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The Arrangement of the Immunoglobulin-like Domains of ICAM-1 and the Binding Sites for LFA-1 and Rhinovirus

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Summary

Intercellular adhesion molecule 1 (ICAM-1, CD54) binds to the integrin LFA-1 (CD11a/CD18), promoting cell adhesion in immune and inflammatory reactions. ICAM-1 is also subverted as a receptor by the major group of rhinoviruses. Electron micrographs show that ICAM-1 is a bent rod, 18.7 nm long, suggesting a model in which the five immunoglobulin-like domains are oriented head to tail at a small angle to the rod axis. ICAM-1 sequences important to binding LFA-1, rhinovirus, and four monoclonal antibodies were identified through the characterization of chimeric ICAM-1 molecules and mutants. The amino-terminal two immunoglobulin-like domains of ICAM-1 appear to interact conformationally. Domain 1 of ICAM-1 contains the primary site of contact for both LFA-1 and rhinovirus; the presence of domains 3–5 markedly affects the accessibility of the binding site for rhinovirus and less so for LFA-1. The binding sites appear to be distinct but overlapping; rhinovirus binding also differs from LFA-1 binding in its lack of divalent cation dependence. Our analysis suggests that rhinoviruses mimic LFA-1 in binding to the most membrane-distal, and thus most accessible, site of ICAM-1.

Introduction

Intercellular adhesion molecule 1 (ICAM-1, CD54) and lymphocyte function-associated antigen 1 (LFA-1, CD11a/CD18) are cell surface adhesion receptors that bind to one another to promote a wide variety of cellular interactions, including T lymphocyte antigen-specific responses and leukocyte binding to endothelium and emigration into inflammatory sites (Rothlein et al., 1986; Marlin and Springer, 1987; Kishimoto et al., 1989; Dustin et al., 1988). ICAM-1 normally has a restricted distribution; however, cytokines and bacterial products released at inflammatory sites induce ICAM-1 mRNA and protein expression in a wide variety of cell types. ICAM-1 is a heavily glycosylated 90,000 M_r single-chain cell surface molecule with five tandem immunoglobulin (Ig)-like domains (Dustin et al., 1986; Simmons et al., 1988; Staunton et al., 1988). The murine homolog of ICAM-1 is 50% identical in amino acid sequence and also has five Ig-like domains (Horley et al., 1989; Siu et al., 1989; Ballantyne et al., 1989). Recently, a second counter-receptor for LFA-1 in the human, ICAM-2, was cloned (Staunton et al., 1989b). ICAM-2 has two Ig-like domains and is more homologous (35%) to the first two domains of ICAM-1 than to other members of the Ig superfamily (~19%); thus, ICAM-1 and ICAM-2 are an Ig-like subfamily specialized for interaction with LFA-1.

LFA-1 expression is confined to leukocytes. LFA-1 contains two noncovalently associated subunits, α of 180,000 M_r and β of 95,000 M_r, and is a member of the integrin family (Kishimoto et al., 1989). LFA-1 and two other adhesion receptors expressed on leukocytes that share the same β subunit, Mac-1 and p150,95, define an integrin subfamily known as the leukocyte integrins. Two other integrin subfamilies share distinct β subunits and primarily recognize extracellular matrix ligands such as fibronectin, laminin, fibrinogen, and collagen. Some but not all of the integrins have been found to recognize the sequence arginine-glycine-aspartic acid (RGD) or closely related sequences within a diverse set of ligands including fibronectin, von Willebrand factor, and fibrinogen (Ruoslahti and Pierschbacher, 1986; Hynes, 1987; Hemler, 1990). Neither ICAM-1 nor ICAM-2 possesses an RGD sequence, however, and binding of ICAM-1 to LFA-1 is not inhibited by RGD peptides (Marlin and Springer, 1987). Thus, the mode of recognition involved in this interesting interaction between members of the Ig and integrin families is unknown.

ICAM-1 has recently been shown to be subverted as a receptor by the major group of rhinoviruses (Greve et al., 1989), and these findings have been confirmed and extended (Staunton et al., 1989a; Tomassini et al., 1989). Rhinoviruses, members of the small, RNA-containing, protein-encapsidated picornavirus family, cause 40% to 50% of common colds (Rueckert, 1985; Sperber and Hayden, 1988). Over 100 immunologically non-cross-reactive rhinoviruses have been defined, of which 90% bind to ICAM-1. X-ray crystallography shows that rhinoviruses are 30 nm in diameter and have icosahedral symmetry with 60 copies of each capsid protein (Rossmann et al., 1985) and hence 60 predicted binding sites for ICAM-1. Based on analysis of amino acid substitution mutants and conformational changes induced by the binding of antiviral drugs, a putative receptor binding site has been defined in a deep region of the depression or "canyon" in the capsid that runs about its 5-fold axes (Rossmann et al., 1985; Colonno et al., 1988; Rossmann and Johnson, 1989). A single ICAM-1 Ig-like domain is predicted to be of the correct size to fit within this region of the canyon (Staunton et al., 1989a); however, an antibody Fab fragment with paired Ig domains is predicted to be excluded (Rossmann et al., 1985). The region of the ICAM-1 molecule that binds to rhinovirus has not been described.

The overall size and shape of ICAM-1 are important for understanding how its Ig domains are arranged; comparison of its dimensions with those of rhinovirus and LFA-1 is important for understanding how the molecules interact. Thus far, X-ray crystal structures for Ig superfamily

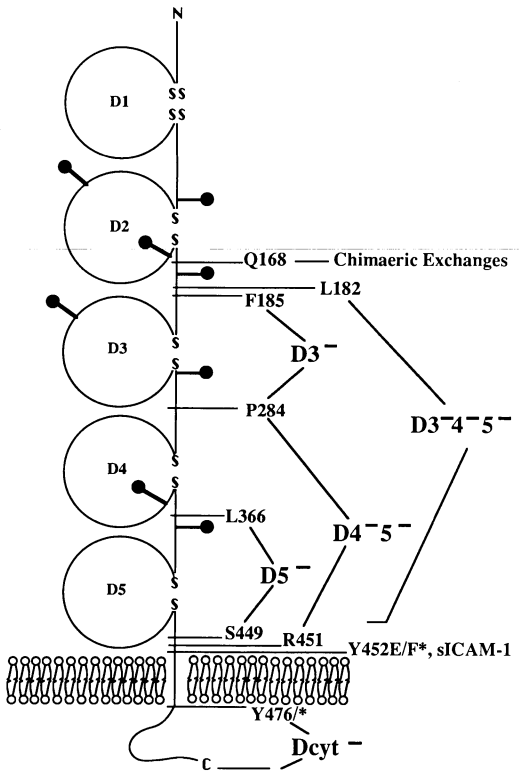

Figure 1. ICAM-1 Schematic with Positions of Chimeric Exchanges and Domain Deletions

Ig-like domains are labeled D1–D5. N-linked glycosylation sites are indicated with "lollipop" structures.

members are available only for immunoglobulins (Alzari et al., 1988), HLA (Bjorkman et al., 1987), and the β_2-microglobulin subunit of HLA (Becker et al., 1977). These molecules have paired Ig domains in their native states, although β_2-microglobulin when released from HLA exists as a free Ig domain (Berggard and Bearn, 1968). Thy-1 contains a single Ig domain (Williams and Barclay, 1988), and electron micrographic studies of neural cell adhesion molecule (NCAM) suggest that its five Ig domains are unpaired (Becker et al., 1989). To understand the arrangement of the Ig domains in ICAM-1, we have examined its structure by electron microscopy.

Cell adhesion and virus adhesion are in principle very similar. Recent findings that a number of cell surface adhesion receptors, including ICAM-1 and CD4, are subverted as virus receptors (White and Littman, 1989) raise the question of what features of these receptors are utilized in common by the cellular counter-receptor and the virus, and whether these features are biologically relevant. Previously, three distinct monoclonal antibodies (MAbs) that block binding of LFA-1 to ICAM-1 were also found to block binding of rhinovirus type 14; this suggests that LFA-1 and rhinovirus-14 may bind to an overlapping region on ICAM-1 (Staunton et al., 1989a). Because the binding sites on ICAM-1 for LFA-1 and rhinovirus are not only interesting in their own right but provide an interesting comparison, we have examined them in parallel using chimeric ICAM-1 molecules and mutant ICAM-1 molecules possessing deletions of Ig-like domains or amino acid substitutions.

Results

Visualization of ICAM-1

A soluble fragment of ICAM-1 truncated at amino acid 453 and possessing essentially the entire extracellular domain (sICAM-1; Figure 1) was purified from the culture media of COS cells. sICAM-1 molecules were further purified for electron microscopy by sedimentation in a glycerol gradient. The sICAM-1 molecules sedimented homogenously with a sedimentation coefficient of 3.3S (±0.2S) and showed a single band of 80 kd in SDS–polyacrylamide gel electrophoresis (SDS–PAGE) (Figure 2A). The mass of 80 kd and sedimentation coefficient of 3.3S suggest a value of f/f_{min} of 2.3, indicative of a highly elongated molecule (Erickson, 1982).

Rotary-shadowed sICAM-1 molecules (Figure 2B) are thin rods that are either straight or contain a single bend. Molecules with a uniform curvature or with two bends were rarely seen, suggesting a rigid rod structure with a single hinge point. Although the angle of the bend was somewhat variable, in most of the obviously bent molecules the angle was close to 90°. In one preparation, 50 clearly identified molecules were classified according to the angle: 55% were straight, 30% had a 90° bend, and 15% had intermediate angles (roughly 110°–160°; any bend within 20° of 180° or 90° was not distinguishable from straight or 90°). Angles smaller than 90° were not observed. The predominance of straight molecules suggests that this is the preferred conformation; the prevalence of a 90° bend, as well as the absence of acute angles, suggests that this may be the limit of flexibility. One additional observation suggests that the bending represents an equilibrium between flexible conformations, rather than a permanent feature of some molecules. If some molecules were permanently bent, these should sediment slightly ahead of straight ones. When fractions across the glycerol gradient peak were examined separately, there was no obvious enrichment of bent molecules in the leading fractions.

Length measurements (after subtracting 1 nm at each end for the shell of metal) gave a value of 16.6 ± 0.24 nm (mean ± SD, n = 25) for the straight molecules. For the bent molecules the long arm was 11.8 ± 0.12 nm, and the short arm was 6.9 ± 0.15 nm (n = 21). The total length of the bent molecules, 18.7 nm, is somewhat greater than that measured for the straight molecules. Since the molecules classified as straight might have included some bent ones, in which the bend was obscured by the limiting resolution of the shadowing, the average length of this population might underestimate the true length. Since the bent molecules necessarily had a clean profile, we prefer the contour length from this population as the best estimate of the total length. The rod appears to have a uniform diameter, on the order of 2–3 nm, but measurements of dimensions this small are not reliable.

The ICAM-1 molecule contains five repeats of Ig-like do-

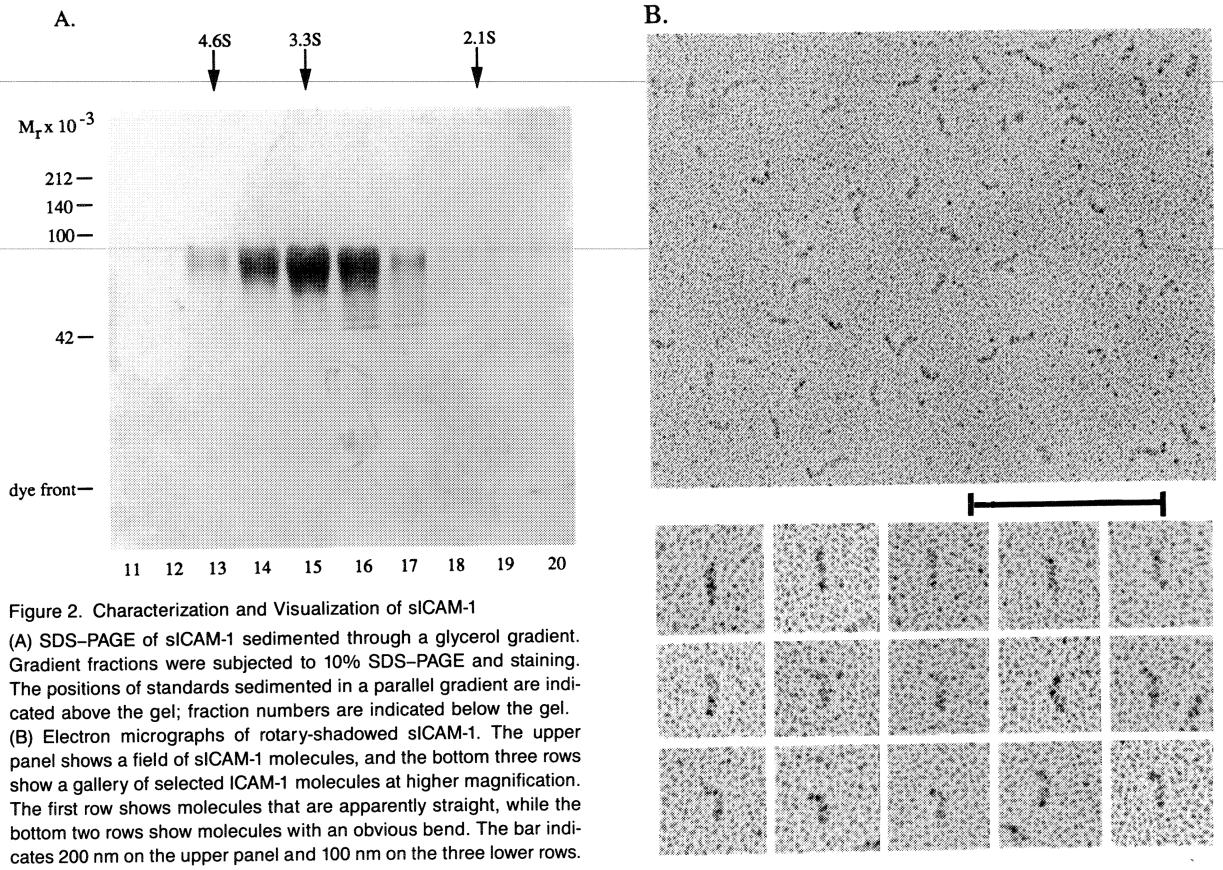

Figure 2. Characterization and Visualization of sICAM-1

(A) SDS–PAGE of sICAM-1 sedimented through a glycerol gradient. Gradient fractions were subjected to 10% SDS–PAGE and staining. The positions of standards sedimented in a parallel gradient are indicated above the gel; fraction numbers are indicated below the gel.

(B) Electron micrographs of rotary-shadowed sICAM-1. The upper panel shows a field of sICAM-1 molecules, and the bottom three rows show a gallery of selected ICAM-1 molecules at higher magnification. The first row shows molecules that are apparently straight, while the bottom two rows show molecules with an obvious bend. The bar indicates 200 nm on the upper panel and 100 nm on the three lower rows.

mains (Staunton et al., 1988; Simmons et al., 1988), labeled D1–D5 in Figure 1. Ig domains have dimensions of 4 × 2.5 × 2.5 nm (Alzari et al., 1988; Becker et al., 1977; Bjorkman et al., 1987; Williams and Barclay, 1988). The total length of the ICAM-1 molecule, 18.7 nm, indicates 3.7 nm per Ig repeat, suggesting that the domains are aligned end to end with their long axes parallel to or at a small angle to the axis of the rod. Models in which two or four of the Ig-like domains are paired with one another side by side are too short. The bend is at a point about two-fifths along the rod, suggesting that it occurs between D2 and D3 or between D3 and D4.

Binding of Chimeric ICAM-1 Molecules to Rhinovirus-14 and LFA-1

A BglI site that is present in a homologous position in murine and human ICAM-1 cDNAs was used to construct chimeric ICAM-1 molecules. The site of exchange corresponds to amino acid 168 in the human sequence (Figure 1). Molecules with human and murine amino-terminal regions were designated hm-168 and mh-168, respectively. The exchanged sequences encompass all of domains D1 and D2 except for the 17 most carboxy-terminal residues of D2.

The efficiency of expression of chimeric and mutant ICAM-1 molecules in these studies was examined with a panel of four MAbs to human ICAM-1 and one MAb to murine ICAM-1. The RR1/1, R6.5, LB-2, and CL203 MAbs to human ICAM-1 do not inhibit binding of one another to cell surface ICAM-1, as shown with biotinylated MAbs (S. D. Marlin, unpublished data), confirming previous results (Makgoba et al., 1989). They thus bind to four distinct epitopes. Following transfection, generally 40%–50% of COS cells expressed chimeric or mutant ICAM-1 molecules at characteristic broad levels as shown by immunofluorescence staining with MAbs (Figures 3A and 3B). The chimeric hm-168 and mh-168 ICAM-1 molecules were expressed at levels comparable to human and mouse wild-type ICAM-1 (Figures 3A and 3C). None of the MAbs showed cross-reactivity for mouse and human ICAM-1. The pattern of staining demonstrates that the epitope for MAb YN1/1 to mouse ICAM-1 is located in D1 or D2, that the epitopes for MAbs RR1/1, R6.5, and LB-2 to human ICAM-1 are located in D1 or D2, and that the epitope for MAb CL203 to human ICAM-1 is located within the last 17 residues of D2 or D3–D5 (Figures 3A and 3C).

COS cells expressing human ICAM-1 bound radiolabeled rhinovirus-14, but COS cells expressing murine ICAM-1 did not (Figure 4). The major group of human rhinoviruses has been reported to bind to human but not murine cell lines (Colonno et al., 1986), although it was not established whether the murine cells that were tested expressed ICAM-1. Our finding demonstrates species specificity at the level of ICAM-1 binding. Furthermore, rhinovirus-14 binds to hm-168 ICAM-1 at levels equivalent to human wild-type ICAM-1, but does not bind to mh-168

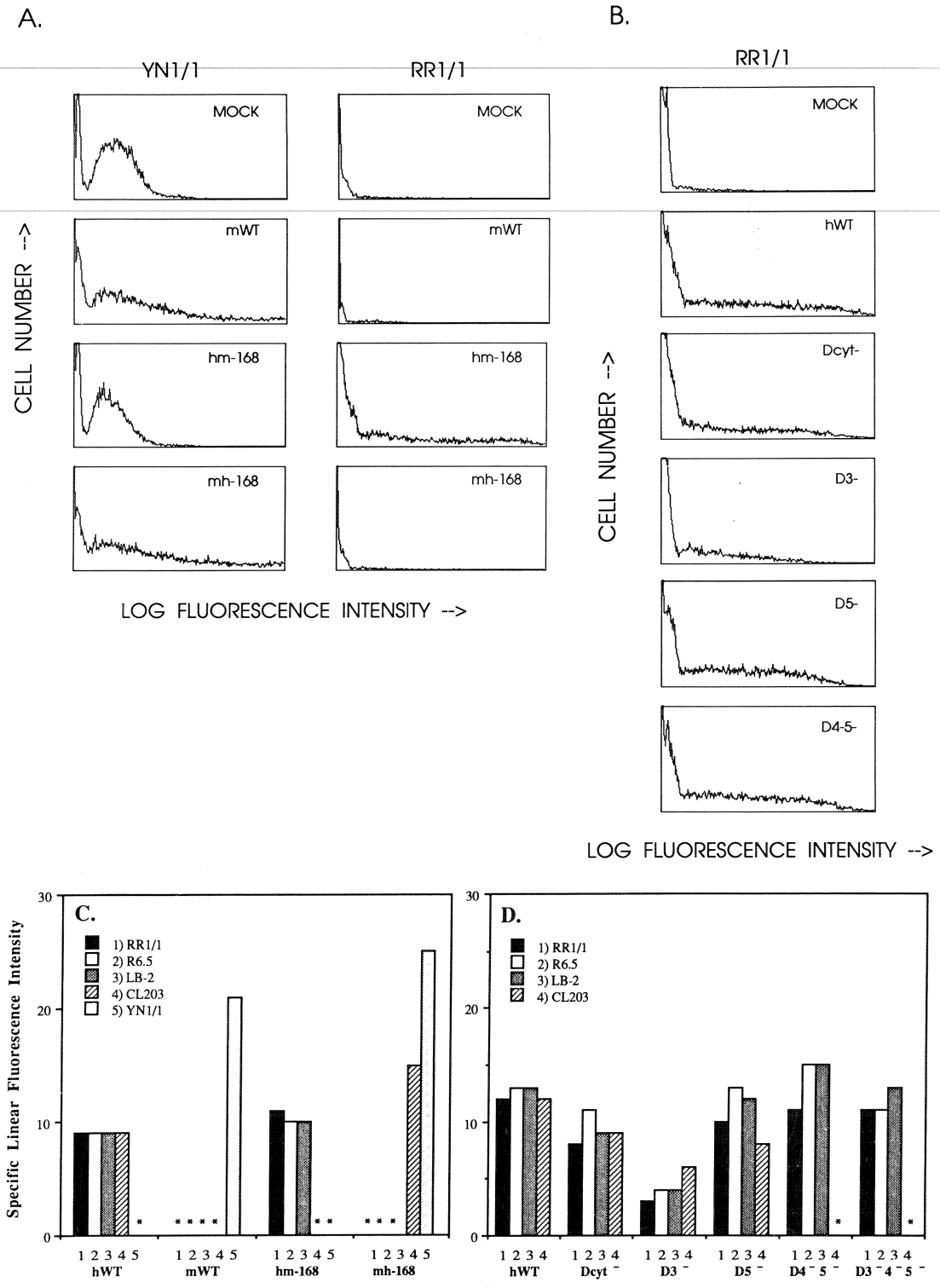

Figure 3. Expression of Chimeric and Deletion Mutant ICAM-1 Molecules in COS Cells

(A and B) COS cells transfected with the indicated ICAM-1 constructs were analyzed by flow cytofluorometry following indirect immunofluorescence staining with the indicated ICAM-1 MAb. hWT = human wild-type ICAM-1 construct; mWT = mouse wild-type ICAM-1 construct.

(C and D) Specific linear fluorescence intensity (SLFI) was determined, with background binding to mock-transfected cells subtracted. Asterisks indicate no staining (SLFI of 0).

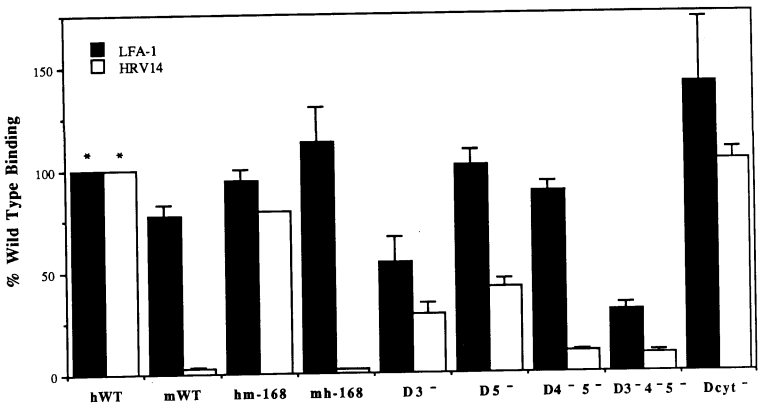

Figure 4. Binding of LFA-1 and Rhinovirus-14 to Chimeric ICAM-1 Molecules and Deletion Mutant ICAM-1 Molecules Expressed in COS Cells

COS cells expressing the indicated ICAM-1 constructs were tested for adherence to plastic-bound LFA-1 (filled boxes) and for binding [^{35}S]methionine- or ^{3}H-labeled rhinovirus-14 (open boxes). LFA-1 binding and rhinovirus-14 binding were normalized as described in Experimental Procedures. Binding to wild-type human ICAM-1 was by definition 100% (asterisks). Standard errors (n = 2–4) are indicated. hWT = human wild-type ICAM-1 construct; mWT = mouse wild-type ICAM-1 construct.

ICAM-1 (Figure 4). Thus D1 and the region of D2 preceding residue 168 of human ICAM-1 confer very efficient rhinovirus binding, whereas the last 17 residues of D2 and D3–D5 of human ICAM-1 demonstrate no ability to bind rhinovirus.

Transfected COS cells expressing murine ICAM-1, human ICAM-1, or the two chimeric ICAM-1 molecules bound equally well to purified LFA-1 (Figure 4). This is in agreement with previous findings of cross-species binding, based on binding of mouse and human cell lines to purified human LFA-1, and inhibition of this binding with MAbs to murine and human ICAM-1 (S. C. Johnston, M. L. D., and T. A. S., submitted).

Binding of ICAM-1 Deletion Mutants to LFA-1 and Rhinovirus-14

To localize the site(s) of LFA-1 contact to a particular ICAM-1 Ig-like domain(s) and to examine the effect of domain deletion on rhinovirus binding, entire domains were deleted by oligonucleotide-directed mutagenesis (Figure 1). In addition, the cytoplasmic domain (Dcyt) was deleted to determine its potential contribution to adhesion. Immunoprecipitation and SDS–PAGE analysis of Dcyt$^-$, D3$^-$, and D4$^-$5$^-$ ICAM-1 molecules from transfected COS cells revealed mass decreases of 3, 24, and 23 kd, respectively, relative to wild-type ICAM-1 (data not shown). Wild-type ICAM-1, approximately 92 kd when expressed in COS cells, has a 55 kd core protein, and thus each of the eight linked glycosylation sites may possess an average of 4 kd of oligosaccharide. Based on the predicted glycosylation of each domain (Figure 1), the observed decreases in mass are reasonably consistent with the expected decreases of 3, 19, and 27 kd, respectively.

The deletion mutant ICAM-1 molecules were examined for MAb epitopes. All MAbs bound at equivalent levels to COS cells transfected with the wild-type, the Dcyt$^-$ mutant, and the D5$^-$ mutant ICAM-1 constructs (Figures 3B and 3D). Binding of MAb CL203 was eliminated upon removal of D4–D5 and D3–D5. Binding of the other three MAbs was unaffected for the D4$^-$5$^-$ mutant and the D3$^-$4$^-$5$^-$ mutant and was decreased for the D3$^-$ mutant. A D4$^-$ mutant, although not expressed well, binds RR1/1, R6.5, and LB-2 at levels of about 25% of wild type, whereas it does not bind MAb CL203 (data not shown). These findings localize the epitopes for RR1/1, R6.5, and LB-2 within D1 or D2, and the epitope for CL203 within D4, consistent with localization in the chimeric molecules. The Dcyt$^-$, D5$^-$, D4$^-$5$^-$, and D3$^-$4$^-$5$^-$ mutants are usually expressed at wild-type levels while the D3$^-$ mutant is consistently expressed at about one-half that of wild type.

Specific binding to LFA-1 was retained after deletion of Ig-like domains D3–D5 or deletion of Dcyt of ICAM-1 (Figure 4, filled bars). Deletion of D5, deletion of D4 and D5, and deletion of Dcyt had no significant effect on LFA-1 binding, and deletion of D3 decreased LFA-1 binding only to an extent comparable to the decreased ICAM-1 expression. LFA-1 binding was retained after deletion of D3, D4, and D5, although it was reduced 3-fold. D1 and D2 are thus sufficient for binding to LFA-1.

Binding of rhinovirus-14 to ICAM-1 domain deletion mutants is consistent with localization of the binding site amino-terminal to residue 168 with chimeric ICAM-1 molecules. Rhinovirus binds significantly to COS cells transfected with D3$^-$, D5$^-$, D4$^-$5$^-$, and Dcyt$^-$ constructs, showing that D1 and D2 are sufficient for this interaction (Figure 4, open bars). However, rhinovirus binding is decreased by deletion of D3, D4, and D5. Although lowered binding to the D3$^-$ mutant may be due in part to its lowered expression, rhinovirus binding is affected more than LFA-1 binding. Binding of rhinovirus is decreased 2.4-fold to the D5$^-$ mutant, and 10-fold to the D4$^-$5$^-$ and D3$^-$4$^-$5$^-$ mutants. Although a direct contribution of D3–D5 to rhinovirus binding cannot be ruled out, such a contribution would have to be extremely weak, because the mh-168 chimeric ICAM-1 molecule contains human domains D3–D5 and yet does not bind rhinovirus. Moreover, a direct contribution by D3, D4, or D5 seems unlikely because deletion of each of these domains has an effect: D3 and D5 by themselves, and D4 when deleted in addition to D5. Therefore, we favor the interpretation that as the binding site on D1 and D2 of ICAM-1 is immersed in the cellular glycocalyx by the shortening of the ICAM-1 molecule caused by deletions within the region of D3–D5, or alternatively, as the ICAM-1 molecule becomes less flexible as a result of dele-

tions in this region, it becomes less accessible to rhinovirus-14.

Further support for the binding of LFA-1 and rhinovirus-14 to D1 and D2 of ICAM-1 is provided by comparison of our MAb epitope localization data to previous MAb blocking data. Thus MAbs RR1/1, R6.5, and LB-2, which recognize epitopes in D1 and D2, block both LFA-1 and rhinovirus-14 binding, whereas MAb CL203, which recognizes an epitope in D4, blocks neither cell adhesion nor virus adhesion (Staunton et al., 1989a; Dustin and Springer, 1989; Maio et al., 1989). MAb YN1/1 to murine ICAM-1, which blocks interaction of murine ICAM-1 and LFA-1 (Horley et al., 1989), also binds to D1 and D2.

ICAM-1 Amino Acid Substitution Mutants

The structural properties of Ig-like domains were used to guide introduction of amino acid substitutions in ICAM-1. The three most amino-terminal Ig-like domains of ICAM-1 are predicted to possess seven β strands each (Staunton et al., 1988, 1989b). These strands are predicted to be arranged in two sheets that are connected by the intradomain disulfide bond to form a "sandwich." The loops connecting the β strands in immunoglobulins form the antigen binding site and are hypothesized to be utilized in intermolecular contacts in other Ig superfamily members (Williams and Barclay, 1988). Our strategy was first to introduce 2 to 4 amino acid substitutions per loop in D1–D3. If effects were found, single substitutions were made in the loops. Finally, in some areas of interest, substitutions were made in the β strands as well. Our notation for the mutations uses the one-letter code for the wild-type sequence followed by a slash and the one-letter code for the corresponding mutant sequence (Figure 5). The position of the first amino acid within the sequence is indicated.

Effects of 2-fold or greater were reproducible in LFA-1, rhinovirus-14, and MAb binding assays and thus were considered significant (boldface and underlined in Figure 5A). Since the epitope of MAb CL203 localizes to D4, whereas D1–D3 were subjected to amino acid substitutions, MAb CL203 was used to determine the level of expression of the mutants. Binding of mutant ICAM-1 molecules to LFA-1 and rhinovirus-14 was normalized with respect to both MAb CL203 binding and binding obtained with wild-type ICAM-1. Furthermore, since MAbs RR1/1, R6.5, and LB-2 bind to distinct epitopes, a loss of two or more of these epitopes indicates a gross disruption of structure and thus corresponding effects on LFA-1 and rhinovirus-14 binding were discounted (Figure 5B). The reduced levels of expression (as measured with MAb CL203) of two mutants, Q27/L and E90/K, were also discounted, as lower expression may reflect decreased efficiency of folding of D1 where these mutations occur.

Amino acid substitution mutants that demonstrate decreased binding of only one ICAM-1 MAb suggest that the corresponding wild-type residues contribute to the MAb epitope (Figure 5A). MAb LB-2 binding was specifically affected by mutations in the sequence at R49 in D1 and R166 in D2. However, these substitutions reduced binding only 2-fold. Although reproducible, this effect may not be of great enough magnitude to signify a binding site. MAb RR1/1 binding was most strongly affected by mutation Q73/H, with a 5-fold reduction, and was reduced 2-fold by the nearby substitution D71/N. The epitope for MAb R6.5 is completely and specifically disrupted by a mutation in the sequence at E111 in D2.

D1 and D2 appear to be conformationally linked. Fourteen of 53 mutations in D1, and a similar proportion in D2 (4/18), disrupt binding of MAbs RR1/1, R6.5, and LB-2 and binding of LFA-1 and rhinovirus-14 (Figure 5B). Since these ligands bind to different sites (the MAbs) or only partially overlapping regions (LFA-1 and rhinovirus-14; see below), the ability of mutations widely spread throughout both D1 and D2 to have common effects suggests that the conformation of D1 is dependent on the conformation of D2 and vice versa. In contrast, none of the mutations in D3 affect binding of these ligands, and none of the mutations in D1–D3 affect binding of MAb CL203 to D4. This suggests that there is substantial contact between D1 and D2 but that D1 and D2 are conformationally independent of D3, i.e., there may be a hinge between D2 and D3. The most disruptive mutations (Figure 5B) involve residues R13 and D60, which would be predicted in an Ig model (Discussion) to come into close proximity to residues in D2. Deletion of D2 (residues P95–A189) has resulted in lack of cell surface expression, further supporting findings that proper folding depends on interaction of D1 and D2.

Some of the mutations that disrupt conformation also cause aberrant disulfide bond formation. Immunoprecipitation and nonreducing SDS–PAGE of ICAM-1 molecules with mutations in 12 different locations in D1 and D2 revealed that two mutants, N103/K and A155N/SV, form disulfide-linked dimers (data not shown). Residues N103 and N156 occur close to C108 and C159, which are predicted to form the intradomain disulfide bridge of D2.

LFA-1 Binding

Mutations with the strongest effect on LFA-1 binding localized to D1. The most dramatic mutations are E34/A, which completely eliminates LFA-1 binding, and Q73/H, which decreases it 10-fold (Figure 5A). A different substitution at Q73, Q73/T, decreases LFA-1 binding 2-fold. The mutations D26QPK/ALPE and G46NN/ASI decrease LFA-1 binding 2- to 3-fold. In the second domain the mutants N118/Q, N156/E, N175/A, and S177/G decrease LFA-1 binding approximately 2-fold. These four mutants affect three of the four N-linked glycosylation sites in D2; there are no N-linked glycosylation sites in D1 (Figure 1). Thus N-linked carbohydrate may have a small but not critical role in LFA-1 binding. The effect of these mutations may be more indirect, however; we believe 2-fold changes should be interpreted with caution. None of the mutations in D3 affected LFA-1 binding, in agreement with the lack of effect of deleting D3.

Comparison of the Rhinovirus-14 and LFA-1 Binding Sites

A number of mutations demonstrate that D1 is more important than D2 in rhinovirus-14 binding and that rhinovirus-14 and LFA-1 binding sites overlap only partially. Seven mutations decrease rhinovirus-14 binding but have

A.

Mutation	LFA-1 Binding (%se)		HRV Binding (%se)		CL203 (%wt se)		RR1/1 (%CL203)	R6.5	LB-2
D1									
Q1T/KA	119	3(2)	**11**	4(2)	230	1(2)	94	115	113
Q1/E	175	3(3)	149	7(3)	135	1(3)	154	145	136
Q1/K	97	0(2)	78	9(2)	168	7(3)	109	121	106
S3/T	149	8(3)	196	2(3)	224	2(3)	111	114	117
S5/T	104	2(2)	125	8(3)	251	4(3)	107	107	120
K8/E	84	6(2)	132	8(2)	111	1(2)	104	121	110
R13/K	98	6(3)	123	3(2)	189	5(4)	133	117	121
G15/SA	120	7(3)	164	3(2)	172	4(2)	89	88	89
T20CS/ACT	91	2(3)	130	6(3)	148	4(3)	86	95	86
S24/A	80	8(2)	99	7(2)	158	4(2)	125	115	125
D26QPK/ALPE	**30**	3(3)	**13**	7(3)	126	0(3)	**52**	89	80
E34/A	**0**	0(3)	**66**	2(4)	132	3(4)	142	150	142
K39KE/ERQ	99	5(3)	**61**	4(3)	84	6(3)	**47**	93	87
K40/A	124	4(2)	89	0(2)	146	4(2)	123	106	106
G46NN/ASI	**49**	5(4)	**9**	5(2)	151	4(4)	140	107	113
N48/H	88	-	81	2(2)	164	1(2)	123	94	100
R49KV/EKL	123	0(2)	**49**	2(2)	233	1(2)	103	97	**52**
Y52/F	72	0(2)	174	6(3)	152	0(2)	90	95	119
Y52E/FA	138	5(2)	125	3(2)	100	0(2)	141	133	117
N56V/HM	121	3(2)	101	2(2)	121	1(2)	106	125	125
Q58/H	109	3(2)	**1**	1(2)	135	5(2)	93	107	93
E59/K	134	0(2)	105	0(2)	130	1(2)	127	109	136
E59/Q	84	8(2)	92	-	195	25	112	106	125
D60/N	92	3(4)	89	4(3)	127	4(3)	100	138	108
S61/I	59	8(3)	111	4(2)	140	8(5)	82	100	100
Q62PM/API	104	8(3)	182	1(2)	200	9(4)	**59**	81	73
M64/I	111	3(2)	107	3(2)	183	0(2)	83	111	116
Y66/T	135	-	204	-	144	-	109	104	113
N68/K	101	1(2)	137	3(2)	153	8(2)	97	96	102
D71/E	118	8(2)	140	7(2)	124	6(2)	89	100	82
D71/N	79	3(3)	**62**	1(2)	109	6(4)	**44**	94	83
Q73/H	**12**	0(4)	117	1(5)	139	7(5)	**21**	80	80
Q73/T	**40**	2(4)	133	6(2)	218	8(2)	71	86	114
S74/A	70	6(2)	156	5(2)	129	9(2)	119	119	113
T75/A	59	8(2)	119	8(2)	153	0(2)	94	94	115
K77T/ES	87	2(4)	**42**	4(4)	151	8(3)	88	80	84
R88V/EA	95	1(2)	152	1(2)	113	4(3)	74	100	121
E90/Q	122	5(3)	157	7(2)	152	7(2)	90	92	112
D2									
L91/A	87	7(2)	105	-	150	-	79	142	133
G101K/AN	97	5(3)	140	0(2)	142	1(3)	85	85	85
E111GGA/KAGS	103	5(3)	162	3(2)	122	-	81	**0**	89
N118/Q	**54**	2(3)	110	-	139	9(3)	93	85	93
R125/E	81	7(3)	157	5(2)	145	7(2)	181	133	104
E127/R	82	9(2)	131	4(2)	191	2(2)	100	119	106
K128/R	109	2(3)	137	7(2)	190	5(2)	100	118	109
V136GE/GVK	92	3(3)	117	1(2)	171	2(2)	220	172	138
R149RD/EEG	81	0(2)	139	6(2)	159	7(2)	166	189	166
H152HGA/EEGS	85	2(2)	82	0(2)	94	-	103	94	112
N156/E	**60**	4(2)	107	-	182	7(2)	84	95	95
R166PQ/EPA	75	8(2)	**25**	8(3)	94	0(3)	100	109	**64**
N175/A	**67**	3(3)	129	6(2)	80	9(2)	162	175	150
S177/G	**59**	1(2)	66	6(2)	87	6(3)	136	118	90
D3									
A189T/SI	91	3(2)	168	-	138	8(2)	175	166	166
D203TQ/TAD	91	3(2)	111	8(2)	125	-	178	116	86
D213GL/HGV	90	2(3)	99	3(2)	128	8(2)	231	175	107
D229QR/HLE	90	7(3)	106	1(2)	210	9(2)	94	92	100
N240DS/KNA	147	9(3)	82	2(2)	142	-	113	125	132
E254DE/KEK	122	3(2)	99	3(2)	180	3(2)	178	129	81
N269QSQI/IQAE	101	9(2)	108	5(2)	95	5(3)	162	150	150

B.

Mutation	LFA-1 Binding (%se)		HRV Binding (%se)		CL203 (%wt se)		RR1/1 (%CL203)	R6.5	LB-2
D1									
S3VS/AGL	18	5(3)	61	2(2)	121	1(2)	5	31	9
V4/G	64	7(3)	30	3(4)	111	9(3)	47	73	58
R13G/EA	2	2(4)	3	1(2)	132	4(2)	0	31	0
R13/E	1	1(3)	10	5(3)	202	4(3)	4	48	4
R13/Q	78	3(3)	60	0(2)	161	6(3)	73	73	47
Q27/L	37	6(3)	57	6(4)	33	5(4)	75	75	125
K50V/EL	29	8(2)	10	8(2)	103	2(2)	23	69	23
Q58EDS/AKDI	3	3(3)	0	0(2)	98	0(3)	10	22	7
D60S/KL	1	1(3)	1	0(2)	105	7(2)	0	21	0
D60/K	14	-	4	0(2)	89	8(2)	0	31	0
D60/Q	18	6(2)	20	8(2)	80	1(2)	30	54	31
D71GQS/NGEL	1	1(4)	21	2(3)	161	4(3)	0	48	26
Y83/S	42	9(2)	86	4(2)	125	-	60	70	50
E87/K	65	0(5)	27	0(3)	94	2(3)	64	64	79
E90/K	34	1(2)	50	2(4)	29	5(4)	100	123	108
D2									
N103/K	12	6(2)	13	-	91	9(2)	17	60	22
A155N/SV	0	0(3)	12	8(2)	60	6(2)	0	0	0
N175TSA/QTLG	26	0(4)	40	8(3)	75	6(3)	19	50	44
A178/G	59	3(2)	102	-	210	4(2)	63	69	74

Figure 5. Binding of Mutant ICAM-1 Molecules Expressed in COS cells to LFA-1, Rhinovirus-14, and MAbs

(A) Mutations with specific effects. (B) Mutations that disrupt overall structure as defined by loss of two or more MAb epitopes. Approximately 2-fold or greater decreases in binding were considered significant (boldface and underlined). ICAM-1 amino acid substitution mutants were generated by oligonucleotide-directed mutagenesis. Wild-type residues precede the slash and are followed by the residues in the mutant. LFA-1 binding and rhinovirus-14 (HRV) binding were normalized to wild-type levels as described in Experimental Procedures and are presented as means ± standard errors (se) for multiple experiments (n = 1–5). MAb CL203 binding is presented as a percentage of wild-type binding. Binding of MAbs RR1/1, R6.5, and LB-2 is presented as the percentage of MAb CL203 binding as measured by SLFI (see Experimental Procedures).

no effect on LFA-1 binding. The two that demonstrate the greatest effect involve amino acid substitutions in D1. Q58/H virtually eliminates rhinovirus-14 binding, and Q1T/KA results in a 10-fold decrease. Four other mutations in D1 demonstrate a specific 2-fold effect on rhinovirus-14 binding: K39KE/ERQ, R49KV/EKL, D71/N, and K77T/ES. One mutation in D2, R166PQ/EPA, results in a 4-fold decrease in rhinovirus-14 binding. Mutations in D3 did not affect rhinovirus-14 binding.

Of the four D1 mutations discussed above that affect LFA-1 binding, three affect rhinovirus-14 binding as well. The mutants D26QPK/ALPE and G46NN/ASI affect rhinovirus-14 binding 10-fold and LFA-1 binding 2- to 3-fold. The mutation E34/A, which totally eliminates LFA-1 binding, decreases rhinovirus-14 binding 2-fold. Four mutations in D2 that decreased LFA-1 binding had little or no effect on rhinovirus-14 binding.

The interaction of LFA-1 and rhinovirus-14 with ICAM-1 was further compared with regard to the requirement for divalent cations. Mg^{2+} dependence has been demonstrated for ICAM-1 and LFA-1-dependent interactions including antigen-specific T cell recognition, homotypic adhesion, binding of cells to purified ICAM-1 or LFA-1 on substrates, and binding of purified LFA-1 to purified ICAM-1 on substrates (Marlin and Springer, 1987; Dustin and Springer, 1989). We compared binding of LFA-1^+ T cells and rhinovirus-14 to purified ICAM-1 adsorbed to a plastic substrate. Confirming previous results, the LFA-1^+ T cell line SKW3 binds to ICAM-1 only in the presence of Mg^{2+} (Figure 6). In contrast, the binding of rhinovirus-14 to ICAM-1 did not significantly differ in the presence of 10 mM Mg^{2+} or 5 mM EDTA. The differences between LFA-1:ICAM-1 and rhinovirus-14:ICAM-1 interactions were confirmed over a range of ICAM-1 densities on the substrate.

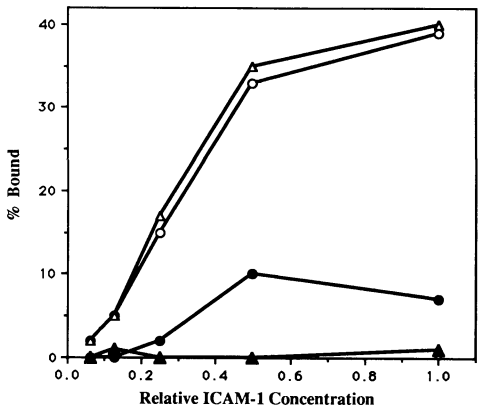

Figure 6. Dependence on Divalent Cations of Rhinovirus-14 and LFA-1 Binding to ICAM-1

Binding of [^{35}S]methionine-labeled rhinovirus-14 to varying concentrations of plastic-bound ICAM-1 occurred in rhinovirus-14 buffer with 10 mM Mg^{2+} (open circles) or rhinovirus-14 buffer with no Mg^{2+} and 5 mM EDTA (open triangles). SKW3 T lymphoma cell line binding to ICAM-1 was in rhinovirus-14 buffer with 0.5 mM Mg^{2+} (filled circles) or 5 mM EDTA (filled triangles).

Discussion

We have demonstrated that the extracellular region of ICAM-1 exists as a 19 nm × 2–3 nm hinged rod. These dimensions suggest that the five predicted Ig-like domains of ICAM-1 are extended and unpaired, and are aligned end to end at a small angle to the rod axis rather than side by side. The estimate of 3.7 nm per Ig domain compares well with the approximate dimensions (4 × 2.5 × 2.5 nm) for Ig domains whose structure has been determined (Alzari et al., 1988; Bjorkman et al., 1987; Becker et al., 1977). The spacing of 3.7 nm per Ig domain is the same as found for NCAM by electron microscopy (Becker et al., 1989). The long arm of NCAM, which comprises five Ig-like domains, has a length of 17.6–18.7 nm (Becker et al., 1989), essentially identical to the total length we have found for the ICAM-1 molecule.

Another striking similarity in the structures of ICAM-1 and NCAM is that both molecules have a bend, typically at 90° but with variation indicating flexibility. In NCAM the bend does not occur within the five Ig-like domains, but at or near the junction with the type III fibronectin repeats (Becker et al., 1989). By contrast, the bend in ICAM-1 is probably between two Ig-like domains, giving a long arm with three domains and a short arm with two. The observation that the conformations of D1 and D2 appear to be dependent on one another and independent of D3 suggests that a hinge region may be located between D2 and D3. The sequence at the D2–D3 border is the most proline-rich region in ICAM-1 (4 prolines within 10 residues). This is consistent with Ig hinge sequences, which are characteristically rich in proline; indeed, the 4 prolines in this region are spaced identically to 4 of the prolines in the hinge region of mouse IgG3 (Kabat et al., 1987). The cell adhesion molecule LCAM, a cadherin that is structurally unrelated to ICAM-1 or NCAM, also has a 90° bend (Becker et al., 1989). This common feature of cell adhesion molecules would appear to have several important functions. It would permit an extended segment of the molecule, rather than just the tip, to face and form a binding interface with its receptor. It would allow binding sites located on the distal, flexible segment to bind to receptors oriented at different angles and located at varying distances with respect to the membrane of the cell bearing the ICAM-1 molecule. Furthermore, segmental flexibility provided by the hinge, and rotation at the membrane of a bent molecule, should increase the rate of diffusion of the binding site within the volume of solvent above the cell surface to which it is limited by its membrane tether, thereby enhancing the kinetics of binding to adhesion receptors or viruses and increasing the efficiency of these interactions.

The binding sites for LFA-1 and rhinovirus-14 were localized to D1 and D2 of ICAM-1 with chimeric ICAM-1 molecules, with ICAM-1 molecules in which specific Ig-like domains were deleted, and by localizing epitopes for MAbs with known effects on LFA-1 and rhinovirus binding. Amino acid substitutions in D1, D2, and D3 further localized the binding sites for LFA-1 and rhinovirus within D1, with perhaps a small contribution from D2.

D3, D4, and D5 of ICAM-1 are important for the accessi-

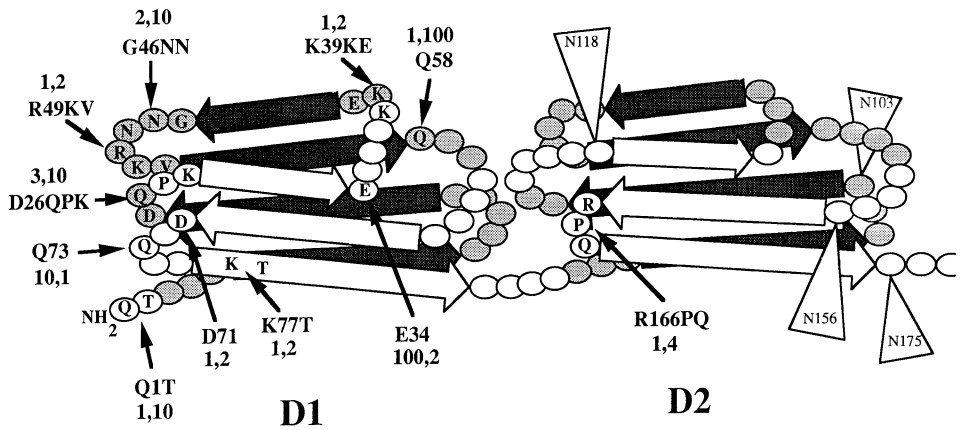

Figure 7. Hypothetical Model of ICAM-1 D1 and D2 Tertiary Structure with Locations of Mutations Affecting LFA-1 and Rhinovirus-14 Binding

The predicted β strands (wide arrows) and β turns of ICAM-1 D1 and D2 (Staunton et al., 1988) are modeled on the basic tertiary structure of an Ig constant region domain (Alzari et al., 1988; Williams and Barclay, 1988). The model makes use of strand predictions for both ICAM-1 and ICAM-2 (Staunton et al., 1989b), since these homologs should have superimposable β strand structures. The alignment for β strand f of D1 (Staunton et al., 1988) was revised to bring C65 into alignment with the conserved Ig intradomain disulfide and to allow C21–C65 and C25–C69 disulfide bonds. Mutations at residues that affect binding and the fold decreases for LFA-1 binding and rhinovirus-14 binding (separated by commas) are shown. N-linked glycosylation sites are indicated by open triangles.

bility of the rhinovirus and LFA-1 binding sites in D1 and D2. Removal of individual domains in the region D3–D5 decreased rhinovirus binding several-fold, and removal of D4 and D5 together or D3, D4, and D5 together decreased binding 10-fold. D3–D5 do not bind to rhinovirus as shown with the mh-168 chimera; therefore, deletion of domains in this region appears to have an indirect effect on accessibility of the rhinovirus binding site in D1 and D2. A similar but less pronounced effect was seen on accessibility of the LFA-1 binding site. Deletion of D3 or D5 alone or deletion of D4 and D5 together had little or no effect, but deletion of D3, D4, and D5 together reduced LFA-1 binding 3-fold. Removal of D4–D5 and of D3–D5 is predicted to shorten ICAM-1 by 7.4 and 11.7 nm, respectively. The effect of these deletions on rhinovirus and LFA-1 binding suggests that the unpaired, end-to-end organization of the domains in ICAM-1 to give an extended, rod-shaped molecule with a hinge is important for its function.

The greater effect of the presence of D3–D5 of ICAM-1 on binding of rhinovirus versus LFA-1 may be related to differences in the size, shape, and valency of these ligands. Rhinovirus is 30 nm in diameter with a depression 2.5 nm deep on the viral capsid surface that is thought to bind ligand (Rossmann et al., 1985), while electron microscopic studies of integrins show a 10 × 8 nm globular domain thought to bind ligand, supported on two stalks that extend 18 nm above the cell surface (Carrell et al., 1985; Nermut et al., 1988). The cellular glycocalyx (Springer, 1990) into which ICAM-1 is submerged by its shortening may repel the bulkier rhinovirus more than LFA-1. Furthermore, binding of multiple ICAM-1 molecules to each rhinovirus (Colonno et al., 1986) would require the ICAM-1 molecules to be in close proximity to one another, and this packing may be hindered by shortening or loss in flexibility.

Because three MAbs to distinct ICAM-1 epitopes blocked both LFA-1 and rhinovirus-14 binding, it was suggested previously that the LFA-1 and rhinovirus-14 contact sites on ICAM-1 are in close proximity (Staunton et al., 1989a). Our present studies show that the binding site on ICAM-1 for LFA-1 is distinct from but partially overlaps the binding site for rhinovirus-14. We have modeled the mutations in D1 and D2 of ICAM-1 that affect binding, showing the decrease in binding for LFA-1 and rhinovirus, respectively (Figure 7). An Ig domain structure has been assumed (Alzari et al., 1988; Williams and Barclay, 1988), although we caution that the Ig fold may differ in some important way for Ig family members with unpaired domains; furthermore, we have not attempted to optimize the model based on our mutational data by placing residues that show similar effects into closer proximity. The predicted locations of contact sequences in the Ig domain model are consistent with close proximity or overlap of the LFA-1 and rhinovirus-14 binding sites. The two binding sites are clearly distinguished, however, by mutations at E34 and Q58; the former abolishes LFA-1 binding, whereas the latter abolishes rhinovirus binding. The overlap of rhinovirus and LFA-1 binding sites in domain 1 may be a consequence of the favorability of this domain as an adhesive interaction site, as outlined above.

ICAM-1 and CD4 are members of the Ig superfamily that demonstrate striking parallels in their function in both cellular and viral adhesion (White and Littman, 1989; Springer, 1990). CD4 is an adhesion receptor on T cells that binds to MHC class II molecules, and is also utilized as a receptor by human immunodeficiency virus (HIV). CD4 has four extracellular domains. Recent studies on CD4 have found that mutations in the amino-terminal Ig-like domain have the strongest effect on binding of MHC class II and HIV, whereas mutations in the second domain have a lesser effect. The binding sites for MHC class II and HIV overlap but are distinct (Peterson and Seed,

1988; Clayton et al., 1989; Lamarre et al., 1989; Landau et al., 1988). Some CD4 MAb epitopes appear to involve residues from both D1 and D2, demonstrating a close physical association of these domains (Landau et al., 1988). In all these respects, findings on the cell adhesion and virus binding sites of ICAM-1 and CD4 are similar.

Our finding that the Ig domains of ICAM-1 are unpaired is consistent with findings that the binding site in rhinovirus is in a canyon that is too narrow to admit the paired Ig domains of an Fab fragment (Rossmann et al., 1985; Colonno et al., 1988; Rossmann and Johnson, 1989). The receptor binding site in the rhinovirus canyon has been implicated to be in a deep cleft that is 3 nm wide at the top, 1.2 nm wide at the bottom, and 2.5 nm deep. These dimensions are appropriate for insertion of the amino-terminal, unpaired Ig-like domain of ICAM-1, which is predicted to be 2.5 × 2.5 nm wide; slightly more than half of the Ig domain length of 4 nm could be inserted. The contact sequences in the distal half of ICAM-1 D1 may form bonds with residues within the cleft. The remaining sequences implicated in rhinovirus-14 contact, which appear not to locate to the distal half of D1, might interact with rhinovirus-14 residues in the rim of the canyon. The distance between the center of each deep cleft around the 5-fold axis, approximately 5 nm, is great enough to allow an ICAM-1 molecule to occupy all five clefts.

The contact site for LFA-1 on ICAM-1 differs from contact sites of many other integrin ligands in sequence and structure. Many integrins that bind extracellular matrix proteins bind to an RGD or an RGD-like sequence in their ligands (Ruoslahti and Pierschbacher, 1986; Hynes, 1987; Hemler, 1990; Springer, 1990). Human ICAM-1 has no RGD sequences, but contains several RGD-like sequences such as RGE (Simmons et al., 1988; Staunton et al., 1988); murine ICAM-1 contains an RGD and an RGE sequence (Horley et al., 1989). However, none of these sites correspond to residues defined by our mutagenesis studies as important in LFA-1 binding to ICAM-1. Instead, a number of noncontiguous sequences in ICAM-1 appear to be recognized. This is similar to Ig binding to protein antigens, in which residues in three noncontiguous complementary-determining regions confer recognition specificity (Alzari et al., 1988).

Residues identified in human ICAM-1 as important to LFA-1 binding are conserved in other ICAMs, whereas the most important residue for binding rhinovirus is not. The residues that are most critical to LFA-1 binding, E34 and Q73, are conserved in mouse ICAM-1 (Horley et al., 1989; Ballantyne et al., 1989; Siu et al., 1989) and in human ICAM-2 (Staunton et al., 1989b), correlating with the ability of mouse ICAM-1 and human ICAM-2 to bind to human LFA-1. The single most important residue for rhinovirus-14 binding, Q58, is not conserved in mouse ICAM-1 or human ICAM-2, consistent with the inability of mouse ICAM-1 and ICAM-2 (D. E. S. and T. A. S., unpublished data) to bind rhinovirus-14.

We found that divalent cations were required for binding of LFA-1 but not rhinovirus-14 to ICAM-1. LFA-1 has been shown to bind Mg^{2+} (Dustin and Springer, 1989); this correlates with the presence of three putative divalent cation binding motifs in its α subunit (Larson et al., 1989) and the Mg^{2+} requirement for binding to ICAM-1 (Marlin and Springer, 1987) and ICAM-2 (Staunton et al., 1989b). Mg^{2+} is included in all rhinovirus buffers (Rueckert, 1985) and could affect stability, because a cation binds at the 5-fold axis of rhinovirus (Rossmann et al., 1985). However, we found no divalent cation requirement for rhinovirus binding to ICAM-1, and previous suggestions of a divalent cation requirement for rhinovirus binding (Rueckert, 1985) appear to be based on work with minor group serotypes, which bind to a distinct receptor.

We have resolved many of the issues concerning the nature of the LFA-1 and rhinovirus binding sites on ICAM-1, and the overall size and shape of ICAM-1 and the arrangement of its Ig-like domains. Further mutagenesis studies might identify additional areas of contact, particularly in the β strands, which were only partially examined in this study. However, we believe that the most significant step in further understanding these interactions would come from X-ray crystallographic studies on ICAM-1:LFA-1 and ICAM-1:rhinovirus complexes. In addition to its inherent biological interest, the identification here of the important binding sites within the first two domains of ICAM-1 suggests that the corresponding fragment of ICAM-1 might have therapeutic utility in preventing rhinovirus infection and leukocyte-mediated tissue damage.

Experimental Procedures

Generation of ICAM-1 Mutants

Oligonucleotide-directed mutagenesis (Kunkel, 1985) with modifications (Peterson and Seed, 1987) was utilized to generate ICAM-1 deletions and amino acid substitutions. Mutations were made using a single-strand template of pCD1.8, an ICAM-1 cDNA expression vector (CDM8) construct (Staunton et al., 1989a). Mutant ICAM-1 oligonucleotides that confer a unique restriction site were used to prime a second-strand synthesis reaction. Following transformation of Escherichia coli, mutants were isolated by screening for the unique restriction sites. In general, two or more isolates of each mutant were expressed in COS cells for binding assays.

Domain deletions were guided by previous assignment of domain boundaries (Staunton et al., 1988) except that the boundary between D4 and D5 was revised to residue 367, which gives each of these domains six predicted β strands and an even number of cysteines. A soluble form of ICAM-1 (sICAM-1), which includes D1–D5, was produced by mutation of the two most carboxy-terminal extracellular residues, Y452 and E453, to F and insertion of a translational stop codon (pCDsD1–5). The entire cytoplasmic domain of ICAM-1 was deleted ($Dcyt^-$) by transforming the codon for the carboxy-terminal transmembrane residue Y476 to a translational stop codon. D3, D5, and D4–D5 were deleted using long (48 bp) mutant oligonucleotides to span distal ICAM-1 sequence such that codons for F185 and P284 ($D3^-$), L366 and S449 ($D5^-$), and P284 and R451 ($D4^-5^-$) would be joined (Figure 1). The mutant $D3^-4^-5^-$ was generated by first introducing, by oligonucleotide-directed mutagenesis, a unique EcoR5 site into ICAM-1 and ICAM-2 cDNAs (Staunton et al., 1988, 1989b) at homologous positions at 685 bp and 702 bp, respectively. EcoR5 fragments were isolated, and ICAM-1 D1 and D2 sequence (residues −27 to 182) was ligated to the transmembrane and cytoplasmic segments of ICAM-2 (residues 194 to 254) with one intervening conservative substitution due to the ligation (ICAM-1 Q183/D). This chimeric molecule is considered analogous to an ICAM-1 deletion mutant, because attachment of the same ICAM-1 D1 and D2 fragment to completely different transmembrane domains gives the same amount of LFA-1 and rhinovirus-14 binding (unpublished data). Deletion mutations were confirmed by DNA sequencing.

Human–Mouse ICAM-1 Chimeras

A 1897 bp mouse ICAM-1 cDNA (Ballantyne et al., 1989) was ligated to BstXI linkers and subcloned into the expression vector CDM8

(pCDmIC1). The conserved and unique BglI site occurring approximately 17 codons from the carboxyl end of domain 2, at Q168 in human and a homologous position in mouse, was used to construct molecules with human amino-terminal and murine carboxy-terminal portions (hm-168) and vice versa (mh-168).

Purification and Visualization of sICAM-1

sICAM-1 was purified as described (Marlin and Springer, 1987) from the supernatants of COS cells transfected with pCDsD1–5, with minor modifications. Supernatants were harvested from 20 × 15 cm dishes between days 4 and 12 after transfection (yielding 2.5 liters of 200 ng/ml sICAM-1) and passed through a 0.22 μm filter and then over RR1/1 MAb–Sepharose (5 ml, 5 mg/ml) at 0.5 ml/min. The column was washed and eluted with 50 mM triethanolamine–HCl, 0.15 M NaCl at pH 10 and pH 12.5, successively, and fractions were neutralized immediately.

sICAM-1 was sedimented through a glycerol gradient (15%–40% [v/v] glycerol, 0.2 M ammonium bicarbonate [pH 8]) at 20°C for 17 hr at 45,000 rpm in a Beckman SW-50.1 rotor, to determine its sedimentation coefficient and as an additional purification step prior to electron microscopy. The sedimentation coefficient was estimated by comparison with standard proteins sedimented in another gradient: bovine serum albumin, 4.6S; ovalbumin, 3.3S; and cytochrome c, 2.1S. sICAM-1 sedimented at the same position as ovalbumin. Rotary-shadowed specimens for electron microscopy were prepared directly from the gradient fractions as described by Fowler and Erickson (1979). Molecule lengths were measured from prints with a 10× lens and eyepiece reticle, and 1 nm was subtracted from each end for the estimated shell of metal.

COS Cell Transfection and Binding Assays

COS cells at 50% confluency were transfected by the DEAE-dextran method (Kingston, 1987) using 0 (mock) or ~4 μg of plasmid per 10 cm plate. COS cells were suspended using trypsin–EDTA 1 day before assay (day 3 or 4) and reseeded. For assays of binding to purified LFA-1 or immunofluorescence staining, the transfected cells were reseeded on 10 cm plates and resuspended 1 day later with 5 mM EDTA in Hanks' balanced salt solution. Transfected COS cells were labeled with $Na_2{}^{51}CrO_4$ for binding to LFA-1-coated plastic as previously described (Staunton et al., 1989b). Indirect immunofluorescence was performed using ICAM-1 MAbs RR1/1 (Rothlein et al., 1986), R6.5 (Rothlein et al., 1988), LB-2 (Clark et al., 1986), CL203 (Maio et al., 1989), and YN1/1 (Horley et al., 1989), and SLFI was determined as previously described (Rothlein et al., 1986).

For rhinovirus-14 binding assays, cells were reseeded in a 24-well plate. One day later the confluent monolayer was washed twice with 1 ml of RPMI 1640, 10 mM $MgCl_2$, 25 mM HEPES (pH 7.3) (rhinovirus-14 buffer). [^{35}S]methionine or ^{3}H-labeled rhinovirus-14 (Sherry et al., 1986), 15,000–25,000 cpm (approximately 10^7 pfu) in 100 μl of rhinovirus-14 buffer, was added to each well. Binding occurred in 1 hr at 35°C in a 5% CO_2 atmosphere with horizontal rotation (100 rpm). Unbound [^{35}S]rhinovirus-14 was aspirated, and COS cells were gently washed with 150 μl of rhinovirus-14 buffer and then solubilized with 1% SDS in phosphate-buffered saline for scintillation counting.

Binding of LFA-1 or rhinovirus-14 to ICAM-1 mutants was corrected for binding to mock-transfected cells and was normalized for the percentage of COS cells staining with MAb CL203 and for the percentage of binding obtained with wild type:

$$\%\ \text{binding} = \frac{(\%\ \text{mut binding} - \%\ \text{mock binding})/\%\ \text{mut CL203 staining}}{(\%\ \text{wt binding} - \%\ \text{mock binding})/\%\ \text{wt CL203 staining}} \times 100.$$

Binding of MAbs RR1/1, R6.5, and LB-2 was normalized to binding of CL203 MAb using the SLFI:

$$\%\ \text{CL203} = \frac{\text{MAb SLFI}}{\text{CL203 MAb SLFI}} \times 100.$$

The percentage of COS cells expressing wild-type ICAM-1 that bound to LFA-1 varied from 11%–63% (mean = 33%); the percentage of mock-transfected COS cell binding varied from 0% to 1%. The percentage of [^{35}S]methionine-labeled rhinovirus-14 that bound to COS cells expressing wild-type ICAM-1 varied from 6%–43% (mean = 21%); the percentage of mock-transfected COS cell binding varied from 0% to 4%.

[^{35}S]rhinovirus-14 binding to ICAM-1-coated plastic was performed as previously described (Staunton et al., 1989a) but with modification of the rhinovirus-14 buffer as indicated. Incubation conditions were 35°C, 5% CO_2 for 1 hr with rotation.

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Note Added in Proof

Recently, the sICAM-1 five-domain fragment has been found to inhibit infection of cells by rhinovirus in vitro (Marlin et al., Nature *344*, 70–72, 1990).