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Functional cloning of ICAM-2, a cell adhesion ligand for LFA-1 homologous to ICAM-1

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THE leukocyte adhesion molecule LFA-1 mediates a wide range of lymphocyte, monocyte, natural killer cell, and granulocyte interactions with other cells in immunity and inflammation[1,2]. LFA-1 (CD11a/CD18) is a receptor for intercellular adhesion molecule 1 (ICAM-1, CD54), a surface molecule which is constitutively expressed on some tissues and induced on others in inflammation[3-5]. Induction of ICAM-1 on epithelial cells, endothelial cells and fibroblasts mediates LFA-1-dependent adhesion of lymphocytes[4,6,7]. Several lines of evidence have suggested the existence of a second LFA-1 ligand: homotypic adhesion of one cell line was inhibited by a monoclonal antibody to LFA-1, but not by one to ICAM-1[8]; there exists an LFA-1-dependent, ICAM-1-independent pathway of adhesion to endothelial cells[6]; and also, there are some types of target cells in which LFA-1-dependent T-lymphocyte adhesion and lysis are independent of ICAM-1[9]. We have cloned this second ligand, designated ICAM-2, using a novel method for identifying ligands of adhesion molecules. ICAM-2 is an integral membrane protein with two immunoglobulin-like domains, whereas ICAM-1 has five[10,11]. Remarkably, ICAM-2 is much more closely related to the two most N-terminal domains of ICAM-1 (34% identity) than either ICAM-1 or ICAM-2 is to other members of the immunoglobulin superfamily, demonstrating the existence of a subfamily of immunoglobulin-like ligands that bind the same integrin receptor.

We have developed a procedure for cloning functional adhesion molecules. LFA-1 purified in the presence of Mg^{2+}, which we have found to be functionally active in binding to both ICAM-1$^+$ and ICAM-1$^-$, putative second ligand$^+$ cells (M.L.D. and T.A.S., manuscript in preparation), was coated on plastic. We used a modified version of the procedure of Aruffo and Seed for selecting complementary DNAs by expression in COS cells[12]. To test the feasibility of this procedure, COS cells were transfected with the previously cloned ICAM-1 cDNA (Fig. 1*a*). ICAM-1 was expressed on 25% of the transfected COS cells. After panning, non-adherent cells were depleted of ICAM-1$^+$ cells, whereas adherent cells released from LFA-1-coated plastic by EDTA were almost completely ICAM-1$^+$. Adherence of ICAM-1$^+$ cells to LFA-1-coated plastic was inhibited with the ICAM-1 monoclonal antibody (mAb) RR1/1. LFA-1 coated on Petri dishes was stable for more than five cycles of COS cell adherence and elution with EDTA.

To clone the second LFA-1 ligand, a cDNA library in the plasmid vector CDM8 was prepared from endothelial cells, which use both the ICAM-1-dependent and ICAM-1-independent components of LFA-1-dependent adhesion[6]. Transfected COS cells were incubated in LFA-1-coated Petri dishes with ICAM-1 mAb present to prevent isolation of ICAM-1 cDNA. Adherent cells were eluted with EDTA and plasmids were isolated and amplified in *Escherichia coli*. After three cycles of transfection, adherence and plasmid isolation and one size fractionation, 30 plasmids were analysed by restriction endonuclease digestion. Of three whose inserts were more than 1 kilobase (kb), one plasmid introduced into COS cells by transfection caused adherence to LFA-1.

This plasmid conferred adherence to LFA-1 in a high percentage of the transfected cells, similar to the percentage seen with ICAM-1 transfection (Fig. 1*b*). Adherence was blocked by LFA-1 mAb, but not by ICAM-1 mAb, in contrast to ICAM-1 transfec-

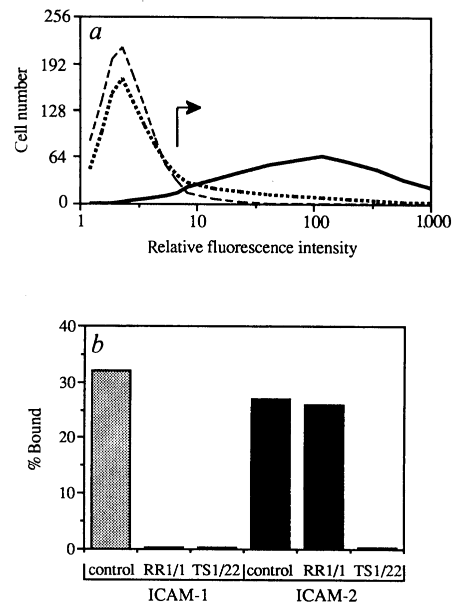

FIG. 1 Binding of transfected COS cells expressing ICAM-1 and ICAM-2 to LFA-1-coated plastic. *a*, COS cells transfected with ICAM-1 cDNA were panned on LFA-1-coated plates and the expression of ICAM-1 was analysed by indirect immunofluorescence flow cytometry with RR1/1 as the primary mAb. Unpanned cells, dotted line; non-adherent cells, dashed line; adherent cells, solid line. *b*, ^{51}Cr-labelled transfected COS cells expressing ICAM-1 or ICAM-2 were bound to LFA-1-coated plastic in the presence of mAb.

METHODS. LFA-1 was purified from SKW-3 lysates[24] by immunoaffinity chromatography on TS2/4 LFA-1 mAb Sepharose and eluted at pH 11.5 in the presence of 2 mM $MgCl_2$ and 1% octylglucoside (M.L.D. and T.A.S., manuscript in preparation). LFA-1 (10 μg per 200 μl per 6-cm plate) was bound to bacteriological Petri dishes by diluting octylglucoside to 0.1% in PBS with 2 mM $MgCl_2$ and overnight incubation at 4 °C. Plates were blocked with 1% BSA and stored in PBS/2 mM $MgCl_2$/0.2% BSA/0.025% azide/50 μg ml^{-1} gentamycin. Synthesis of a cDNA library from lipopolysaccharide-stimulated umbilical vein endothelial cells was as described previously[10]. After second-strand synthesis, the cDNA was ligated to *Bst*XI adaptors[12] and cDNAs longer than 600 bp were selected by low-melting point agarose gel electrophoresis. The cDNA was then ligated to CDM8 (ref. 25), introduced into *E. coli* host MC1061/P3 and plated to obtain 5×10^5 colonies. The colonies were suspended in LB medium, pooled and plasmid prepared by standard alkali-lysis method[26]. Ten 10-cm plates of COS cells at 50% confluency were transfected with 10 μg per plate of the plasmid cDNA library using DEAE-dextran[27]. The putative second LFA-1 ligand is trypsin-resistant on endothelial and SKW-3 cells (M.L.D., unpublished results). COS cells three days after transfection were suspended by treatment with 0.025% trypsin/1 mM EDTA/HBSS (Gibco) and panned[12] on LFA-1 coated plates as described below for ^{51}Cr-labelled COS cells. Adherent cells were released by addition of 10 mM EDTA. Plasmid was recovered from the adherent population of COS cells in Hirt supernatants[28]. The *E. coli* strain MC1061/P3 was then transformed with the plasmid, colonies on plates were suspended in LB medium, pooled and plasmid prepared by the alkali-lysis method. Selection of LFA-1-adherent transfected COS cells and plasmid recovery was repeated twice. Pooled colonies obtained after the third cycle were grown to saturation in 100 ml LB medium with 18 μg ml^{-1} tetracycline and 20 μg ml^{-1} ampicillin. Plasmid was prepared and fractionated by 1% low-melting point agarose gel electrophoresis and MC1061/p3 was transformed separately with plasmid from nine different size fractions. Individual plasmids from the fraction with greatest activity in promoting COS cell adhesion to LFA-1 were examined for uniqueness by restriction enzyme digestion and tested in the COS cell adherence assay. This yielded one plasmid with an ICAM-2 cDNA insert of 1.1 kb, pCDIC2.27. For adhesion assays, the ICAM-2 plasmid pCDIC2.27 or an ICAM-1 construct containing the 1.8-kb *Sal*I-*Kpn*I fragment[10] in CDM8 (2 μg per 10-cm plate) was introduced into COS cells using DEAE-Dextran. COS cells were suspended with 0.025% trypsin/1 mM EDTA/HBSS 3 days after transfection and labelled with ^{51}Cr. Approximately 2×10^5 ^{51}Cr-labelled COS cells in 2 ml PBS/5% FCS/2mM $MgCl_2$/0.025% azide (buffer) with 5 μg ml^{-1} of the mAb indicated were incubated in LFA-1-coated 6-cm plates at 25 °C for 1 hour. Non-adherent cells were removed by gentle rocking and three washes with buffer. Adherent cells were eluted by the addition of EDTA to 10 mM and γ-counted.

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                              CTAAAGATCTCCCTCCAGGCAGCCCTTGGCTGGTCCCTGCGAGCCCGTGGAGACTGCCAGAG   62

ATGTCCTCTTTCGGTTACAGGACCCTGACTGTGGCCCTCTTCACCCTGATCTGCTGTCCAGGATCGGATGAGAAGGTATTCGAGGTACAC 152
M  S  S  F  G  Y  R  T  L  T  V  A  L  F  T  L  I  C  C  P  G  S  D  E  K  V  F  E  V  H    9

GTGAGGCCAAAGAAGCTGGCGGTTGAGCCCAAAGGGTCCCTCGAGGTCAACTGCAGCACCACCTGTAACCAGCCTGAAGTGGGTGGTCTG 242
V  R  P  K  K  L  A  V  E  P  K  G  S  L  E  V  N  C  S  T  T  C  N  Q  P  E  V  G  G  L   39

GAGACCTCTCTAAATAAGATTCTGCTGGACGAACAGGCTCAGTGGAAACATTACTTGGTCTCAAACATCTCCCATGACACGGTCCTCCAA 332
E  T  S  L  N  K  I  L  L  D  E  Q  A  Q  W  K  H  Y  L  V  S  N  I  S  H  D  T  V  L  Q   69

TGCCACTTCACCTGCTCCGGGAAGCAGGAGTCAATGAATTCCAACGTCAGCGTGTACCAGCCTCCAAGGCAGGTCATCCTGACACTGCAA 422
C  H  F  T  C  S  G  K  Q  E  S  M  N  S  N  V  S  V  Y  Q  P  P  R  Q  V  I  L  T  L  Q   99

CCCACTTTGGTGGCTGTGGGCAAGTCCTTCACCATTGAGTGCAGGGTGCCCACCGTGGAGCCCCTGGACAGCCTCACCCTCTTCCTGTTC 512
P  T  L  V  A  V  G  K  S  F  T  I  E  C  R  V  P  T  V  E  P  L  D  S  L  T  L  F  L  F  129

CGTGGCAATGAGACTCTGCACTATGAGACCTTCGGGAAGGCAGCCCCTGCTCCGCAGGAGGCCACAGCCACATTCAACAGCACGGCTGAC 602
R  G  N  E  T  L  H  Y  E  T  F  G  K  A  A  P  A  P  Q  E  A  T  A  T  F  N  S  T  A  D  159

AGAGAGGATGGCCACCGCAACTTCTCCTGCCTGGCTGTGCTGGACTTGATGTCTCGCGGTGGCAACATCTTTCACAAACACTCAGCCCCG 692
R  E  D  G  H  R  N  F  S  C  L  A  V  L  D  L  M  S  R  G  G  N  I  F  H  K  H  S  A  P  189

AAGATGTTGGAGATCTATGAGCCTGTGTCGGACAGCCAGATGGTCATCATAGTCACGGTGGTGTCGGTGTTGCTGTCCCTGTTCGTGACA 782
K  M  L  E  I  Y  E  P  V  S  D  S  Q  M  V  I  I  V  T  V  V  S  V  L  L  S  L  F  V  T  219
                                                                     T M
TCTGTCCTGCTCTGCTTCATCTTCGGCCAGCACTTGCGCCAGCAGCGGATGGGCACCTACGGGGTGCGAGCGGCTTGGAGGAGGCTGCCC 872
S  V  L  L  C  F  I  F  G  Q  H  L  R  Q  Q  R  M  G  T  Y  G  V  R  A  A  W  R  R  L  P  249

CAGGCCTTCCGGCCATAGCAACCATGAGTGGCATGGCCACCACCACGGTGGTCACTGGAACTCAGTGTGACTCCTCAGGGTTGAGGTCCA 962
Q  A  F  R  P  *                                                                           254
GCCCTGGCTGAAGGACTGTGACAGGCAGCAGAGACTTGGGACATTGCCTTTTCTAGCCCGAATACAAACACCTGGACTTAAAAAAAAAAA1052
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FIG. 2 Nucleotide and amino-acid sequence of ICAM-2. The amino-acid sequence is numbered from first residue after the predicted cleavage site of the signal peptide. The hydrophobic putative signal peptide and transmembrane sequences (TM) are underlined. Potential *N*-linked glycosylation sites are boxed. The putative polyadenylation signal AATACA is overlined. Both strands of the ICAM-2 cDNA were sequenced within CDM8 by sequential synthesis of complementary oligonucleotide primers and dideoxynucleotide chain termination sequencing[29] according to the manufacturer's recommendations (Sequenase, US Biochemical).

tants (Fig. 1*b*). Furthermore, cells transfected with this plasmid did not react with a panel of four ICAM-1 mAbs. Thus, all functional criteria for a cDNA encoding a second LFA-1 ligand were fulfilled, and we designated this ligand ICAM-2.

The ICAM-2 cDNA sequence of 1,052 base pairs (Fig. 2) contains a 62-bp 5′ untranslated region and a 165-bp 3′ untranslated region. An AATACA polyadenylation signal at position 1,022, which, in contrast to AATAAA, occurs in approximately 2% of vertebrate messenger RNAs[13], is followed at 1,041 bp by a poly(A) tail. The longest open reading frame begins with the first ATG at position 63 and ends with a TAG termination codon at position 885. Hydrophobicity analysis[14] and usage of amino acids around cleavage sites[15] predict a 21-residue signal peptide (Fig. 2). The predicted mature sequence contains a putative extracellular domain between amino acids 1 and 202, followed by a 26-residue hydrophobic putative transmembrane domain and a 26-residue cytoplasmic domain. Four turns of the putatively α-helical transmembrane segment are amphipathic, with threonine and serine residues falling on one side, suggesting the possibility of self-association or association with other membrane proteins in the plane of the membrane. The cytoplasmic domain is unusually basic. The predicted relative molecular mass (M_r) of the mature polypeptide is 28,393 which, if the six predicted *N*-linked glycosylation sites are used, would result in a ICAM-2 glycoprotein of M_r about 46,000.

The 1.1-kb ICAM-2 cDNA hybridizes to a 1.4-kb poly(A)$^+$ mRNA and weakly to a 3-kb mRNA (Fi. 3*a*), both of which are distinct from the 3.3-kb and 2.4-kb ICAM-1 mRNA (Fig. 3*b*). We examined mRNA in cells that have been characterized functionally for ICAM-1-dependent and second-ligand-dependent binding to LFA-1. ICAM-1 mRNA is strongly induced in edndothelial cells by lipopolysaccharide (Fig. 3*b*, lanes 2 and 3). In contrast, the basal expression of ICAM-2 mRNA is high in endothelial cells and is not induced further by lipopolysaccharide (Fig. 3*a*, lanes 2 and 3).This correlates with the strong basal and non-inducible expression of the LFA-1-dependent, ICAM-1-independent, pathway in endothelial cells and the inducibility of the ICAM-1-dependent pathway[6]. ICAM-2 mRNA is present in a wide variety of cell types including Ramos and BBN B lymphoblastoid, U937 monocytic and SKW3 T lymphoblastoid cell lines (Fig. 3*a*: lanes 1, 4, 6 and 8, moderate or long autoradiograph exposure). Of these, SKW3, U937 and BBN have been shown to adhere to LFA-1$^+$ cells in an LFA-1-dependent, ICAM-1-independent way[8,9], and to LFA-1-coated plastic (M.L.D., unpublished results). The HeLa epithelial cell line, which has only the ICAM-1-dependent component of LFA-1-dependent adhesion[9], shows no ICAM-2 mRNA (Fig. 3*a*, lane 5), even after prolonged autoradiograph exposure. The distribution of mRNA for ICAM-2 could therefore account for all of the observed ICAM-1-independent LFA-1-dependent adhesion, but confirmation that it does must await the production of a function-blocking mAb to ICAM-2, as additional

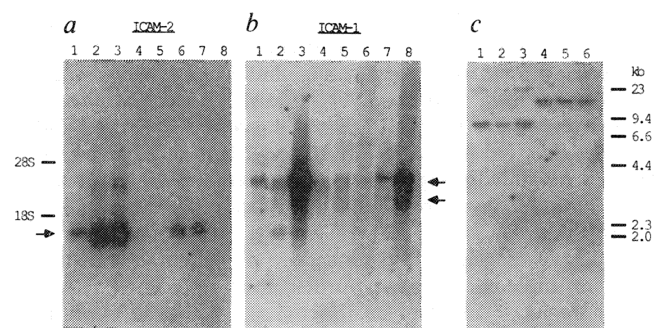

FIG. 3 RNA and DNA blots. Northern (*a*, *b*) and Southern (*c*) blots were hybridized to the 1.1-kb ^{32}P-labelled ICAM-2 cDNA (*a*, *c*) and rehybridized to the 3-kb ^{32}P-labelled ICAM-1 cDNA (*b*). *a*, *b*, Poly(A)$^+$ RNA (6 μg) from the Burkitt lymphoma cell line, Ramos (lane 1), endothelial cells (lane 2), endothelial cells stimulated for 3 hours with lipopolysaccharide (lane 3), an Epstein–Barr virus immortalized B-lymphoblastoid cell line, BBN (lane 4), epithelial carcinoma cell line, HeLa (lane 5), T lymphoma cell lines, Jurkat (lane 6) and SKW-3 (lane 7) and a promonocyte cell line, U937 (lane 8). ICAM-2 and ICAM-1 mRNAs are indicated by arrows. (*c*), Genomic DNA (6 μg) from B-cell lines BL-2 (lanes 1 and 4), ER-LCL (lanes 2 and 5) and Raji (lanes 3 and 6) digested with *Eco*R1 (lanes 1–3) or *Hin*dIII (lanes 4–6).

METHODS. Northern blots used 6 μg of poly(A)$^+$ RNA which was denatured and electrophoresed through a 1% agarose-formaldehyde gel[26] and electrotransferred to a nylon membrane (Zeta Probe, BioRad). Completion of transfer was confirmed by ultraviolet transillumination of the gel and fluorescent photography of the blot. The genomic DNAs were digested with five times the manufacturer's recommended quantity of *Eco*RI and *Hin*dIII endonucleases (New England Biolabs). Following electrophoresis through a 0.8% agarose gel, the DNAs were transferred to Zeta Probe. RNA and DNA blots were prehybridized and hybridized following standard procedures[26] using ICAM-2 or ICAM-1 cDNAs labelled with [α^{32}P]dNTP's by random priming (Boehringer Mannheim).

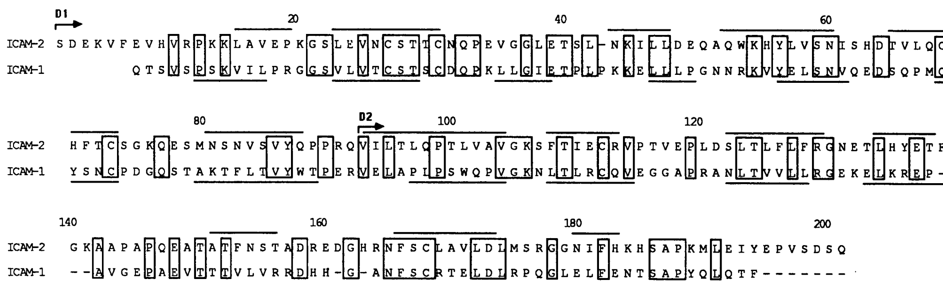

FIG. 4 ICAM-2 homology to ICAM-1. The entire 202-residue extracellular sequence of ICAM-2 was aligned with ICAM-1 residues 1–185 using the ALIGN program[30] and by inspection. ICAM-2 residues are numbered; identities are boxed. D1 and D2 indicate the boundary of immunoglobulin-like domains of ICAM-2 and ICAM-1. β-strand predictions[31] of ICAM-2 are overlined and those of ICAM-1 are underlined.

LFA-1 ligands might exist. Southern blots of genomic DNA (Fig. 3*c*) hybridized with the ICAM-2 cDNA showed a single predominant *Eco*RI fragment of 8.2 kb and *Hin*dIII fragment of 14 kb, suggesting that there is a single gene with most of the coding information present in 8 kb.

Because of their functional similarity as LFA-1 ligands, the amino-acid sequences of ICAM-2 and ICAM-1 were compared. ICAM-1 is a member of the immunoglobulin superfamily and its extracellular domain consists entirely of five constant region (C)-like domains. The 202-amino-acid extracellular domain of ICAM-2 consists of two immunoglobulin C-like domains, with putative intradomain disulphide-bonded cysteines spaced 43 and 56 residues apart and a predicted β-strand structure (Fig. 4). Remarkably, the two immunoglobulin-like domains of ICAM-2 have 34% identity in their amino acid sequences with the two most N-terminal immunoglobulin-like domains of ICAM-1 (Fig. 4), with an ALIGN score 15 s.d. above the mean, and 27% identity to ICAM-1 domains 3 and 4, with an ALIGN score 3 s.d. above the mean. A search of the NBRF and SWISS-PROT protein databases yielded only partial domain homologies with other members of the immunoglobulin superfamily, primarily with histocompatibility complex (HLA) class II antigens. ICAM-2 has somewhat fewer conserved residues characteristic of immunoglobulin domains than ICAM-1. ICAM-2 is 17% and 19% identical to the two N-terminal domains of the adhesion molecules NCAM[16] and MAG[17], respectively, whereas ICAM-1 identity is 19% and 20%, respectively.

In contrast to LFA-1 and ICAM-1, which were identified by selecting mAbs that blocked T-lymphocyte-mediated killing, and homotypic adhesion, respectively[8,18], ICAM-2 has been defined using a functional cDNA cloning procedure that requires no previous identification of the protein by biochemical or immunological techniques. Previously, we and other groups have not succeeded in defining ICAM-2 with mAbs, and screening 350 mAbs in non-lineage T-cell and myeloid panels of the Fourth International Leukocyte Workshop did not reveal any that were reactive with ICAM-2 expressed on COS cells. Our approach should be applicable to other adhesion molecules, and has the advantage over selection of function-blocking mAb in that it is not limited by immunogenicity.

ICAM-2 and the two N-terminal domains of ICAM-1 are much more similar to each other than to other members of the immunoglobulin superfamily. Significantly, the LFA-1-binding region of ICAM-1 has been mapped to domains 1 and 2 by domain deletion and systematic amino-acid substitution (D.E.S., M.L.D. and T.A.S., unpublished results). Thus, the homology is both structural and functional. Although there is little precedence among cell adhesion receptors, several integrin receptors for extracellular matrix components have been shown to recognize multiple ligands[19,20]. Neither ICAM-1 or ICAM-2 contains an Arg-Gly-Asp (RGD) sequence, and thus the mode of recognition by LFA-1 may differ from integrins that bind extracellular matrix components[19,20]. It will be interesting to determine whether the cellular ligands recognized by Mac-1 and p150,95, leukocyte integrins closely related to LFA-1[2], belong to the same immunoglobulin subfamily. ICAM-1 has recently been shown to be a receptor for the major group of rhinoviruses which cause 50% of common colds[21,22]; whether ICAM-2 also functions as a receptor for rhinoviruses or other picornaviruses remains to be determined.

The identification of a family of LFA-1 ligands emphasizes the importance of this recognition pathway and may be a mechanism for imparting fine specificity and functional diversity. Several differences between ICAM-1 and ICAM-2 are of potential importance. ICAM-1 is inducible on most cells, whereas ICAM2 expression is not affected by cytokines on the cells thus far tested. The three additional domains on ICAM-1 are expected to project its LFA-1 binding site further from the cell surface than that of ICAM-2, suggesting that closer cell-cell contact would be required for the LFA-1/ICAM-2 than the LFA-1/ICAM-1 interaction. ICAM-2-transfected COS cells are more readily detached from LFA-1-coated plastic, compared with ICAM-1 transfected COS cells, as the washing shear force is increased. This may be due to the smaller size of ICAM-2 which may make it less accessible to LFA-1 on the artificial substrate, or to differences in sequence which impart differences in affinity. LFA-1 and ICAM-1 are receptors for each other[23], and although we have arbitrarily referred to LFA-1 as a receptor and to the ICAMs as ligands, both are cell surface molecules and may function as signalling receptors which inform cells of their environments. Thus, the distinct cytoplasmic domains of ICAM-1 and ICAM-2 may impart different signals or may cause differing localization on the cell surface; likewise, signalling or interaction with the cytoskeleton by LFA-1 may differ depending on whether ICAM-1 or ICAM-2 is bound. □

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