N68, and Q73 did not bind sLFA-1 at concentrations between 66- and 2645-fold of the EC_{50} for wild-type 5dICAMIg and displayed antibody binding similar to wild type. When G32 was changed to Ala, binding to sLFA-1 was also significantly reduced (Table 2). In our model of ICAM-1, the side chain of Y66 is positioned over the backbone of G32 (Figure 5). In this conformation, substitution of G32 by Ala would result in the Y66 side chain being in close proximity to the Ala side chain, forcing Y66 to change conformation, thereby possibly disrupting the conformations of one or more additional residues in the binding site. An alternative explanation for the observed reduction in binding of G32A places G32 in direct contact with LFA-1 and would require a different side chain conformation of Y66.

DISCUSSION

We have established a cell-free system in which functional binding of recombinant extracellular domains of LFA-1 and ICAM-1 was used to study the LFA-1-binding site on ICAM-1. The binding of heterodimeric sLFA-1 to ICAM-1 immunoadhesin was found to be sensitive to divalent cations and was inhibited by function-blocking antibodies to LFA-1 and excess ICAM-1. Thus, this system allows the analysis of LFA-1/ICAM-1 binding without the complication of cellular modulation of ligand receptor affinity/avidity. In this system, saturable, dose-dependent binding of 5dICAMIg is observed over a 10–50-fold concentration range. This has allowed for the quantitative evaluation of the effects of mutants in the first Ig domain of ICAM-1 on binding to sLFA-1.

Our ICAM-1 domain 1 model comprises a β-sandwich Ig-like fold, with one β-sheet including β-strands A, B, and E and the other β-sheet including β-strands C, D, F, and G. The disulfide bond between the two β-sheets, such as that found in VCAM-1 domain 1, is conserved in ICAM-1 domain 1. However, the Trp residue, which abuts the disulfide, found in many Ig-like domains (including VCAM-1 domain 1) is absent. In our model, ICAM-1 residue I33 is positioned in place of this conserved Trp.

The CD11a-binding site on ICAM-1 forms a rectangle consisting of residues E34, K39, M64, Y66, N68, and Q73. These residues are exposed on four antiparallel β-strands comprising one β-sheet: E34 is on β-strand C, K39 on β-strand D, M64, Y66, and N68 on β-strand F, and Q73 on β-strand G. All residues that make up the binding site had a 67-fold or greater reduction in binding to LFA-1 when changed to Ala or to other amino acids. Another mutation, G32A, was also evaluated. G32 is in β-strand C and changing it to Ala would position the Ala side chain to the exterior of the β-strand. The G32A change resulted in a large decrease in binding to LFA-1, but binding to the panel of anti-ICAM antibodies remained similar to native ICAM-1. Thus, it is possible that LFA-1 interacts directly with the backbone of G32, and that this interaction is intolerant of the perturbation resulting from introduction of a methyl group when Gly is changed to Ala. An alternative explanation is that the Y66 side chain, which is important for LFA-1 binding, is positioned over the G32 backbone (Figure 5) and introduction of Ala forces the Y66 side chain to move, thereby disrupting the interaction with LFA-1. The data pre-

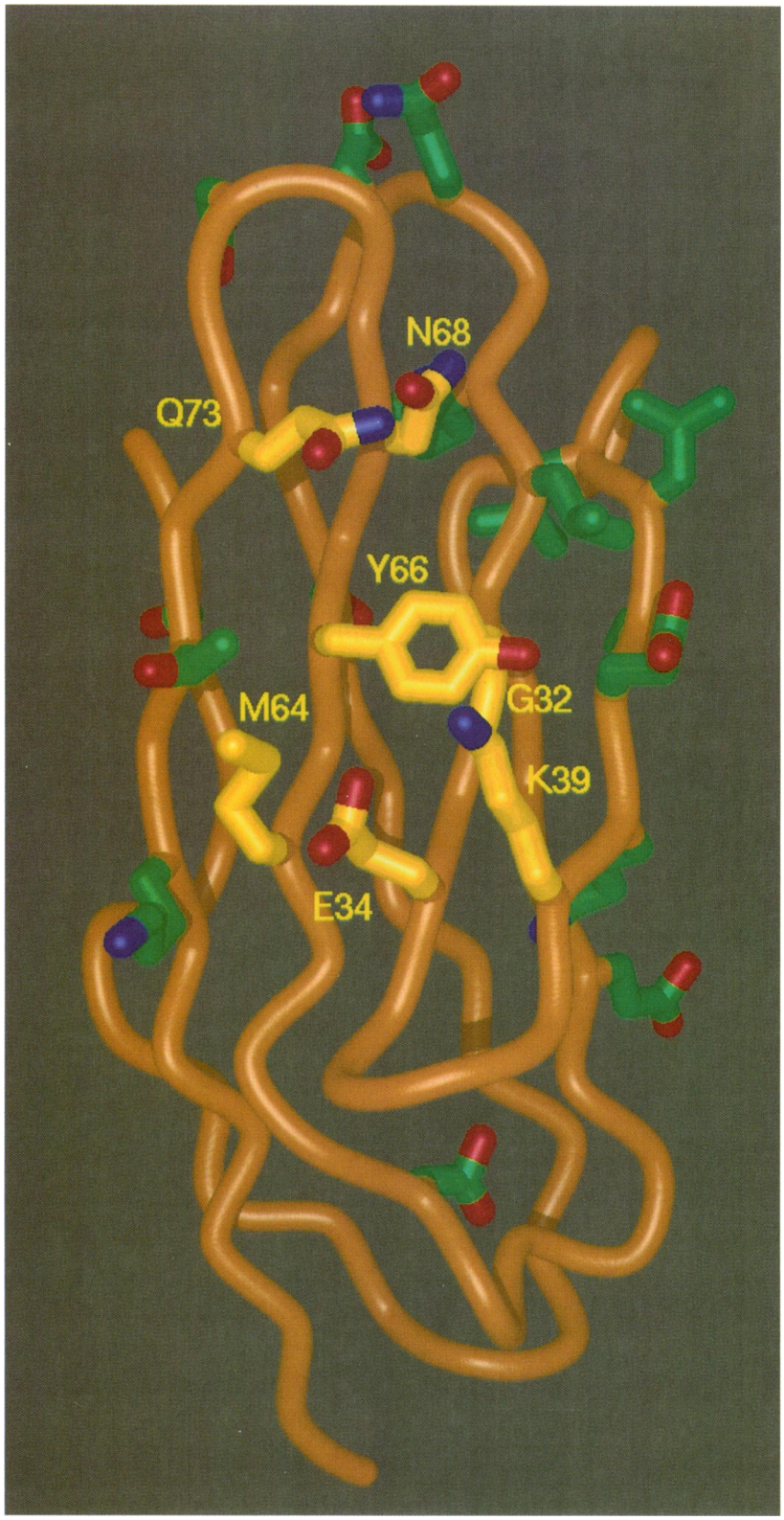

Figure 5. Model of domain 1 of ICAM-1. Proposed model of domain 1 of ICAM-1 based on the structure of domain 1 of VCAM-1 and ICAM-1 mutagenesis data. The side chains of point mutants that exhibited wild-type-like binding to sLFA-1 are shown in green whereas those that had extremely low or no binding are highlighted in yellow.

sented are consistent with both of these interpretations.

Mutation of Y66 to Ser resulted in loss of binding to three (BBIG-I1, 1B9, and 4D3) of the four function-blocking antibodies that appear to share a common epitope, based on antibody competition and mutant-binding data (Table 2). Binding to the 84H10 function-blocking antibody, as well as to the nonblocking antibodies (except 1E8), was retained. These data, along with the greater than 60-fold decrease in LFA-1 binding, suggest that Y66 is part of the epitope for several of the function-blocking antibodies and participates directly in binding to LFA-1. The extent of change tolerated at residue E34 was evaluated using two conservative mutations: E34D and E34Q. In each case binding to LFA-1 was abrogated even at very high concentrations. This suggests that both the charge and length of the E34 side chain are critical for interaction with LFA-1. K39 also appears to be intolerant of changes in charge and side chain length as the mutants K39A, K39M, and K39E displayed a nearly 100-fold reduction in binding to LFA-1. Although the K39E mutant showed loss of binding to all function-blocking antibodies and antibody 1E8, mutants K39A and K39M retained binding to two of the four function-blocking antibodies. It is unlikely, therefore, that K39 is a strictly structural residue, and we propose that it participates directly in the ICAM-1 interaction with LFA-1. M64 and N68 also showed reduced binding to LFA-1 when changed to Ala, but bound the panel of antibodies in a manner similar to native ICAM-1. When these residues were mutated by Staunton *et al.* (1990), they displayed wild-type activity. Staunton's change M64I is conservative, which may account for the difference in our results. One explanation for the disparate binding capacities of Staunton's N68K and our N68A mutants is that the Lys could be acting as a hydrogen bond donor in place of the Asn.

Although mutants of many of the binding site residues bound all or most of the antibodies in a manner similar to wild-type ICAM-1, mutations at other residues occasionally resulted in decreased binding of one or more antibodies. Binding to four antibodies (BBIG-I1, 1B9, 1E8, and 4D3) was reduced to each of three mutations at K29, yet all three mutations retained binding to sLFA-1. Thus, K29 forms part of the epitope of these four antibodies, three of which are function-blocking antibodies, but is not part of the LFA-1 binding site on ICAM-1. Likewise, L30 and E41 form part of the epitope of 1B9 and 4D3, yet retained binding to sLFA-1. The epitope for 84H10 appears to include residue K40, which retained binding to sLFA-1 and all of the other mAbs. Thus, residues that define the epitope of multiple function-blocking antibodies do not necessarily correspond to the LFA-1-binding site on ICAM-1.

All of the residues that we identify as part of the binding site for LFA-1 are on the same face of the first domain of ICAM-1. To investigate whether additional residues on the opposite face of this domain were important for binding, a number of mutations were made. Three residues (S3, S22, and E53), that lie on the opposite face are conserved in ICAM-1 across different species and are located on β-strands A, B, and E, respectively, were chosen for mutagenesis to Ala. R49, which is located in β-strand E and is highly conserved as a Trp in ICAM-2 and ICAM-3 and across species, was mutated to Trp. In ICAM-3, mutation to Ala of W51, which is analogous to R49 in ICAM-1, was found to perturb the overall structure based on the abrogation of binding of many anti-ICAM-3 antibodies to this mutant (Klickstein *et al.*, 1996). We chose to mutate R49 to Trp in ICAM-1 to determine whether this residue plays a role in binding of LFA-1 to ICAM-1. When tested for binding to sLFA-1, all four mutants displayed wild-type binding to sLFA-1 and bound the mAbs at wild-type levels. These data in conjunction with data previously published by Staunton *et al.* (1990) show that every residue on the opposite face that has been mutated has had no effect on LFA-1 and antibody binding to ICAM-1. Based on our model, the remaining residues that were not mutated are predicted to be buried, with the exception of S7, L18, and K50 on β-strands A, B, and E respectively. Since K50 is predicted to be partially buried and S7 and L18 differ in ICAM-2, ICAM-3, and across species, these residues are unlikely to be involved in binding. Using this highly directed approach, we were unable to identify residues in the ABE β-sheet of ICAM-1 domain 1 that affected the interaction with LFA-1, thus it seems unlikely that this face participates in ICAM-1 binding to LFA-1.

When data from the structure/function analysis of ICAM-1 (Staunton *et al.*, 1990) was compared with that from studies of ICAM-3 (Sadhu *et al.*, 1994; Holness *et al.*, 1995; Klickstein *et al.*, 1996), three common residues were identified as important for binding to LFA-1: E34, M64, and Q73 in ICAM-1 and E37, L66, and Q75 in ICAM-3. The mutation E32K/AS in ICAM-3 resulted in an approximately sixfold decrease in binding to LFA-1 (Sadhu *et al.*, 1994); in contrast the residue analogous to E32 in ICAM-1, K29, had no effect on binding. Three mutations in ICAM-3, N23A, S25A, and F54A, resulted in an approximately four- to fivefold decrease in binding to LFA-1 (Klickstein *et al.*, 1996); the residue analogous to S25 in ICAM-1, S22, had no effect on binding while the other two analogous residues were not tested. Four other mutations in ICAM-3, K42E/AS, S68A, and Y70A (Sadhu *et al.*, 1994) and K42A (Klickstein *et al.*, 1996) had no effect on LFA-1 binding while the analogous residues in ICAM-1, K39 (aligns with K42 in ICAM-3), Y66 (aligns with S68), and N68 (aligns with Y70), are critical for

LFA-1 binding. Although mutagenesis of S68 to Ala in ICAM-3 did not affect binding (Sadhu *et al.*, 1994), substitution of Lys in this position abrogated binding to LFA-1 (Holness *et al.*, 1995). To investigate the role of these residues in ICAM-1/LFA-1 interactions and to determine whether mutating the ICAM-1 sequence to that of ICAM-3 (A35/S68) would restore LFA-1 binding, the double mutation, G32A/Y66S, was constructed. This double mutant, as well as the single mutants G32 to Ala and Y66 to Ser, abrogated binding. In ICAM-3 the difference in binding of S68A and S68K may be explained by the difference in size of the substituted side chain. In contrast, the difference in the effects on LFA-1 binding of Y66T (Staunton *et al.*, 1990) and Y66S is unexpected due to the structural similarities of Thr and Ser. Thus, we have defined six residues (E34, K39, M64, Y66, N68, and Q73) in ICAM-1 that are critical for LFA-1 binding. At least two of these (K39 and N68) are novel contact sites. It will be of interest to determine the role of these residues in the binding of other Ig superfamily members to their integrin receptors.

A residue in VCAM-1, D40, which aligns with E34 of ICAM-1, has been shown in mutagenesis studies to be critical for binding to $\alpha 4\beta 1$ (Osborne *et al.*, 1994). Residue D40 of VCAM-1 is located in a loop between β-strands C and D. In contrast, E34 of ICAM-1 is predicted to be within β-strand C. Thus, while the binding site on ICAM-1 for LFA-1 is predicted to be a relatively flat surface, the binding site on VCAM-1 for $\alpha 4\beta 1$ is present in a loop. The crystal structure of the I domain of LFA-1, which is thought to interact with ICAM-1, has been determined (Qu and Leahy, 1995). Mutagenesis studies of the I domain have revealed several residues that are critical for binding to ICAM-1 (Edwards *et al.*, 1995; Huang and Springer, 1995), and these residues all lie on a relatively flat side of the I domain. The distance between S245 and E146, two critical residues in the I domain that are separated by the greatest distance, is 25 Å as determined by the crystal structure (Qu and Leahy, 1995). This is in close agreement with the distance between K39 and Q73 of ICAM-1, which is predicted from our model to be 17 Å. Thus, although it is premature to align specific residues of the model of domain 1 of ICAM-1 and the LFA-1 I domain, a tantalizing view of the interaction of these two flat surfaces is beginning to emerge.

ACKNOWLEDGMENTS

We thank P. Jhurani, P. Ng, and M. Vasser for oligonucleotides; M. Hamner and A. Goddard for sequence analysis; D. Leung, M. Champe, G. Nakamura, and P. Berman for providing cDNAs; and D. Eastman and C. Ladner for cell-based assays. Dr. J. Hildreth generously provided the PLM-2, MHM23, and MHM24 antibodies.

REFERENCES

Arnaout, M.A., Gupta, S.K., Pierce, M.W., and Tenen, D.G. (1988). Amino acid sequence of the alpha subunit of human leukocyte adhesion receptor Mo1 (complement receptor type 3). J. Cell Biol. *106*, 2153–2158.

Berendt, A.R., McDowall, A., Craig, A.G., Bates, P.A., Sternberg, M.J.E., Marsh, K., Newbold, C.I., and Hogg, N. (1992). The binding site on ICAM-1 for plasmodium falciparum-infected erythrocytes overlaps, but is distinct from, the LFA-1-binding site. Cell *68*, 71–81.

Bernstein, F.C., Koetzle, T.F., Williams, G.J.B., Meyer, E.F., Jr., Brice, M.D., Rodgers, J.R., Kennard, O., Shimanouchi, T., and Tasumi, M. (1977). The Protein Data Bank: a computer-based archival file for macromolecular structures. J. Mol. Biol. *112*, 535–542.

Briskin, M.J., McEvoy, L.M., and Butcher, E.C. (1993). MAdCAM-1 has homology to immunoglobulin and mucin-like adhesion receptors and to IgA1. Nature *363*, 461–464.

Carter, P., Nilsson, B., Burnier, J.P., Burdick, D., Wells, J.A. (1989). Engineering subtilisin BPN' for site-specific proteolysis. Proteins Struct. Funct. Genet. *6*, 240–248.

Chuntharapai, A., Kim, K.J. (1997). Generation of monoclonal antibodies to chemokine receptors. Enzymology (*in press*).

Connolly, M.K., Kitchens, E.A., Chan, B., Jardieu, P., and Wofsy, D. (1994). Treatment of murine lupus with monoclonal antibodies to lymphocyte function-associated antigen-1: dose-dependent inhibition of autoantibody production and blockade of the immune response to therapy. Clin. Immunol. Immunopathol. *72*, 198–203.

Corbi, A.L., Kishimoto, T.K., Miller, L.J., and Springer, T.A. (1988). The human leukocyte adhesion glycoprotein Mac-1 (complement receptor type 3, CD11b) α subunit. J. Biol. Chem. *263*, 12403–12411.

Corbi, A.L., Miller, L.J., O'Connor, K., Larson, R.S., and Springer, T.A. (1987). DNA cloning and complete primary structure of the α subunit of a leukocyte adhesion glycoprotein, p150,95. EMBO J. *6*, 4023–4028.

Diamond, M.S., and Springer, T.A. (1994). The dynamic regulation of integrin adhesiveness. Curr. Biol. *4*, 506–517.

Dransfield, I., and Hogg, N. (1989). Regulated expression of Mg^{2+} binding epitope on leukocyte integrin α subunits. EMBO J. *8*, 3759–3765.

Dustin, M.L., Rothlein, R., Bhan, A.K., Dinarello, C.A., and Springer, T.A. (1986). Induction by IL 1 and Interferon-γ: tissue distribution, biochemistry, and function of a natural adherence molecule (ICAM-1). J. Immunol. *137*, 245–254.

Dustin, M.L., and Springer, T.A. (1989). T-cell receptor cross-linking transiently stimulates adhesiveness through LFA-1. Nature *341*, 619–624.

Dustin, M.L., and Springer, T.A. (1991). Role of lymphocyte adhesion receptors in transient interactions and cell locomotion. Annu. Rev. Immunol. *9*, 27–66.

Eaton, D.L., Wood, W.I., Eaton, D., Hass, P.E., Hollingshead, P., Wion, K., Mather, J., Lawn, R.M., Vehar, G.A., and Gorman, C. (1986). Construction and characterization of an active factor VIII variant lacking the central one-third of the molecule. Biochemistry *25*, 8343–8347.

Edwards, C.P., Champe, M., Gonzalez, T., Wessinger, M.E., Spencer, S.A., Presta, L.G., Berman, P.W., and Bodary, S.C. (1995). Identification of amino acids in the CD11a I-domain important for binding of the leukocyte function-associated antigen-1 (LFA-1) to intercellular adhesion molecule-1 (ICAM-1). J. Biol. Chem. *270*, 12635–12640.

Ellison, J.W., Berson, B.J., and Hood, L.E. (1982). The nucleotide sequence of a human immunoglobulin C gamma-1 gene. Nucleic Acids Res. *10*, 4071–4079.

Fawcett, J., Holness, C.L.L., Needham, L.A., Turley, H., Gatter, K.C., Mason, D.Y., and Simmons, D.L. (1992). Molecular cloning of ICAM-3, a third ligand for LFA-1, constitutively expressed on resting leukocytes. Nature *360*, 481–484.

Giranda, V.L., Chapman, M.S., and Rossmann, M.G. (1990). Modeling of the human intercellular adhesion molecule-1, the human rhinovirus major group receptor. Proteins Struct. Funct. Genet. *7*, 227–233.

Gorman, C., Padmanabhan, R., and Howard, B.H. (1983). High efficiency DNA-mediated transformation of primate cells. Science *221*, 551–553.

Graham, F.L., Smiley, J., Russell, W.C., and Nairn, R. (1977). Characteristics of a human cell line transformed by DNA from human adenovirus type 5. J. Gen. Virol. *36*, 59–74.

Heagy, W., Walterbangh, C., and Martz, E. (1984). Potent ability of anti-LFA-1 monoclonal antibody to prolong allograft survival. Transplantation *37*, 520–523.

Hession, C., Moy, P., Tizard, R., Chisholm, P., Williams, C., Wysk, M., Burkly, L., Miyake, K., Kincade, P., and Lobb, R. (1992). Cloning of murine and rat vascular cell adhesion molecule-1. Biochem. Biophys. Res. Commun. *183*, 163–169.

Hildreth, J.E.K., Gotch, F.M., Hildreth, P.D.K., and McMichael, A.J. (1983). A human lymphocyte-associated antigen involved in cell-mediated lympholysis. Eur. J. Immunol. *13*, 202–208.

Hildreth, J.E. K., Holt, V., August, J.T., and Pescovitz, M.D. (1989). Monoclonal antibodies against procine LFA-1: Species cross-reactivity and functional effects of β-subunit-specific antibodies. Mol. Immunol. *26*, 883–895.

Holness, C.L., Bates, P.A., Littler, A.J., Buckley, C.D., McDowall, A., Bossy, D., Hogg, N., and Simmons, D.L. (1995). Analysis of the binding site on intercellular adhesion molecule 3 for the leukocyte integrin lymphocyte function-associated antigen 1. J. Biol. Chem. *270*, 877–884.

Huang, C., and Springer, T.A. (1995). A binding interface on the I domain of lymphocyte function-associated antigen-1 (LFA-1) required for specific interaction with intercellular adhesion molecule 1 (ICAM-1). J. Biol. Chem. *270*, 19008–19016.

Hynes, R.O. (1992). Integrins: versatility, modulation, and signaling in cell adhesion. Cell *69*, 11–25.

Isobe, M., Yagita, H., Okumura, K., and Ihara, A. (1992). Specific acceptance of cardiac allograft after treatment with antibodies to ICAM-1 and LFA-1. Science *255*, 1125–1127.

Jones, E.Y., Davis, S.J., Williams, A.F., Harlos, K., and Stuart, D.I. (1992). Crystal structure at 2.8 Å resolution of a soluble form of the cell adhesion molecule CD2. Nature *360*, 232–239.

Jones, E.Y., Harlos, K., Bottomley, M.J., Robinson, R.C., Driscoll, P.C., Edwards, R.M., Clements, J.M., Dudgeon, T.J., and Stuart, D.I. (1995). Crystal structure of an integrin-binding fragment of vascular cell adhesion molecule-1 at 1.8 Å resolution. Nature *373*, 539–544.

Kawasaki, K., Yaoita, E., Yamamoto, T., Tamatani, T., Miyasaka, M., and Kihara, I. (1993). Antibodies against intercellular adhesion molecule-1 and lymphocyte function-associated antigen-1 prevent glomerular injury in rat experimental crescentic glomerulonephritis. J. Immunol. *150*, 1074–1083.

Kim, K.J., Alphonso, M., Schmelzer, C.H., and Lowe, D. (1992). Detection of human leukemia inhibitory factor by monoclonal antibody based ELISA. J. Immunol. Methods *156*, 9–17.

Kishimoto, T.K., Larson, R.S., Corbi, A.L., Dustin, M.L., Staunton, D.E., and Springer, T.A. (1989). The leukocyte integrins. Adv. Immunol. *46*, 149–182.

Kishimoto, T.K., O'Connor, K., Lee, A., Roberts, T.M., and Springer, T.A. (1987). Cloning of the β subunit of the leukocyte adhesion proteins: homology to an extracellular matrix receptor defines a novel supergene family. Cell *48*, 681–690.

Klickstein, L.B., York, M.R., de Fougerolles, A.R., and Springer, T.A. (1996). Localization of the binding site on intercellular adhesion molecule-3 (ICAM-3) for lymphocyte function-associated antigen 1 (LFA-1). J. Biol. Chem. *271*, 23920–23927.

Kunkel, T.A. (1985). Rapid and efficient site-specific mutagenesis without phenotypic selection. Proc. Natl. Acad. Sci. USA *82*, 488–492.

Larson, R.S., Corbi, A.L., Berman, L., and Springer, T. (1989). Primary structure of the leukocyte function-associated molecule-1α subunit: An integrin with an embedded domain defining a protein superfamily. J. Cell Biol. *108*, 703–712.

Newman, P.J., Berndt, M.C., Gorski, J., White, G.C., Lyman, S., Paddock, C., and Muller, W.A. (1990). PECAM-1 (CD31) cloning and relation to adhesion molecules of the immunoglobulin gene superfamily. Science *247*, 1219–1222.

Osborn, L., Hession, C., Tizard, R., Vassallo, C., Luhowskyj, S., Chi-Rosso, G., and Lobb, R. (1989). Direct expression cloning of vascular cell adhesion molecule 1, a cytokine-induced endothelial protein that binds to lymphocytes. Cell *59*, 1203–1211.

Osborn, L., Vassallo, C., Browning, B.G., Tizard, R., Haskard, D.O., Benjamin C.D., Dougas, I., and Kirchhausen, T. (1994). Arrangement of domains, and amino acid residues required for binding of vascular cell adhesion molecule-1 to its counter-receptor VLA-4 (α4β1). J. Cell. Biol. *124*, 601–608.

Ponder, J.W., and Richards, F.M. (1987). Tertiary templates for proteins. Use of packing criteria in the enumeration of allowed sequences for different structural classes. J. Mol. Biol. *193*, 775–791.

Qu, A., and Leahy, D.J. (1995). Crystal structure of the I-domain from the CD11a/CD18 (LFA-1, αLβ2) integrin. Proc. Natl. Acad. Sci. USA *92*, 10277–10281.

Renz, M.E., Chiu, H.H., Jones, S., Fox, J., Kim, K.J., Presta, L.G., and Fong, S. (1994). Structural requirements for adhesion of soluble recombinant murine vascular cell adhesion molecule-1 to α4β1. J. Cell Biol. *125*, 1395–1406.

Rothlein, R., and Springer, T. (1986). The requirement for lymphocyte function-associated antigen 1 in homotypic leukocyte adhesion stimulated by phorbol ester. J. Exp. Med. *163*, 1132–1149.

Sadhu, C., Lipsky, B., Erickson, H.P., Hayflick, J., Dick, K.O., Gallatin, W.M., and Staunton, D.E. (1994). LFA-1 binding site in ICAM-3 contains a conserved motif and non-contiguous amino acids. Cell Adhesion Commun. *2*, 429–440.

Simmons, D., Makgoba, M.W., and Seed, B. (1988). ICAM, an adhesion ligand of LFA-1, is homologous to the neural cell adhesion molecule NCAM. Cell *331*, 624–627.

Simmons, D.L., Walker, C., Power, C., and Pigott, R. (1990). Molecular cloning of CD31, a putative intercellular adhesion molecule closely related to carcinoembryonic antigen. J. Exp. Med. *171*, 2147–2152.

Simmons, D.L. (1995). The role of ICAM expression in immunity and disease. Cancer Surv. *24*, 141–155.

Staunton, D.E., Dustin, M.L., Erickson, H.P., and Springer, T.A. (1990). The arrangement of the immunoglobulin-like domains of ICAM-1 and the binding sites for LFA-1 and rhinovirus. Cell *61*, 243–254.

Staunton, D.E., Dustin, M.L., and Springer, T.A. (1989). Functional cloning of ICAM-2, a cell adhesion ligand for LFA-1 homologous to ICAM-1. Nature *339*, 61–64.

Staunton, D.E., Marlin, S.D., Stratowa, C., Dustin, M.L., and Springer, T.A. (1988). Primary structure of ICAM-1 demonstrates interaction between members of the immunoglobulin and integrin supergene families. Cell *52*, 925–933.

Stockinger, H., Gadd, S.J., Eher, R., Majdic, O., Schreiber, W., Kasinrerk, W., Strass, B., Schnabl, E., and Knapp, W. (1990). Molecular characterization and functional analysis of the leukocyte surface protein CD31. J. Immunol. *145*, 3889–3897.

Tanaka, Y., Kobayashi, K., Takahashi, A., Arai, I., Higuchi, S., Otomo, S., Habu, S., and Nishimura, T. (1993). Inhibition of inflammatory liver injury by a monoclonal antibody against lymphocyte-function-associated antigen-1. J. Immunol. *151*, 5088–5095.

Van der Vieren, M., Trong, H.L., Wood, C.L., Moore, P.F., St. John, T., Staunton, D.E., and Gallatin, W.M. (1995). A novel leukointegrin, $\alpha d\beta 2$, binds preferentially to ICAM-3. Immunity *3*, 683–690.

Vazeux, R., Hoffman, P.A., Tomita, J.K., Dickinson, E.S., Jasman, R.L., St. John, T., and Gallatin, W.M. (1992). Cloning and characterization of a new intercellular adhesion molecule ICAM-R. Nature *360*, 485–488.

Wang, J.H., Yan, Y.W., Garrett, T.P., Liu, J.H., Rodgers, D.W., Garlick, R.L., Tarr, G.E., Husain, Y., Reinherz, E.L., and Harrison, S.C. (1990). Atomic structure of a fragment of human CD4 containing two immunoglobulin-like domains. Nature *348*, 411–418.

Weiner, S.J., Kollman, P.A., Case, D.A., Singh, U.C., Ghio, C., Alagona, G., Profeta, S., Jr., and Weiner, P. (1984). A new force field for molecular mechanical simulation of nucleic acids and proteins. J. Am. Chem. Soc. *106*, 765–784.

Weiner, S.J., Kollman, P.A., Nguyen, D.T., and Case, D.A. (1986). An all atom force field for simulations of proteins and nucleic acids. J. Computational Chem. *7*, 230–252.

Wurm, F.M., Gwinn, K.A., and Kingston, R.E. (1986). Inducible overproduction of the mouse c-myc protein in mammalian cells. Proc. Natl. Acad. Sci. USA *83*, 5414–5418.