Brief Definitive Report

Cloning and Expression of Intercellular Adhesion Molecule 3 Reveals Strong Homology to Other Immunoglobulin Family Counter-receptors for Lymphocyte Function-associated Antigen 1

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Summary

Based on protein sequence, we have isolated a cDNA for intercellular adhesion molecule 3 (ICAM-3), the most recently defined counter-receptor for lymphocyte function-associated antigen 1 (LFA-1). Expression of the cDNA yields a product that reacts with monoclonal antibody to ICAM-3 and functions as a ligand for LFA-1. The deduced 518–amino acid sequence of the predicted mature protein defines a highly glycosylated type I integral membrane protein with five immunoglobulin (Ig)-like domains. The five Ig-like domains of ICAM-3 are highly homologous with those of human ICAM-1 (52% identity) and human ICAM-2 (37% identity).

Three counter-receptors have been described for the leukocyte integrin LFA-1, intercellular adhesion molecule 1 (ICAM-1), ICAM-2, and ICAM-3 (1–5). Both ICAM-1 and ICAM-2 have been cloned and are members of the Ig supergene family. ICAM-3 was defined with a mAb that inhibited a pathway of lymphoblastoid cell adhesion to purified LFA-1 that was independent of ICAM-1 and ICAM-2 (5). In contrast to ICAM-1 and ICAM-2, ICAM-3 is absent from endothelium and expressed solely on leukocytes. ICAM-3 is a heavily glycosylated protein of 124,000 M_r that is well expressed on resting lymphocytes, monocytes, and neutrophils, representing the major LFA-1 ligand on these cells (5). Although an exact role for ICAM-3 is yet to be established, the finding that adhesion of resting T lymphocytes to purified LFA-1 occurs primarily via ICAM-3, combined with the fact that ICAM-3 is much better expressed than the other LFA-1 ligands on monocytes and resting lymphocytes, implies an important role in the initiation of immune responses. We now report the cloning and characterization of human ICAM-3.

Materials and Methods

Monoclonal Antibodies. The following previously described murine mAbs to human antigens were used: TS1/22 (anti-CD11a, IgG1) (6), CBR-IC3/1 (anti-ICAM-3, IgG1) (5), W6/32 (anti-HLA, A, B, C, IgG2a) (7), and X63 (nonbinding antibody, IgG1). CBR-IC3/2 (anti-ICAM-3, IgG2a) was generated by immunizing mice with purified ICAM-3 and screening hybridomas for reactivity to purified ICAM-3 and ICAM-3-bearing cells (A. de Fougerolles et al., manuscript in preparation). All mAbs were used at a saturating concentration of 20 μg/ml for flow cytometry and adhesion assays.

Purification of ICAM-3 and Peptide Sequencing. ICAM-3 was purified from detergent lysates of human tonsil by immunoaffinity chromatography at 4°C using the anti-ICAM-3 mAb CBR-IC3/1. mAb CBR-IC3/1 was purified from ascites on a protein A affinity column (8) and then coupled to cyanogen bromide–activated Sepharose CL-4B (Pharmacia Fine Chemicals, Piscataway, NJ) (9). Frozen human tonsils (30 g) were finely diced and lysed in 450 ml lysis buffer (100 mM Tris HCl, pH 8.0, 150 mM NaCl, 2% Triton X-100, 5 mM iodoacetamide, 1 mM PMSF, 0.24 trypsin inhibitor units (TIU)/ml aprotinin, 0.025% azide) for 90 min while stirring gently. The resultant lysate was centrifuged at 10,000 *g* for 2 h; the supernatant was decanted and subjected to ultracentrifugation at 100,000 *g* for 1 h. The clarified lysate was precleared with human Ig–coupled Sepharose (40 μl of a 1:1 slurry/ml of lysate) during rotation overnight. The human Ig–coupled Sepharose was pelleted and the precleared lysate passed over the CBR-IC3/1 immunoaffinity column (bed volume, 6 ml; 3 mg/ml CBR-IC3/1) at a rate of 30 ml/h. The column was sequentially washed at 30 ml/h with 20-bed volumes 50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.1% Triton X-100, and with 10-bed volumes 50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% octyl β-D-glucopyranoside (OG). ICAM-3 was eluted from the immunoaffinity column with 10-bed volumes of 50 mM glycine, pH 3.8, 150 mM NaCl, 1% OG, and neutralized with 1:10 vol 1 M Tris, pH 8.6, 1% OG, aliquoted, and stored frozen at −70°C for 3–6 mo without loss of activity.

ICAM-3 fractions were pooled and concentrated by centrifugal ultrafiltration (Centricon-30; Amicon, Beverly, MA), subjected to SDS-7.5% PAGE under reducing conditions, and protein electroblotted onto nitrocellulose (Schleicher & Schuell, Inc., Keene, NH). The nitrocellulose-blotted proteins were excised and subjected to in situ enzymatic cleavage with lysyl endoproteinase (Lys-C) (10).

Cleaved fragments were separated by reverse-phase HPLC (11). Peptides were subjected to microsequence analysis on a gas phase microsequenator (Applied Biosystems, Inc., Foster City, CA).

Construction and Screening of cDNA Libraries. A human tonsil cDNA library in λgt11 (accession no. 37546; American Type Culture Collection, Rockville, MD) (12) with inserts of 2 kb or greater was screened as described (13). Partially degenerate antisense oligonucleotides were based on the ICAM-3 peptide sequences, the codons used in homologous sequences in ICAM-1 and ICAM-2, and general human codon usage frequency (14): peptide 10, the 25mer 5′ TTNAG(G/A)TG(C/T)TGNGG(G/A)CANGTNGCNC 3′; peptide 17, the 23mer 5′ A(G/A)NGANGTCTCCAG(G/A)GC-(G/A/T)AT(T/C)TT 3′ (N = inosine). End labeling and hybridization of oligonucleotides was as described (13). Inserts from hybridizing phage were excised using EcoRI and subcloned into plasmid pBluescript KS$^-$ (Stratagene, San Diego, CA) for restriction mapping and sequence analysis.

Sequencing and Homology. Nucleotide sequence was determined by the dideoxynucleotide chain termination method with modified T7 DNA polymerase (U.S. Biochemical Corp., Cleveland, OH). T7 and RM13 primers (New England Biolabs, Beverly, MA) were used to sequence the 5′ and 3′ ends of the ICAM-3 clones and internal primers were synthesized based on sequencing results. The sequence on both strands was determined for the entire 11.2 cDNA clone, for the 5′ end and the 3′ SacI-EcoRI fragment of clone 7.3.1, and for the 5′ EcoRI-EcoNI fragment of clone 14a2.2. The ends of all clones were sequenced on one strand.

cDNA Transfection. A full-length ICAM-3 cDNA was constructed by ligating in 5′ to 3′ order the fully sequenced 0.24-kb HindIII-EcoNI fragment of clone 14a2.2, the 1.24-kb EcoNI-SacI fragment of clone 11.2, and the 0.35-kb SacI-EcoRI fragment of clone 7.3.1.. The HindIII and EcoRI sites were present in the pBluescript polylinker. The fragments were subcloned into the transient expression vector Ap^{r}M9 (a derivative of CDM8 containing the β-lactamase from pBluescript and a polylinker from pSP64; L. B. Klickstein, unpublished results) cut with HindIII and EcoRI to yield pCDIC-3. The pCDIC-3 or Ap^{r}M9 plasmids were transfected into COS cells using DEAE-dextran (15). 3 d after transfection, cells were detached with HBSS, 10 mM EDTA, washed three times in 10% FCS, RPMI 1640, and used for flow cytometry or binding to LFA-1-coated plates.

Flow Cytometry. Indirect immunofluorescence and flow cytometry were performed as previously described (3).

Adhesion Assay. Adherence of COS cell transfectants to purified LFA-1 absorbed onto 96-well polystyrene microtiter plates was quantitated by fluorescence as previously described (3). Site density of LFA-1, determined by radioimmunoassay using saturating amounts of ^{125}I-TS1/22 mAb and calculated assuming monovalent binding of the mAb, was 700 sites/μm^2.

Fluorescently labeled COS cells were pretreated with 20 μg/ml of purified mAb for 30 min at room temperature, and 5×10^4 cells in 50 μl were transferred to each well. Some wells were pretreated for 30 min at room temperature with 20 μg/ml of purified TS1/22 mAb to LFA-1. Cells were allowed to settle and adhere to the solid-phase LFA-1 for 1 h at 37°C, and washed with four aspirations through a 25-gauge needle. Bound cells were quantitated in the 96-well plate using a Pandex fluorescence concentration analyzer (IDEXX Corp., Westbrook, ME).

Results

ICAM-3 purified from human tonsil by mAb affinity chromatography was homogeneous by SDS-PAGE (Fig. 1). Attempts to obtain NH_2-terminal sequence were unsuccessful, suggesting that the NH_2-terminus was blocked. To obtain internal amino acid sequence, blotted material was digested with Lys-C protease. Reverse-phase HPLC yielded a small number of peptide peaks that were subjected to gas-phase microsequencing. The sequence of peptide 17, IALETSLSK, was significantly homologous to ICAM-1 and ICAM-2 in a region of the first Ig-like domain. The sequence of peptide 10, IDRATCPQHLK, was weakly homologous to the fifth Ig-like domain of human ICAM-1.

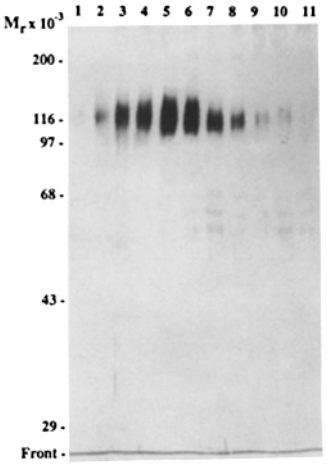

Figure 1. SDS-PAGE of ICAM-3 purified from human tonsil. Aliquots of successive fractions of the pH 3.8 eluate from the CBR-IC3/1 mAb Sepharose (10 μl) were subjected to SDS-7.5% PAGE under reducing conditions and silver stained. Fractions shown in lanes *2–8* were pooled, and subjected to preparative SDS-PAGE for sequencing studies. Molecular weight markers were myosin (200,000), β-galactosidase (116,000), rabbit muscle phosphorylase b (97,000), BSA (68,000), hen egg OVA (43,000), and bovine carbonic anhydrase (29,000).

The sequence of each peptide was used to design an oligonucleotide probe. One clone (11.2) was isolated from a size-selected λgt11 tonsil cDNA library that hybridized to both oligonucleotides probes, and yielded an insert of 1.6 kb. This clone and its 5′ EcoRI-EcoNI fragment were utilized to isolate cDNA clones that extended further in the 5′ and 3′ directions. The overlapping cDNA clones were subjected to restriction mapping (Fig. 2) and sequencing (EMBL/GenBank/DDBJ accession number X69819). The two peptide sequences were in perfect agreement with the translated amino acid sequence (Fig. 3, *thin overlining*), establishing the authenticity of the cDNA as that of ICAM-3. Two potential ATG initiation codons at nucleotide positions 9 and 18 are present, both of which fit Kozak's criteria for a translation initiation site (16). The first initiation codon is followed by an open reading frame of 1,641 bp. An in-frame stop codon (TGA) at position 1650 is followed by an untranslated region of 79 bp. The poly(A) tail is present 15 bp after a consensus polyadenylation sequence at position 1710.

The deduced amino acid sequence of the mature protein is shown in Fig. 3. Hydrophobicity analysis (17) suggests the presence of an NH_2-terminal signal peptide sequence and a 25–amino acid transmembrane segment near the COOH terminus, and thus ICAM-3 is a type I membrane protein. A consensus signal peptide cleavage site (18) predicts that glutamine is NH_2 terminal in the mature polypeptide chain. This is consistent with our inability to obtain an NH_2-terminal amino acid sequence as glutamine may cyclize to pyroglutamic acid, resulting in a blocked NH_2 terminus. The mature ICAM-3 polypeptide chain contains a 456–amino acid NH_2-terminal region predicted to be extracellular and a

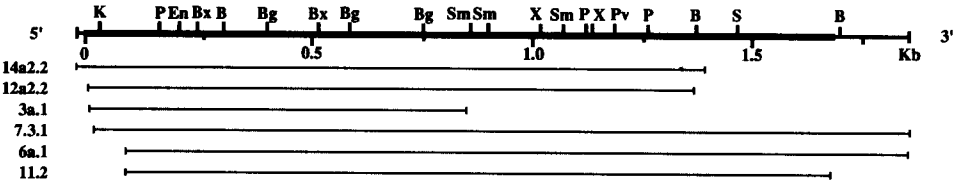

Figure 2. Restriction map of ICAM-3 cDNA clones. The top line is the map of the full-length cDNA assembled as described in Materials and Methods. (K, KpnI; P, PstI; En, EcoNI; Bx, BstXI; B, BamHI; Bg, BglI; Sm, SmaI; X, XhoI; Pv, PvuII; and S, SacI). EcoRI and HindIII sites present in the pBluescript KS⁻ polylinker are not shown. The open reading frame is shown as a thick line.

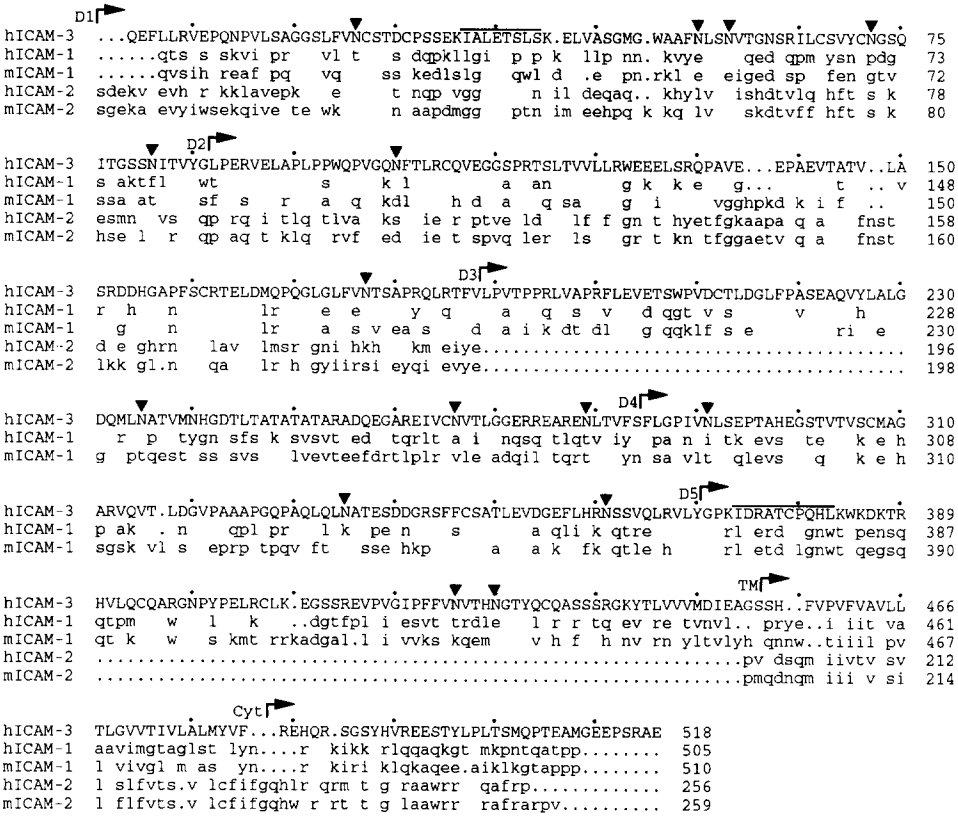

Figure 3. Homology of ICAM-3 with ICAM-1 and ICAM-2. The sequences of ICAM-3, mouse and human ICAM-1 (23, 24), and mouse and human ICAM-2 (19, 25) were aligned using the GCG PileUp (26) program and by inspection. The amino acid sequence of ICAM-3 was deduced from the cDNA sequence. The amino acid sequences of Lys-C-cleaved peptides are overlined, and potential N-linked glycosylation sites are indicated by inverted triangles. The entire mature sequence of ICAM-3, and residues in ICAM-1 and ICAM-2 that differ from ICAM-3, are shown. Gaps appear as dots. Boundaries of the Ig-like domains and boundary of D5 with the transmembrane domain are based on exon boundaries of ICAM-1 (27) and ICAM-2 (19).

Table 1. *Identities between ICAM Ig-like Domains*

	ICAM-1					ICAM-2		ICAM-3				
ICAM-3	D1	D2	D3	D4	D5	D1	D2	D1	D2	D3	D4	D5
D1	**38**	16	27	21	23	**36**	18	–				
D2	30	**77**	22	23	28	26	**38**	16	–			
D3	15	26	**52**	22	20	25	23	29	21	–		
D4	21	25	23	**51**	18	9	22	17	24	20	–	
D5	14	13	22	20	**37**	16	13	19	20	14	20	–

Percent amino acid identity between Ig-like domains was determined with the GAP program from GCG (26). Bold numbers represent comparison of ICAM-3 domains with the corresponding ones in ICAM-1 or -2.

37–amino acid putative cytoplasmic region. The predicted 518–amino acid mature polypeptide chain backbone of ICAM-3 is 56,980 M_r. Native ICAM-3 was found to migrate as a protein of 124,000 M_r in SDS-PAGE, and upon N-glycanase treatment yielded a broad band of 87,000 M_r (5). In parallel experiments, ICAM-2 and HLA were cleaved to the size predicted for their polypeptide chain backbones; they were converted from diffuse to sharp bands in SDS-PAGE, whereas the ICAM-3 band remained diffuse. ICAM-3 has 15 putative N-linked glycosylation sites. The discrepancy with the predicted polypeptide backbone and the apparent heterogeneity after N-glycanase treatment suggests either the presence of additional posttranslational modifications, or the resistance of some N-linked sites to N-glycanase. No obvious proteoglycan or O-linked glycosylation sites are present. The frequency of N-linked sites, with 1 every 30 amino acids, is

unusually high for a cell surface glycoprotein. Some of these sites, particularly closely paired sites at residues 55 and 58, and at residues 424 and 428, may be resistant to N-glycanase. If so, the average size of the N-linked chains is 4,500 M_r.

The extracellular region of ICAM-3 contains five Ig-like domains and is strikingly homologous to ICAM-1 and ICAM-2 (Fig. 3). Like ICAM-1 and ICAM-2, the Ig-like domains of ICAM-3 are of the C2 set, and the first Ig-like domain contains two putative intradomain disulfide bonds. The extracellular five Ig-like domains of ICAM-3 are 52% identical in amino acid sequence to the corresponding region in human ICAM-1, with the highest identity contained within domain 2 and the first half of domain 3. The homology with ICAM-2 is lower (Table 1). All ICAM-3 Ig-like domains are most closely related to the domains with the corresponding position in ICAM-1 and ICAM-2 (Table 1). The transmembrane and cytoplasmic domains of ICAM-3 show little if any conservation with those of ICAM-1 and ICAM-2.

To express ICAM-3, cDNA segments from clones 14a2.2, 11.2, and 7.3.1 were ligated together to obtain a full-length cDNA clone. COS cells transfected with the ICAM-3 cDNA reacted specifically with the anti-ICAM-3 mAb, CBR-IC3/1 (Fig. 4). Transfectants expressing human ICAM-3 bound efficiently to purified human LFA-1 on plastic (Fig. 5). By contrast, mock-transfected cells bound poorly, showing equivalent binding to substrates bearing LFA-1 and BSA. Binding to LFA-1 of COS cells transfected with ICAM-3 was blocked by LFA-1 mAb, and by a combination of anti-ICAM-3 mAbs.

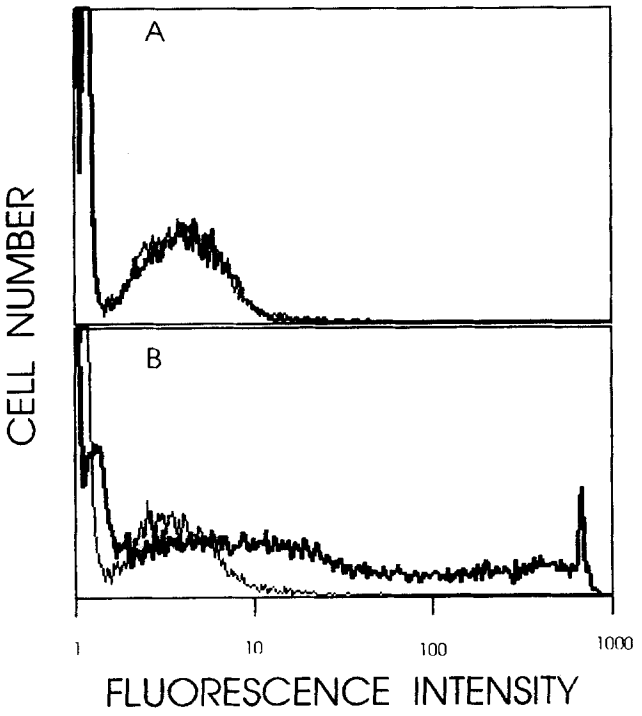

Figure 4. Surface expression of ICAM-3 on COS cells. COS cells transfected with (*A*) Ap^{r}M9 vector alone (mock) or (*B*) pCDIC-3 were labeled with saturating amounts of control mAb X63 (*thin line*) or mAb CBR-IC3/1 (anti-ICAM-3), followed by FITC-anti-mouse Ig. Cells were subjected to immunofluorescent flow cytometry.

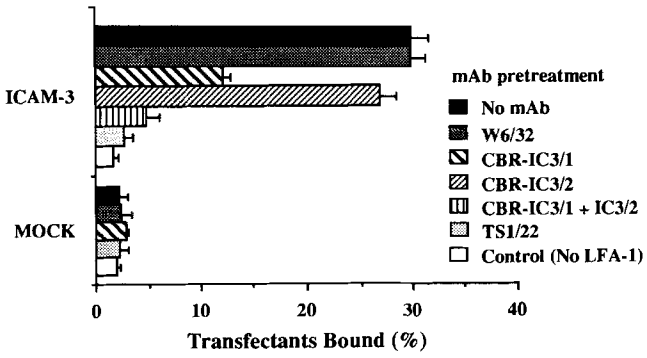

Figure 5. Adhesion of transfected COS cells to purified LFA-1. Cells transfected with pCDIC-3 or Ap^{r}M9 alone (mock) were allowed to bind to LFA-1-coated microtiter wells for 60 min at 37°C and then washed four times by aspiration. Control wells lacked LFA-1. Cells were pretreated with the indicated mAb or, alternatively, the absorbed purified LFA-1 was pretreated with mAb TS1/22 (anti-LFA-1α). To achieve effective blockade of the ICAM-3/LFA-1 adhesion pathway both anti-ICAM-3 mAbs are required. One representative experiment of four is shown and error bars indicate 1 SD.

Discussion

We have characterized a cDNA clone for ICAM-3. Its authenticity is proven by the presence in the translated amino acid sequence of the two Lys-C-cleaved ICAM-3 peptide fragments. Additionally, when expressed, the ICAM-3 cDNA is recognized by anti-ICAM-3 mAbs and is functionally active in binding to purified LFA-1.

The cloning and analysis of the human ICAM-3 cDNA demonstrates ICAM-3 to be a type I integral membrane protein belonging to the Ig superfamily. ICAM-3 is the most heavily glycosylated LFA-1 ligand with 15 putative N-linked glycosylation sites, as compared with eight and six for ICAM-1 and -2, respectively. The apparent size of the carbohydrate is high for ICAM-3 at 4,500 M_r per site, as it is for ICAM-1 and ICAM-2, which average 5,000 and 5,250 M_r per site, respectively.

The sequence relationships among the extracellular domains of the three ICAMs define them as a subfamily of the Ig superfamily. The homology between ICAM-1 and ICAM-3 is particularly striking, with 52% amino acid identity and the same number of Ig domains. The first two domains of ICAM-3 are closely related to the two domains of ICAM-2, with 37% identity. The relationship with all other Ig superfamily members is much weaker, on the order of 20%. Compar-

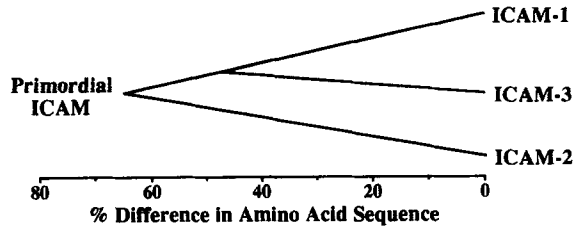

Figure 6. Evolutionary relationship of the ICAMs. The horizontal axis represents the percent difference in amino acid sequence of the extracellular domains.

isons of individual Ig-like domains of ICAM-3 with those in ICAM-1 and ICAM-2 show that the ICAM-3 Ig-like domains are most closely related to the corresponding domains in ICAM-1 or ICAM-2. These relationships suggest that ICAM-1, -2, and -3, evolved from a primordial ICAM gene in two steps (Fig. 6).

In contrast to the extracellular domains, there is little conservation of the transmembrane and cytoplasmic domains among ICAM-1, -2, and -3. However, these domains are well conserved between mouse and human ICAM-1, and between mouse and human ICAM-2. Thus, there may be important differences among ICAMs in localization on the cell surface, interaction with the cytoskeleton, and signaling.

The ICAM molecules were identified and named based on their ability to bind LFA-1 (3, 5, 19). Since all three ligands are homologous, we predict that the ligand recognition sites lie in homologous positions and contain key conserved residues. The binding site for LFA-1 has been mapped in ICAM-1 to the first Ig domain and key residues have been identified as E34 and Q73 (20). Both of these residues are conserved in ICAM-3 and in ICAM-2 (Fig. 3). However, compared with other domains in ICAM-3, domain 1 is one of the least well conserved with ICAM-1. The reason for the high level of identity (77%) between the second Ig-like domains of ICAM-1 and ICAM-3 remains unclear, because no ligand binding sites have been mapped to this domain. The third Ig-like domain of ICAM-1 is the binding site for the integrin Mac-1 (21); whether ICAM-3 also binds to Mac-1 is not known.

The existence of a subfamily of Ig-related molecules containing three members, ICAM-1, -2, and -3, that has evolved to bind to the integrin LFA-1 attests to the importance of this adhesion pathway. The multiplicity of ICAMs may allow finer regulation of this adhesion pathway since there are significant differences in tissue distribution and inducibility. ICAM-1 is highly inducible in immune and inflammatory reactions on many cell types (1), whereas ICAM-2 is constitutively expressed on endothelium (3). Thus, these molecules may regulate leukocyte circulation and localization patterns in disease and health, respectively. ICAM-3 is well expressed on all leukocytes and absent from nonhematopoietic cells. As ICAM-3 represents the major LFA-1 ligand on resting lymphocytes (5), it may play an important role in initiating immune responses. The distinct transmembrane and cytoplasmic domains of the ICAMs provide further diversity. The cytoplasmic regions of ICAM-1, -2, and -3 may impart different signals when LFA-1 is bound or cause differing localizations on the cell surface (22).

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Note added in proof: While this paper was in review, two other groups reported a cDNA encoding an LFA-1 ligand that was similar in tissue distribution and molecular weight, but not tested for identity with ICAM-3 (28, 29). The sequence of one (29) is identical to that reported here; the other (28) has two silent substitutions at nucleotide positions 187 and 1268 in our sequence and a 2-bp deletion after the polyadenylation signal.

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