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Localization of the Binding Site on Intercellular Adhesion Molecule-3 (ICAM-3) for Lymphocyte Function-associated Antigen 1 (LFA-1)*

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Intercellular adhesion molecule 3 (ICAM-3; CD50) is the predominant counter-receptor on resting T cells and monocytes for the leukocyte integrin, lymphocyte function-associated antigen 1 (LFA-1; CD11a/CD18), and may play an important role in the initial stages of the T cell-dependent immune response. Deletion of individual immunoglobulin superfamily (IgSF) domains of ICAM-3 and ICAM-3 IgSF domain chimeras with CD21 showed there is a single LFA-1 binding site in ICAM-3 and that IgSF domain 1 is necessary and sufficient for LFA-1 binding. Epitope mapping and functional studies performed with 17 anti-ICAM-3 monoclonal antibodies demonstrated that only some monoclonal antibodies, with epitopes wholly within domain 1 of ICAM-3, were able to block binding of ICAM-3 bearing cells to purified LFA-1, in agreement with the data obtained from the domain deletion mutants and CD21 chimeras. Analysis of a panel of 45 point mutants of domain 1 of ICAM-3 identified five residues that may contact LFA-1 as part of the binding site, Asn^{23}, Ser^{25}, Glu^{37}, Phe^{54}, and Gln^{75}. These five residues are predicted by molecular modeling, based on the structure of vascular cell adhesion molecule 1 (VCAM-1), to cluster in two distinct locations on domain 1 of ICAM-3 on the BED face (Asn^{23} and Ser^{25}) and on the C strand or CD loop (E37), the E strand (F54), and the FG loop (Q75). The residues, Asn^{23} and Ser^{25}, comprise a consensus *N*-linked glycosylation site.

Intercellular adhesion molecule 3 (ICAM-3, CD50)[1] is a 120-kDa single chain glycoprotein found exclusively on leukocytes (1, 2) and most highly expressed on T cells, where it is the predominant LFA-1 counter-receptor (3, 4). The existence of ICAM-3 was inferred by the observation that anti-LFA-1 monoclonal antibodies (mAb) completely inhibited the PMA-stimulated homotypic aggregation of a T cell line, whereas a combination of blocking anti-ICAM-1 and anti-ICAM-2 mAb only partially inhibited aggregation (5). ICAM-3 was subsequently characterized with the mAb, IC3/1 (1), and it was later determined that the previously anonymous leukocyte antigen, CD50, is identical to ICAM-3 (6). ICAM-3 is constitutively expressed on leukocytes in contrast to the inducibly expressed ICAM-1 and is not present on endothelium or platelets as is ICAM-2 (7). Functional activities of anti-ICAM-3 mAb include partial blocking of the allogeneic mixed lymphocyte reaction (2, 8) and co-stimulatory activity for resting T cells (2, 9, 10).

ICAM-3 recently was cloned independently by three groups (11–13), which revealed a type 1 integral membrane protein with a 37-amino acid cytoplasmic region containing 5 serine and 2 tyrosine residues in contrast to ICAM-1 and ICAM-2, which have no serine and only 1 tyrosine in the cytoplasmic domain (14–16). ICAM-3 contains a 25-residue transmembrane region and a 456-residue mature extracellular domain comprising five immunoglobulin superfamily (IgSF) domains. ICAM-3 is 52% identical to ICAM-1 and 37% identical to ICAM-2 in the corresponding regions.

In previous studies of ICAM-1, domain transfer and deletion mutagenesis revealed that the LFA-1 binding site was located within the amino-terminal two IgSF domains (17, 18). Expression of IgSF domain 1 or domain 2 of ICAM-1 in the absence of one another has not been achieved, and point mutations that disrupt conformation suggest that these two domains are conformationally interdependent. Point mutations were identified within domains 1 and 2 that affected binding of transfected cells to purified LFA-1, although the E34A and Q73H mutations within domain 1 had the greatest effect (17). The epitopes of mAb that blocked ICAM-1-dependent binding to purified LFA-1 were mapped within domain 1 (*e.g.* RR1/1) or domain 2 (*e.g.* R6.5) (17). Studies with synthetic peptides of ICAM-2 found that a 22-mer oligopeptide derived from the sequence of domain 1 of ICAM-2, residues Gly^{21} through Ser^{42} inclusive, could inhibit by 50% the binding of endothelial cells to purified LFA-1 at a concentration of 15 μM (19). Peptides shortened from either end were significantly less active. The corresponding peptide based on the ICAM-1 sequence was 10-fold less active. Others have reported inhibitory function for peptides derived from the second domain of ICAM-2 (20), the second domain of ICAM-1 (21), and the fourth domain of ICAM-1 (22). Domain deletion, point mutagenesis, and epitope mapping studies of vascular cell adhesion molecule 1 (VCAM-1) (23, 24), an IgSF member that binds to the β-1 integrin VLA-4, found two distinct homologous binding sites, in IgSF domains 1 and 4, and the existence of a 5-residue motif important for the binding of CAMs to integrins in both of these domains was proposed (23, 24).

In this study of ICAM-3, in contrast to what has been reported for ICAM-1, we demonstrate that IgSF domain 1 of ICAM-3 is sufficient for functional expression of the LFA-1 binding site. Furthermore, in contrast to what has been reported for VCAM-1 and ICAM-2, ICAM-3 does not contain a

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[1] The abbreviations used are: ICAM, intercellular adhesion molecule; mAb, monoclonal antibody(ies); IgSF, immunoglobulin superfamily; LFA-1, lymphocyte function-associated antigen 1; VCAM, vascular cell adhesion molecule; CAM, cell adhesion molecule; BCECF, 2′,7′-bis-(2-carboxyethyl)-5-(and -6)-carboxyfluorescein, acetoxymethyl ester.

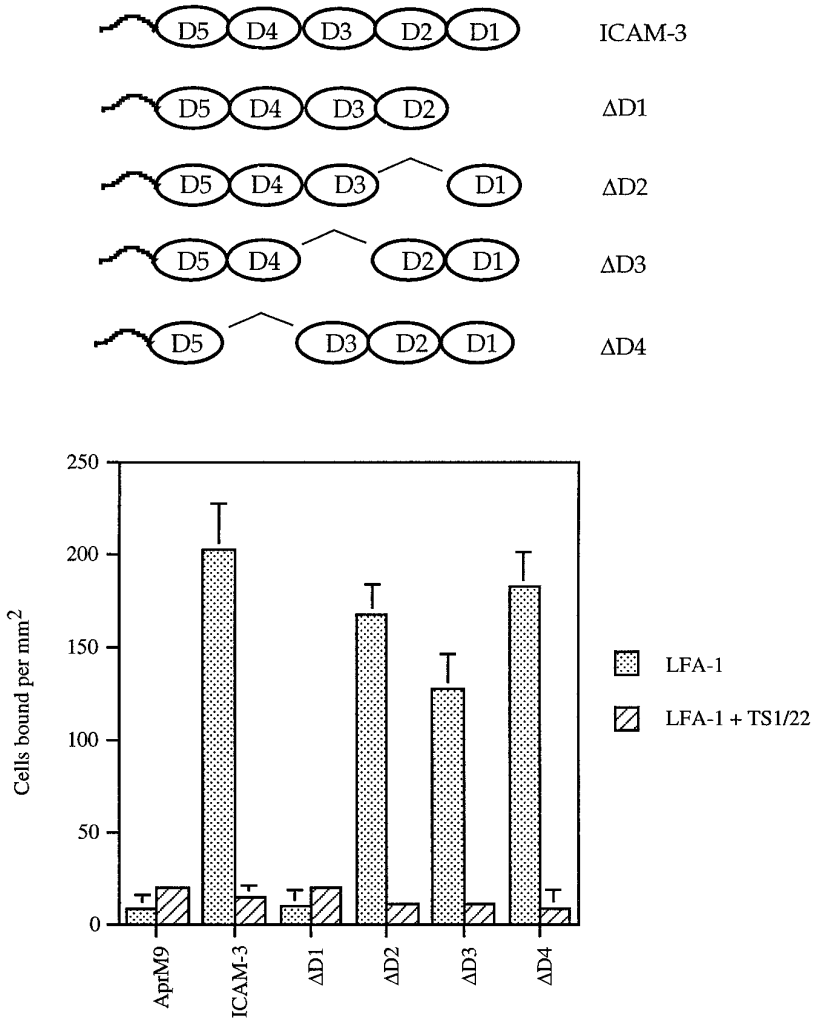

FIG. 1. **Binding of COS cells expressing ICAM-3 deletion mutants to purified LFA-1 on plastic.** *Upper panel*, ICAM-3 domain deletion mutants. *Lower panel*, representative assay of COS cells transiently expressing the indicated cDNAs binding to purified LFA-1 immobilized on plastic. Binding was in the presence of an isotype matched control myeloma, X63, or LFA-1 mAb TS1/22. The *error bars* indicate one standard deviation of triplicate cell counts.

linear sequence centered around Glu^{37} (corresponding to residue Glu^{34} of ICAM-1, residue Asp^{40} of VCAM-1, and residue Glu^{40} of ICAM-2) that is necessary for binding to LFA-1, although Glu^{37} itself is essential. Mutation of five noncontiguous residues within domain 1 of ICAM-3 disrupts binding to LFA-1 without affecting mAb epitopes in domain 1. These residues are predicted to localize at two distinct regions on the three-dimensional model of IgSF domain 1 of ICAM-3.

MATERIALS AND METHODS

Plasmids—The expression vector used throughout this study was AprM9, a derivative of AprM8, which was in turn derived from CDM8 (25). AprM8, 5.6 kilobase pairs, was constructed by ligating the large 2.9-kilobase pair *Sal*I-linkered *Stu*I-*Mlu*I fragment of CDM8 to an *Xho*I-linkered 2.7-kilobase pair *Pvu*I fragment of pBluescript KS+. An orientation was chosen such that the M13 intergenic sequence directed the rescue of the noncoding strand upon co-infection of an *Escherichia coli* strain bearing the plasmid with a helper phage. Thus, the *supF* gene of CDM8 was replaced by a β-lactamase gene, and the piVX ori and M13 intergenic sequences from CDM8 were replaced by the corresponding sequences from Bluescript. In AprM9, the sequence between the *Hin*dIII and *Pst*I sites of AprM8 was replaced by the pSP64 (Promega, Madison WI) nucleotides, GGGCTGCAGGTCGACTCTAGAGGATCCCCGGGCGAGCTCGAATTCCGAGCTCGCCCGGGGATCC, to introduce new *Eco*RI and *Sal*I cloning sites. The plasmid pCDIC-3 was constructed by inserting the full coding sequence of ICAM-3 into the *Hin*dIII and *Eco*RI sites in AprM9 (13).

Mutagenesis—Site-directed mutagenesis, performed by the method of Kunkel (26) with Sequenase v1.0 (U. S. Biochemical Corp.), was used to create the domain deletion mutants and the point mutants in the AprM9 vector. The target mutation was identified by restriction mapping and sequence analysis of the region of the mutation. Reversion of point mutants to wild type was performed as above, after preparing single stranded uracil-containing DNA from each mutant to be reverted.

The plasmids, D1-CD21, D12-CD21, and D123-CD21 were constructed by ligation of polymerase chain reaction fragments of sequence encoding domain 1 (to residue 87), domains 1 and 2 (to residue 188), or domains 1, 2, and 3 (to residue 283), respectively, of ICAM-3 to the *Spe*I and *Xho*I sites of the plasmid piCR2X (27), resulting in replacement of the sequence encoding the leader peptide and first two short consensus repeats of CD21 with the sequence encoding the leader peptide and the indicated domains of ICAM-3. The 5′ oligonucleotide for the polymerase chain reactions was GGACTAGTCCCTGTCAGAATGGCCACCATGTGAC and the 3′ oligonucleotides were CTCCTCGAGCCCGTACACGGTGATGTTAGAGG, GGGCTCGAGAAAGGTTCGGAGCTGGCGGGGG, and TAGCTCGAGAAAGACCGTCAAGTTCTCCCGGG for D1, D12, and D123, respectively. *Spe*I-*Apa*I fragments containing the chimeric molecules were ligated to the 4.3-kilobase pair *Xba*I-*Apa*I fragment of AprM8 to restore the CMV promoter and transfer the chimeras to the same expression vector used for ICAM-3.

The structures of all deletion mutants, chimeric constructs, and point mutations were confirmed by restriction mapping and sequence analysis of the regions of alterations. DNA sequencing was performed as described (28). All restriction and modification enzymes were from U. S. Biochemical Corp. or New England Biolabs.

Transfection—COS cells at 30–50% confluence were transfected in 15-cm dishes using DEAE-dextran (29) with DNA prepared from 1.5-ml cultures by alkaline lysis, and transfected cells were harvested after 72 h. Transfection efficiency, which varied from 25 to 80% between experiments, was relatively uniform within an experiment and was assessed by indirect immunofluorescence and flow cytometry or by a fluorescent cell binding assay with immobilized monoclonal anti-ICAM-3 antibody (see below).

Antibody Binding Assays—Epitope mapping was performed by indi-

TABLE I

Monoclonal antibody epitope localization by immunofluorescent flow cytometry of COS cell transfectants analyzed

COS cells were transfected with the AprM8 vector as control or clones with cDNA for full-length ICAM-3. ICAM-3 clones containing deletions of IgSF domain 1 (ΔD1), domain 2 (ΔD2), domain 3 (ΔD3) or domain 4 (ΔD4). IC2/2 is a mAb to ICAM-2 used as a negative control. All other mAb are to ICAM-3. Values are linear fluorescence intensity units of the total population analyzed and in parentheses the percentage of positive cells. The results are the average of two experiments. Significantly lower values are in bold type and underlined.

mAb name	Vector control	ICAM-3	ΔD1	ΔD2	ΔD3	ΔD4
CBR-IC2/2	33 (7)	35 (9)	32 (7)	31 (6)	33 (7)	33 (7)
BRIC79	36 (9)	445 (67)	**29 (5)**	199 (46)	196 (47)	155 (44)
CG106	40 (12)	288 (60)	**33 (6)**	178 (43)	163 (44)	161 (49)
WDS 3A9	43 (15)	354 (64)	**45 (10)**	171 (43)	168 (46)	155 (47)
ICO-60	42 (14)	361 (64)	**36 (10)**	169 (43)	158 (45)	173 (48)
CBR-IC3/1	34 (7)	227 (56)	**31 (5)**	106 (35)	106 (38)	129 (41)
CBR-IC3/6	37 (10)	156 (56)	**32 (6)**	69 (30)	71 (31)	79 (33)
BY44	37 (12)	285 (59)	**33 (7)**	137 (39)	132 (40)	157 (45)
HP2/19	46 (18)	261 (59)	**41 (12)**	153 (44)	153 (45)	166 (51)
140–11	37 (11)	306 (47)	**33 (7)**	157 (41)	160 (45)	137 (45)
CBR-IC3/2	34 (7)	378 (63)	227 (55)	**32 (7)**	142 (42)	159 (45)
TP1/24	38 (12)	353 (65)	260 (59)	**34 (13)**	139 (39)	156 (47)
101–1D2	36 (8)	289 (63)	200 (53)	**36 (98)**	164 (43)	143 (46)
KS128	38 (10)	324 (62)	**39 (13)**	**26 (7)**	112 (37)	165 (47)
152–2D11	36 (9)	366 (63)	**31 (6)**	**33 (6)**	137 (41)	149 (44)
CBR-IC3/3	32 (5)	152 (52)	117 (45)	73 (34)	67 (31)	**32 (6)**
CBR-IC3/4	35 (8)	116 (46)	108 (43)	74 (32)	62 (29)	**32 (5)**
CBR-IC3/5	35 (8)	215 (57)	156 (50)	95 (37)	83 (35)	**31 (6)**

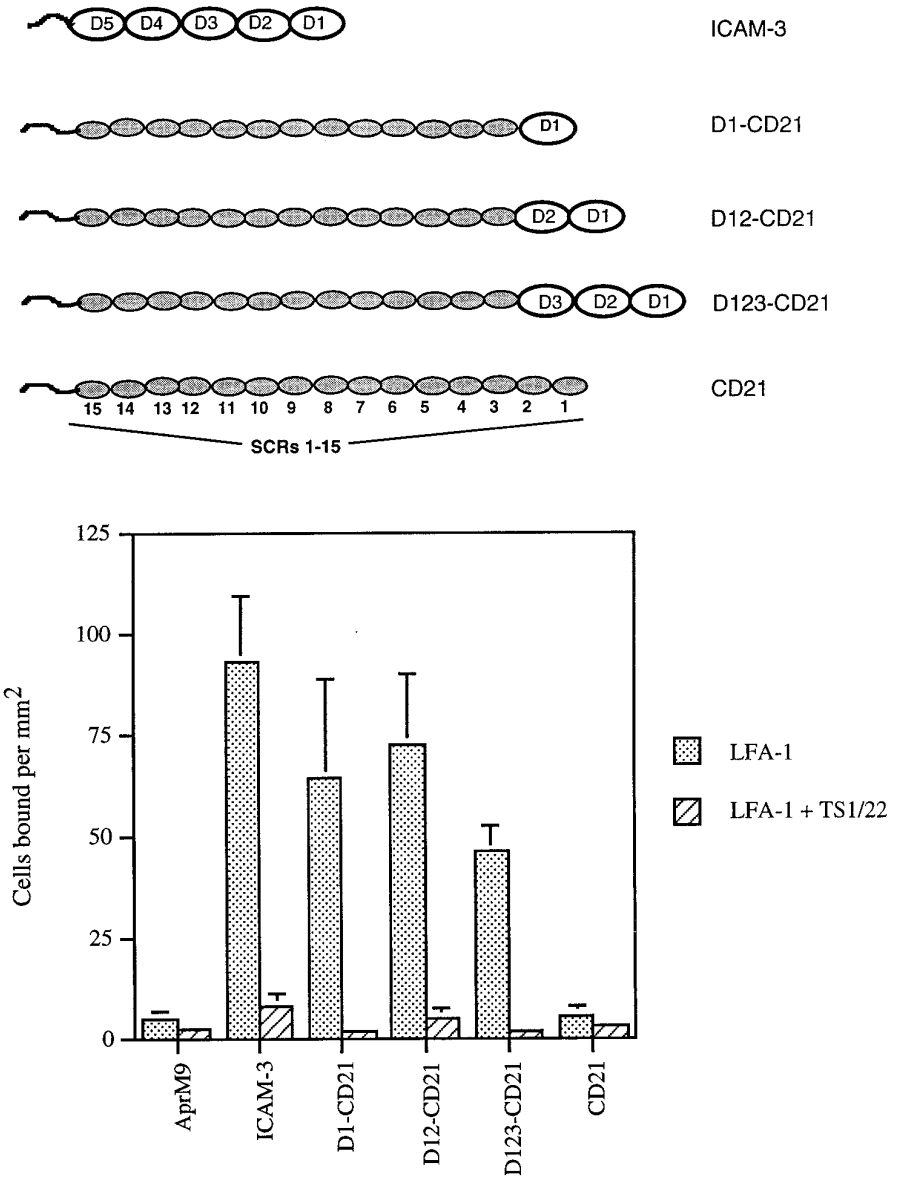

FIG. 2. **Binding of COS cells expressing ICAM-3-CD21 chimeras to purified LFA-1 on plastic.** The structures of the chimeras and the parent molecules are shown in the *upper panel*. The *large ovals* represent IgSF domains, and the *smaller, shaded ovals* represent the short consensus repeats (*SCRs*) comprising the CD21 extracellular domain. The *lower panel* shows the results of a representative LFA-1 binding assay of COS cells transiently expressing the indicated cDNAs, as described in the legend to Fig. 1.

rect immunofluorescence and flow cytometry of transfected cells or by an immobilized antibody binding assay. In the latter, 50 μl of purified monoclonal antibody at 10–20 μg/ml in 50 mM Tris, pH 9.0, or ascites at 1:100 dilution in the same buffer were placed in duplicate wells of Linbro Titertek flat bottom 96-well plates. After 1–2 h at 21 °C, the antibody was removed and the wells were blocked with assay buffer (phosphate-buffered saline with 1.5% bovine serum albumin, 3 mM $MgCl_2$, 0.02% NaN_3) for an additional hour. After two additional washes, transfected cells labeled with BCECF (Molecular Probes) (10^4–10^5 cells in 100 μl of assay buffer) were added and allowed to bind for 45 min at room temperature. Nonbound cells were removed with two to three washes with 200 μl of assay buffer, and the percentage of cell binding was determined by fluorimetry.

LFA-1 Binding Assays—LFA-1 was purified from human tonsil and human spleen lysates by immunoaffinity chromatography as described (30). The binding assay was a tip plate assay (31), where purified protein was spotted onto Falcon 35-mm Petri dishes in 50 mM Tris, pH 9.0, allowed to bind for 1–2 h at room temperature, and then removed, and the plate was blocked with assay buffer and then washed twice with assay buffer. Transfected cells (1×10^6) were added in a final volume of 2.0 ml and allowed to settle and bind for 45 min at 37 °C. Unbound cells were removed by tipping the plate and aspirating the medium and cells. After one wash of 3 ml, adherent cells were then fixed with 2% paraformaldehyde in phosphate-buffered saline and counted to determine cells bound per mm^2 for each immobilized protein. For mAb inhibition experiments, the cells or plates (after blocking) were pretreated with mAb at 10 μg/ml in assay buffer for 1 h prior to use. In every case, each plate also contained spots of purified IC3/2 mAb and control IC2/2 mAb to allow normalization for transfection efficiency or small variations in cell number. Bovine serum albumin spots served as negative controls for LFA-1 binding.

For binding of Jurkat cells, purified LFA-1 was coated on 96-well flat bottom microtiter plates (Linbro). Jurkat cells were labeled with BCECF and were pretreated for 15 min at 0 °C with RR1/1 and IC2/2 at 20 μg/ml to block ICAM-1 and ICAM-2, respectively, in addition to the test anti-ICAM-3 mAb. Phorbol myristate acetate was added to a final concentration of 50 ng/ml, and the cells were added to wells in duplicate and allowed to settle under gravity and bind for 45 min at 37 °C. The wells were washed three times by aspiration with a 25 gauge needle. Percent binding was determined by microfluorimetry.

Computer-aided Sequence and Structure Analysis—DNA sequences were stored and manipulated with the University of Wisconsin Genetics Computer Group package (32) implemented on a VAX microcomputer. Molecular modeling was performed with the Hyperchem and Grasp software running on a Silicon Graphics Indigo workstation and Prekin and Mage, running on a Macintosh. VCAM-1 coordinates (33) were obtained from the Brookhaven National Laboratories via anonymous file transfer protocol at the address pdb.pdb.bnl.gov.

RESULTS

Oligonucleotide-directed mutagenesis was used to create a panel of four IgSF domain deletions (Fig. 1). All four deletion mutants directed the expression in transfected COS cells of antigenic ICAM-3 at comparable levels (Table I). COS cell transfectants were assayed for binding to purified LFA-1 on plastic (Fig. 1). The construct, ΔD1, in which domain 1 was deleted, did not direct binding of transfected COS cells to purified LFA-1. In contrast, the constructions lacking domains 2, 3, or 4 directed binding to purified LFA-1 at near wild type levels (Fig. 1). All ICAM-3-mediated binding was inhibitable by pretreating the coated plates with an LFA-1-specific mAb, confirming the specificity of the assay.

The above results demonstrated that domain 1 of ICAM-3 is required for binding to LFA-1 but did not rule out a contribution from the other IgSF domains. To determine whether domain 1 of ICAM-3 was able to mediate specific binding to purified LFA-1 out of the context of adjacent IgSF domains, sequences encoding domain 1 of ICAM-3, domains 1 and 2, or domains 1, 2, and 3 were transferred to the sequence encoding the amino-terminal end of short consensus repeat 3 of CD21 (Fig. 2). The short consensus repeat sequences comprising the entire extracellular region of CD21 have no homology with IgSF members and are independent structural units (27). COS cells transiently expressing the chimeric proteins displayed the

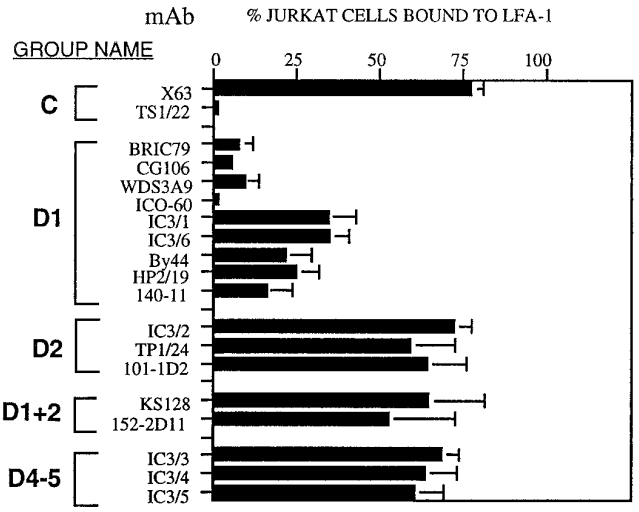

FIG. 3. **Effect of anti-ICAM-3 monoclonal antibodies on binding of Jurkat cells to purified LFA-1 on plastic.** Jurkat cells labeled with the fluorescent dye BCECF were preincubated with RR1/1 + IC2/2 mAb to block ICAM-1 and ICAM-2, respectively, and with the indicated anti-ICAM-3 antibody and then added to the wells of a 96-well plate coated with purified LFA-1. The percentage of binding was determined by analysis with a fluorescence concentration analyzer before and after washing. X63 is a nonbinding negative control myeloma, and TS1/22 is a blocking anti-LFA-1 antibody used as a positive control. Antibodies were used at 10 μg/ml or at a 1:100 dilution of ascites. The *error bars* indicate the standard deviation of the average of six experiments, except that BRIC79 was studied in three experiments.

CD21 epitope for the HB-5 mAb and the expected ICAM-3 mAb epitopes (data not shown). COS cells expressing wild type ICAM-3 but not COS cells transfected with the AprM9 vector alone or with a cDNA encoding the full-length CD21 bound to purified LFA-1 (Fig. 2). COS cells expressing the CD21 chimeras containing domain 1 of ICAM-3, domains 1 and 2, and domains 1, 2, and 3 bound to LFA-1 at 50–78% of wild type levels (Fig. 2). The lower binding of the chimeras with respect to wild type ICAM-3 is likely accounted for by the lower expression levels of the chimeras on COS cells observed in all five experiments (not shown). These results demonstrate that an LFA-1 binding site is wholly contained within domain 1 of ICAM-3.

The ICAM-3 domain deletion mutants were transiently expressed in COS cells and examined for the presence of the epitopes defined by 17 ICAM-3 mAb clustered in the 5th Leukocyte Typing Workshop (7) by indirect immunofluorescence and flow cytometry. Four mAb groups were identified (Table I). Nine mAb failed to bind to cells expressing the mutant lacking domain 1 but bound well to cells expressing all the other domain deletions, suggesting that the epitope(s) for these mAb are within domain 1 of ICAM-3 (Table I). Three mAb failed to bind to cells expressing the mutant lacking domain 2 but bound well to cells expressing all the other domain deletions, suggesting that the epitope(s) for these mAb are within domain 2 of ICAM-3 (Table I). Two mAb required the presence of both domains 1 and 2 for expression of their epitopes. Three mAb required the presence of domain 4 but not domains 1, 2, or 3 for epitope expression (Table I). Similar results were obtained with stably transfected L cells (data not shown).

The 17 anti-ICAM-3 mAb were assayed in the presence of mAb to ICAM-1 and ICAM-2 for the ability to inhibit binding of fluorescently labeled Jurkat T cells to purified LFA-1 adsorbed to plastic. Four mAb with epitopes that mapped to domain 1 of ICAM-3 completely blocked binding of Jurkat cells to purified LFA-1 (Fig. 3). The five other mAb to domain 1 of ICAM-3 inhibited by approximately 50% the binding of Jurkat cells to

mutation	LFA-1 binding % of control	BRIC79	CG106	IC3/1	IC3/6	WDS3A9	HP2/19	140-11	ICO-60	Epitope map n=
AprM9	0 *(11)	0	0	0	0	0	0	0	0	7
ICAM-3	100 *(11)	106	90	105	85	97	85	108	104	7
V7/S	70 ±42(3)	63	56	31	35	54	75	105	142	3
V7/F	117 ± 7(2)	86	73	79	47	73	100	120	133	2
P9/A	62 ±12(2)	93	52	54	42	77	39	104	99	2
Q10/A	60 ± 7(2)	101	79	92	66	90	96	118	103	2
L14/A	84± 20(3)	93	71	92	44	92	98	159	90	2
S19/A	125 ±12(2)	150	90	59	51	79	104	110	120	2
F21/A	78 ±17(2)	112	86	83	49	83	91	100	105	2
N23/A	**24±5**(3)	95	77	66	36	93	75	86	122	2
N23/Q	88 ±42(3)	111	66	30	52	71	86	108	124	2
N23/Arev	101 ±23(3)	§	86	56	35	81	110	111	142	2
S25/A	**19±3**(4)	113	46	56	36	76	46	95	95	2
S25T/AA†	4 ±1(2)	19	0	0	0	0	0	6	20	2
S25/Arev	107 ±26(2)	94	69	84	73	100	95	107	105	3
P29/A	71 ±32(2)	119	63	85	42	83	92	128	89	1
S31/A	90 ±16(2)	136	39	92	93	70	93	152	105	2
E32/A	83 ± 9(3)	100	68	60	30	85	121	114	118	2
A35/S	92 ±17(2)	76	46	68	24	66	117	101	134	2
L36/A	83 ±15(2)	124	72	132	70	71	108	111	96	2
ΔL36-K42†	1 ± 1(1)	17	0	0	0	0	0	6	20	1
E37/A	**4 ±1**(5)	16	17	21	7	2	101	49	121	4
E37/S	**11±3**(3)	23	16	15	2	9	75	42	91	2
E37/K	**5 ±2**(3)	3	1	3	3	5	92	41	102	2
E37/Arev	109 ±17(3)	111	56	73	58	74	96	102	103	3
T38/A	65 ±11(2)	114	52	93	52	84	97	86	121	2
S39/A	71 ±14(2)	110	51	91	47	75	88	105	109	2
L40/A	64 ± 8(2)	115	73	78	41	69	99	91	117	2
S41/A	66 ±10(2)	119	54	76	39	59	100	98	111	2
K42/A	82 ±18(2)	104	86	96	91	81	94	104	114	2
L44/A	103 ±47(3)	92	82	59	55	77	99	103	102	2
G48/A	136 ±22(2)	134	66	107	73	91	96	109	124	2
W51/A†	0 ± 1(4)	18	0	0	0	0	120	0	21	3
F54/A	**17± 2**(5)	§	61	41	32	66	83	91	118	4
F54/Arev	100 ±18(3)	107	60	54	34	74	77	105	115	3
L56/A	71 ±14(2)	74	57	46	12	65	66	97	117	3
S57N/AA	127 ±35(3)	118	84	90	57	80	107	102	112	2
V59/S	56 ±20(5)	§	73	48	30	57	85	70	87	3
G61/A	57 ±31(2)	118	69	58	55	70	99	96	111	2
N62S/AA	121 ±26(3)	109	69	76	46	76	101	105	108	2
N72/A	85 ±37(2)	108	66	82	59	67	84	100	108	2
Q75/H	**22±13**(3)	132	31	41	6	7	124	39	121	2
Q75/Hrev	125 ±31(3)	81	63	50	43	54	83	93	103	2
I76T/LA	125 ±21(2)	107	65	47	40	67	95	83	112	1
S79S/AA	53 ±14(2)	§	130	79	72	120	144	79	166	2
T83/A	109 ±24(2)	139	78	67	58	67	141	93	90	1
Y85/A	107 ±24(2)	102	75	47	36	48	76	67	103	1

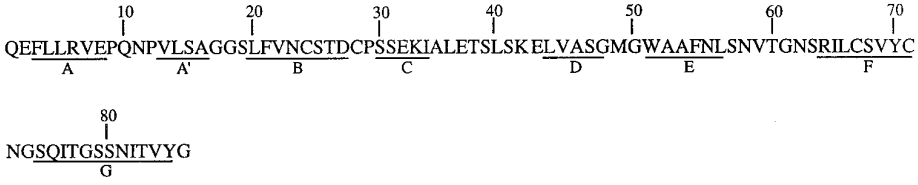

FIG. 4. **Effect of point mutations of ICAM-3 on binding to purified LFA-1 and to immobilized mAb.** Mutations are indicated using the one letter code and numbered after de Fougerolles *et al.* (13). The residues after the slash indicate the change(s) made. The suffix "rev" indicates a reversion to wild type sequence of the designated mutant. Δ*L36-K42* represents a deletion of the range of residues specified, inclusively. LFA-1 binding is expressed in the following format: percentage of control binding ± standard deviation (number of experiments). Each experiment was counted in triplicate. Binding of transfected COS cells to immobilized mAb was quantitated by a fluorescence concentration analyzer and is expressed as the percentage of binding to IC3/2 mAb, a mAb with an epitope within domain 2 of ICAM-3 that was highly expressed in all mutants, even those judged to have substantial loss of overall domain 1 structure. Binding for all transfectants was less than 100% for some mAb, *e.g.* IC3/6, an IgE mAb supplied as ascites that apparently was not immobilized as efficiently as others, *e.g.* 140–11. Mutations with significant effects on binding to purified LFA-1 are in *bold* and *underlined*. *, COS cells transfected with AprM9, the expression vector, and wild type ICAM-3 were used to normalize other data and gave 0% binding and 100% binding by definition. † indicates point mutations where nearly all mAb epitopes were lost and were judged to have caused a large scale disruption of domain 1 structure. § indicates binding data not available for BRIC79 mAb. The sequence of domain 1 of ICAM-3 is indicated at the *bottom*. Predicted β strands are *underlined* and labeled after Jones *et al.* (33). Strands A′ and C were shortened to exclude proline residues.

purified LFA-1, and all other mAb did not significantly inhibit binding (Fig. 3). These data confirm that domain 1 of ICAM-3 is necessary for binding to LFA-1 and support the finding that the other ICAM-3 domains do not contribute to the binding site. Furthermore, unlike ICAM-1 (17, 18), domain 1 of ICAM-3 may be expressed independently of domain 2.

The sequence of domain 1 of ICAM-3 was aligned with the sequence of domains of other IgSF members that bind integrins: domain 1 of ICAM-2, ICAM-3, mucosal addressin CAM-1, and VCAM-1 and domain 4 of VCAM-1. All residues conserved among the CAMs, except for the four invariant cysteines, and residues conserved among ICAMs but not among VCAMs or mucosal addressin CAM were subjected to site-directed mutagenesis. Additionally, residues previously suggested to be important in interactions with integrins and all five potential *N*-linked glycosylation sites were changed (Fig. 4). In general, residues were substituted with alanine, except alanine and valine were substituted with serine. Mutants were named with the one-letter code, with a slash separating the wild type and mutated residues. A "Δ" indicates deletion of the residues that follow, and the suffix "rev" refers to a wild type revertant of the indicated mutation. All mutant cDNAs were expressed at comparable levels and directed the expression of ICAM-3 with an intact domain 2 epitope, as assessed by binding of transfected cells to purified IC3/2 mAb on plastic or by indirect immunofluorescence and flow cytometry (not shown). Studies with eight mAb to domain 1 showed that mutants S25T/AA and ΔL36ETSLSK lost all eight epitopes, and W51/A lost 7 of 8 epitopes, indicative of a large scale disruption of domain structure. Mutants E37/A, E37/S, and E37/K lost five mAb epitopes, Q75/H lost two mAb epitopes and partially lost three others and L56/A partially lost one epitope (Fig. 4). All mAb that recognize domain 1 of ICAM-3 have overlapping epitopes (7), thus loss of multiple epitopes must be interpreted with caution because it may indicate loss of a localized epitope rather than overall domain disruption.

The point mutants N23/A, S25/A, E37/A, F54/A, and Q75/H mediated binding of transfected COS cells to purified LFA-1 at 24, 19, 4, 17, and 22% of wild type binding, respectively ($p < 0.01$; Fig. 4). Two of these, E37/A and Q75/H, correspond to the

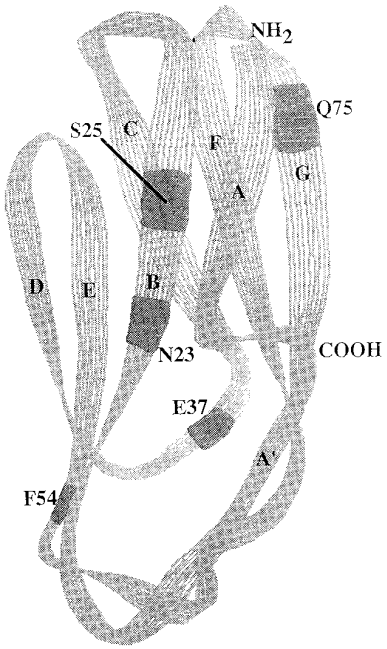

FIG. 5. **Model of domain 1 of ICAM-3.** The predicted alpha-carbon backbone of ICAM-3 is based on the reported crystal structure of domains 1 and 2 of VCAM-1 (33, 37). The CD loop has been shortened to account for the 3-amino acid deletion in that region of ICAM-3 relative to the corresponding region of VCAM-1. The locations of the 5 residues in domain 1 of ICAM-3 found to be important for LFA-1 binding are indicated.

E34/A and Q73/H mutations of ICAM-1 that were shown previously to be important in binding to LFA-1 (17, 18). Loss of binding to LFA-1 seen with the E37/A mutation was confirmed with two additional mutations, E37/S and E37/K (Fig. 4). The F54/A and Q75/H mutations represent two of the nine positions where a residue is conserved among the ICAMs but is distinct from residues in the corresponding positions in VCAMs or mucosal addressin CAM. The other seven positions had no significant effect on binding, except W51/A, where domain structure likely was perturbed. The N23/A and S25/A mutations both disrupt a potential *N*-linked glycosylation site. To address whether the decreased LFA-1 binding was due to alteration of the protein or loss of *N*-linked glycosylation, another mutation, N23/Q, with a conservative substitution of glutamine for asparagine was made. COS cells expressing ICAM-3 with the N23Q mutation bound to purified LFA-1 at levels not significantly different from wild type (Fig. 4). This suggests either that the residue at position 23 itself is important for LFA-1 binding, not a putative *N*-linked carbohydrate, or that hydrogen bonds from the glutamine side chain can substitute for hydrogen bonds from *N*-linked carbohydrate residues to the protein. None of the other mutations that disrupted consensus *N*-linked glycosylation sites, S57N/AA (two sites), N72/A, or T83/A, had significant effects on LFA-1 binding (Fig. 5). None of the mutations that targeted sites conserved among all CAMs, including the mutations S19/A and Y85/A, which are the only residues other than cysteine that are 100% identical among all CAMs, had a significant effect on binding of transfected cells to purified LFA-1 (Fig. 4).

The cDNAs encoding the five point mutants that had a significant functional effect, N23A, S25A, E37A, F54A, and Q75H, were individually reverted to wild type using the same method by which they were initially prepared (26). COS cells expressing the revertants bound LFA-1 at levels equivalent to wild type and regained antibody epitopes that had been lost (Fig. 4). These results confirmed that the loss of function and epitopes was due to the identified mutation and not due to an unrecognized change elsewhere in the molecule.

DISCUSSION

This work has demonstrated that IgSF domain 1 of ICAM-3 is necessary and sufficient for expression of an LFA-1 binding site. Domain 1 of ICAM-3 is an independently folded domain, because it was successfully expressed as an amino-terminal fragment in the CD21 chimera. By contrast, domain 1 of ICAM-1 cannot be expressed in the absence of domain 2 (17, 18). Point mutations within domain 2 of ICAM-1 have been defined that result in diminished binding to LFA-1. Furthermore, the mAb R6.5 recognizes an epitope in domain 2 of ICAM-1 yet blocks binding to LFA-1. It is possible that LFA-1 binds to similar regions in domain 1 of ICAM-1 and ICAM-3 and binds to an additional site present on domain 2 of ICAM-1 but not ICAM-3; this would be consistent with the stronger binding observed to ICAM-1 (1). Alternatively, the R6.5 mAb may sterically hinder binding of LFA-1 to domain 1 of ICAM-1, or mutations in domain 2 that diminish binding to LFA-1 may affect domain 1 conformation. In ICAM-1, mutations in domain 1 can affect expression of mAb epitopes in domain 2 and vice versa, showing the domains are closely linked structurally (17, 18, 34). In contrast, complete deletion of domain 1 of ICAM-3 or point mutations that caused loss of all epitopes in domain 1 of ICAM-3 did not affect the expression of domain 2 epitopes. Nevertheless, domains 1 and 2 of ICAM-3 are predicted to be closely associated, as shown for other adhesion molecules with IgSF domains, CD4 (35) and CD2 (36). Indeed, two ICAM-3 mAb, KS128 and 152–2D11, required the presence of both ICAM-3 domains 1 and 2, which suggests an epitope that spans both domains.

Epitope mapping studies identified ICAM-3 mAb with epitopes that were wholly within domain 1 that required the presence of both domain 1 and 2, that were wholly within domain 2, and that were dependent on the presence of domain 4. All nine mAb to domain 1 of ICAM-3 cross-block the mAb IC3/1, HP2/19, or 140–11 (7), indicating that the mAb epitopes substantially overlap. The extensive glycosylation of domain 1 (1) might limit the number of antibody epitopes. In contrast to the R6.5 and 8.4A6 mAb to domain 2 of ICAM-1, which block interaction with LFA-1 (7, 17, 18), none of three mAb to domain 2 of ICAM-3, IC3/2, 101–1D2, and TP1/24, which represent two nonoverlapping epitopes,[2] blocked binding of ICAM-3 bearing cells to purified LFA-1; nor did mAb that required presence of both domain 1 and 2 interfere with LFA-1 binding. The nine mAb to domain 1 of ICAM-3 must recognize at least five overlapping but not identical epitopes, because five of the nine anti-ICAM-3 mAb did not bind to the Glu^{37} mutants, and only four of the nine mAb blocked binding of ICAM-3 bearing cells 100%, whereas the others inhibited approximately 50%. Three of the nine mAb stimulated strong homotypic aggregation (7), whereas the others did not. A summary of these features is presented in Table II.

The panel of point mutants studied resulted in the identification of 5 residues within domain 1 of ICAM-3 important for binding to LFA-1: Asn^{23}, Ser^{25}, Glu^{37}, Phe^{54}, and Gln^{75}. We modeled ICAM-3 (Fig. 5) based on the crystal structure of VCAM-1 (33, 37). Mutational analysis of VCAM-1 together with the structure of VCAM-1 suggest that integrin binding occurs to the CFG face of domain 1, with the acidic residue in VCAM-1 homologous to Glu^{37} in ICAM-3 present on a prominent loop between β strands C and D (33, 37). Glu^{37} corre-

[2] L. B. Klickstein, M. R. York, A. R. de Fougerolles, and T. A. Springer, unpublished observations.

TABLE II
Characteristics of mAb to domain 1

Homotypic aggregation triggered by mAbs was studied and reported in the 5th Leukocyte Typing Workshop (7). Similar mAbs are grouped together.

mAb name	Epitope wholly within domain 1	Epitope retained in E37/A and E37/S	Promoted strong homotypic aggregation	Completely blocked binding to LFA-1 on plastic
BRIC79 CG106 WDS3A9	Yes	No	No	Yes
ICO-60	Yes	Yes	No	Yes
IC3/1 IC3/6	Yes	No	Yes	No
BY44 140–11	Yes	Yes	No	No
HP2/19	Yes	Yes	Yes	No
KS128 152–2D11	No	Yes	No	No

sponds to Glu^{34} of ICAM-1, previously shown to be the most important residue for binding to LFA-1 (17, 18). E37 of ICAM-3 also corresponds to residue Asp^{229} in domain 3 of ICAM-1, a residue shown to be important in binding to Mac-1 (34), and Glu^{37} corresponds to Asp^{40} and Asp^{328} of VCAM-1, mutation of which affects binding of VCAM-1 to $\alpha 4$ integrins (23, 24). Thus, the acidic residue corresponding to Glu^{37} of ICAM-3 is an essential component of all integrin-binding CAMs identified to date. The residue of ICAM-1 corresponding to Gln^{75} is Gln^{73} and has previously been shown to be important for binding of ICAM-1 bearing cells to LFA-1 (17). Residue Gln^{75} is also predicted to lie on the CFG face on the FG loop. Interestingly, although Gln^{75} is consistently found to be functionally important in recognition of ICAM-1 and ICAM-3 by LFA-1, the homologous residue in VCAM-1 is not; however, a nearby Glu residue in the G strand of VCAM-1 is important. The residue of ICAM-1 corresponding to Phe^{54} is Tyr^{52}. Tyr^{52} in ICAM-1 was tested but was conservatively changed to Phe, another aromatic residue, and no significant effect on LFA-1 binding was seen (17). Alignment with the VCAM-1 structure suggests that Phe^{54} of ICAM-3 is in β strand E; and is not far from Glu^{37}; thus these residues may be part of a common recognition site.

The most curious mutational results to emerge from this study concern residues Asn^{23} and Ser^{25}, which have not been previously studied and are predicted to be surface exposed in β strand B. This strand is located in the BED face and thus is on the opposite face of domain 1 from Glu^{37} and Gln^{75}. The S25/A mutation is conservative, resulting in loss of only one hydroxyl, yet consistently reduced binding to LFA-1 by 5-fold. Reversion of the mutation (S25/A rev) resulted in full recovery of function. Mutation of the neighboring Asn^{23} residue to Ala but not the conservative substitution to Gln also significantly diminished LFA-1 binding. Residues Asn^{23} and Ser^{25} constitute a consensus *N*-linked glycosylation site in ICAM-3. The results suggest either that the glycan predicted to be *N*-linked to Asn^{23} interacts with domain 1 in a manner that can be mimicked by the N23Q but not N23A or S25A mutations and has a localized effect on conformation important for binding to LFA-1 or that Asn^{23} and Ser^{25} may be part of a second binding site for LFA-1. The residue of ICAM-1 that aligns with Asn^{23} is Thr^{20}, which was changed to an alanine by Staunton *et al.* (17) in the mutant T20CS/ACT, and had no significant effect on binding to LFA-1. Ser^{22} of ICAM-1, which corresponds to Ser^{25} of ICAM-3, has not been tested. These findings may suggest that the residues mediating binding of ICAM-1 and ICAM-3 to LFA-1 are similar but not identical. It remains to be determined whether this is related to the observation that two anti-LFA-1 mAb that block binding of LFA-1 bearing cells to ICAM-3 do not block binding to ICAM-1 and that the activating LFA-1 mAb, MEM-83, stimulates binding to ICAM-1 but not to ICAM-3 (38).

Individual mutagenesis of each residue in ICAM-3 from positions 35 to 42 showed that only mutation of E37A had a significant effect, although there was a slight but not insignificant reduction in binding by mutations at residues 38–41. This is in contrast to human VCAM-1, where Gln^{38}, Asp^{40}, and Leu^{43} were individually shown to be important for transfected COS binding to VLA-4 on Ramos cells, and a 5-residue consensus sequence was proposed to be important for integrin binding (23). This was subsequently proven when a peptide encompassing VCAM-1 residues Trp^{35}-Lys^{46} was shown to block binding of Ramos cells to purified, recombinant soluble VCAM-1 on plastic (37). Similarly, a synthetic peptide from the corresponding region of ICAM-2 has been shown to bind LFA-1, whereas the corresponding region from ICAM-1 or ICAM-3 did not bind (19). Clearly, the ICAM-3 binding site for LFA-1 is not represented by a short, linear stretch of amino acids similar to the canonical RGD sequence recognized by some β_1 integrins such as VLA-5 or to the LDV sequence in the CS-1 peptide of fibronectin recognized by VLA-4 (39, 40) but rather is comprised of residues more widely distributed over the first IgSF domain.

Complementary studies were recently published in which mutant ICAM-3 Ig chimeras were adsorbed to plastic and tested for binding to cells bearing LFA-1 (41, 42), *i.e.* the binding assay was in the opposite orientation to that reported here. Sadhu *et al.* (41) also found that domain 1 of ICAM-3 was structurally independent of domain 2. Sadhu *et al.* (41) found that three of six mAb to domain 1 of ICAM-3 did not bind to an E37T/AS double mutant, and two of six did not bind to a Q75I/AS double mutant, similar to the results reported here with single E37/A, E37/S, E37/K, and Q75/H mutants. In contrast, all eight mAb to domain 1 of ICAM-3 studied by Holness *et al.* (42) in an enzyme-linked immunosorbent assay bound at wild type levels to both E37A and Q75H mutants, including the mAb CG106 and By44, which were evaluated in this study and found not to bind to the same mutants (Fig. 5 and data not shown). The discrepancy in these results remains unexplained.

All three groups found Glu^{37} and Gln^{75} to be important for ICAM-3 function. Sadhu *et al.* (41) identified T38/A as an ICAM-3 mutant that supported only 35% of wild type binding; in the present study, the same mutant supported 65% of wild type binding. Holness *et al.* (42) also found L66/K and S68/K mutants that did not support binding to LFA-1 bearing cells; however, Sadhu *et al.* (41) prepared the S68/A mutation and reported no loss of epitopes or LFA-1 binding function. Neither of these other studies examined Asn^{23}, Ser^{25}, or Phe^{54}.

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