PRIMARY STRUCTURE OF LYMPHOCYTE FUNCTION–ASSOCIATED ANTIGEN 3 (LFA-3)

The Ligand of the T Lymphocyte CD2 Glycoprotein

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The organization of specialized cells within tissues during embryogenesis and development, as well as in inflammation, is thought to be due to a collection of cell surface receptors that interact with molecules in the extracellular matrix and on the surface of other cells, thereby imparting positional information. Recently, we found (1) that lymphocyte function–associated antigen 3 (LFA-3),[1] a surface glycoprotein expressed on many cell types, binds to the T lymphocyte surface glycoprotein CD2 with a high affinity (10^7–10^8/M). This interaction is important in mediating thymocyte interactions with thymic epithelial cells, antigen-independent and -dependent interactions of T lymphocytes with target cells and antigen-presenting cells, and the T lymphocyte rosetting with erythrocytes (1–7). In addition to mediating cell adhesion, it has been suggested that the LFA-3/CD2 interaction may prime responses by both the $CD2^+$ and LFA-3^+ cells (8–10). Although receptors for extracellular matrix components (11), and receptors that appear to engage in like-like interactions (N-CAM) (12), have been defined, to our knowledge CD2 and LFA-3 are the first example in which an integral membrane protein on one cell binds to a distinct integral membrane protein on another cell. These molecules thus represent an ideal model system for studying cell–cell adhesion. To understand the structural basis for cognate interactions between cell adhesion molecules, we have cloned an LFA-3 cDNA and determined the primary structure of the LFA-3 protein.

Materials and Methods

LFA-3 Purification and Sequencing. LFA-3 was purified by immunoaffinity chromatography from human erythrocytes as described (6). LFA-3 purified by one cycle of affinity chromatography was deglycosylated using peptide:N–glycosidase F (*N*-glycanase) according to the supplier's instructions (Genzyme, Boston, MA). The glycosylated and deglycosylated forms were further purified by preparative SDS-PAGE and electroeluted exactly

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[1] *Abbreviations used in this paper:* LFA-3, lymphocyte function–associated antigen 3; PIPLC, phosphatidylinositol-specific phospholipase C.

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as described (13). Deglycosylated LFA-3 was digested with trypsin and the tryptic peptides were separated by HPLC (14). Intact LFA-3 (300 pmol), *N*-glycanase-treated LFA-3 (50 pmol), and tryptic fragments (5–50 pmol) were subjected to automated Edman degradation on a gas-phase sequencer (model 470A; Applied Biosystems, Inc., Foster City, CA) in the presence of polybrene (15). PTH-amino acids were identified on-line using a PTH analyzer (model 120A; Applied Biosystems, Inc.).

Construction of the PBL cDNA Library. PBL from leukapheresis were stimulated with 100 U/ml IFN-γ and 10 μg/ml PHA for 24 h. RNA was prepared by phenol extraction (16), and double-stranded cDNA was synthesized from poly(A)-selected RNA by the methods of Okayama and Berg (17) and Gubler and Hoffman (18). The cDNA was ligated to an Eco RI/blunt-ended linker, designed to allow the cDNA insert to be excised by Not I. Size-selected (800 bp and longer) cDNA was ligated to Eco RI–digested λgt10 arms, and aliquots of the ligation mixture were packaged in Gigapak (Stratagene, San Diego, CA). The packaged phage was used to infect *Escherichia coli.* The resulting library contained 1.1×10^6 independent recombinant phages.

Isolation of the cDNA Clones. 10^6 recombinants of either the human PBL λgt10 cDNA library or a human tonsil λgt11 cDNA library (a generous gift of Lloyd Klickstein, Harvard Medical School, Boston, MA) (19) were screened with ^{32}P-labeled oligonucleotide probes as described (20). Antisense oligonucleotide probes were synthesized on an Applied Biosystems, Inc., 30A DNA Synthesizer. Oligonucleotide probe pool LF1 was a 20 mer, 32-fold degenerate, corresponding to amino acids 28–34 of the NH_2-terminal sequence of LFA-3 (Table I). Probe pools LF2 to LF5 were synthesized to residues 3–7 of the NH_2-terminus and were a set of four subpools, each 20 bp long and 96-fold degenerate. The nucleotide sequence of each subpool was identical except for the nucleotide in the third position of the codon for glycine. Probes were 5′ endlabeled using γ-[^{32}P]ATP (7,000 Ci/mmol; New England Nuclear, Boston, MA or ICN Pharmaceuticals, Inc., Irvine, CA) and polynucleotide kinase according to the protocol of Maxam and Gilbert (21).

DNA Sequencing. DNA sequences were determined by the method of Maxam and Gilbert (21) and in some cases by the related procedure of Church and Gilbert (22).

Northern Blot Analysis. A431, CEM, THP1, U937, HL60, Jurkat, HT1080 cell lines were obtained from the American Type Culture Collection, Rockville, MD. L428 and U266 cell lines were a gift of Steve Shaw, NIH, Bethesda, MD (23, 24), and the ROHA-9 cell line was obtained from A. Muchmore, NIH (25). RNA from human tissues and from 10^7 cells of all cell lines was prepared by the guanidinium thiocyanate–CsCl method (26). Poly(A) RNA was selected by passage over an oligo(dT)–cellulose column. Aliquots of 10 μg of total RNA or 3 μg of poly(A) RNA were electrophoresed on a 1% agarose-formaldehyde gel, transferred to a GeneScreen membrane (New England Nuclear) and hybridized to the respective ^{32}P-labeled probe, essentially as described (27).

Genomic Southern Blot Analysis. DNA was extracted from the human lymphoblastoid cell line GM1416,48,XXXX, obtained from the Human Mutant Cell Repository, Camden, NJ, as previously described (28). Digestion of 20-μg aliquots of DNA with restriction endonucleases Eco RI, Bam HI, Pvu II, or Pst I, separation of the digested DNA on an agarose gel, transfer to nylon membrane, and hybridization to ^{32}P-labeled cDNA, were performed essentially as described (29).

Results and Discussion

LFA-3 was purified from human erythrocytes by mAb affinity chromatography (6). In SDS-PAGE it migrated as a diffuse band of 45–66,000 M_r (lane *1* in Fig. 1, *A–C*), as previously reported (6). One batch of LFA-3 was subjected to a second affinity chromatography step to remove minor contaminants. No contaminants were detectable by SDS-PAGE, in 10% (Fig. 1*A*) or 15% gels (not shown). This batch of LFA-3 was directly subjected to gas-phase microsequencing. The NH_2-terminal 38 amino acid residues, except for one unidentifiable residue at position 12, were determined (Table I). A second batch of LFA-3 purified by one cycle of affinity chromatography was divided into two aliquots, and *N*-linked

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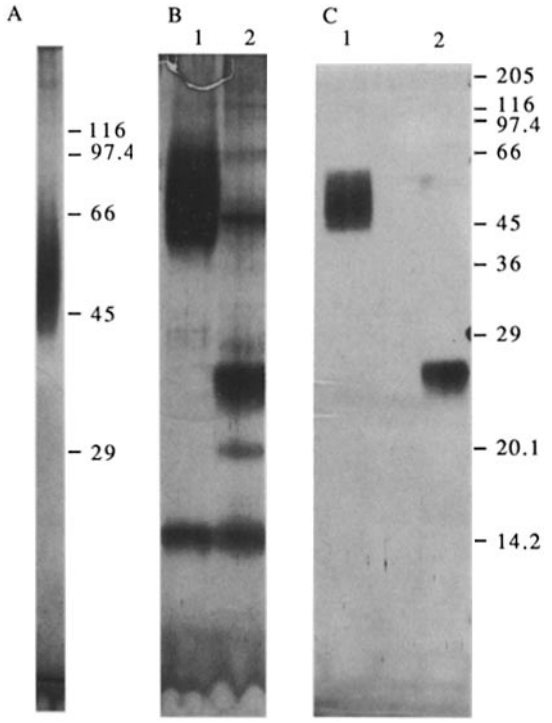

FIGURE 1. Purified LFA-3. (*A*) LFA-3 (150 ng) purified by two cycles of affinity chromatography and run on a SDS 10% PAGE under nonreducing conditions. (*B*) LFA-3 (600 ng) purified by one cycle of affinity chromatography (lane *1*) and the same material treated with *N*-glycanase (lane *2*). SDS 12% PAGE under reducing conditions. (*C*) Same LFA-3 as in B (150 ng) after electroelution from SDS 12% preparative PAGE and run on SDS 12% PAGE under reducing conditions. All samples were visualized by silver staining.

TABLE I

Sequence Data for Human LFA-3 Peptides

Peptide		LFA-3 Protein Sequence
		10 * 20 30
NH_2-Terminus, intact		F S Q Q I Y G V V Y G X V T F H V P S N V P L K E V L W K K Q K D K V A E L
		*
NH_2-Terminus, deglycosylated		X S Q Q I Y G V V Y G D V T F H V P S N V P L
Tryptic peptides:	T72-73	D K V A E L E N S E F
		*
	T91	V L Y D T V S G S L T I Y D L T S
	T105	F F L Y V L E S L P S P T L X X A L
	T68	G L I M Y S

Intact, deglycosylated LFA-3 or tryptic fragments were subjected to NH_2-terminal protein sequence analysis. Tryptic peptide designations correspond to peaks off the HPLC. Amino acid sequences corresponding to oligonucleotide probes are underlined. *N*-glycosylated residues are indicated by asterisks.

carbohydrate was removed from one aliquot with *N*-glycanase. The glycosylated and deglycosylated forms (Fig. 1*B*, lanes *1* and *2*) were purified by preparative SDS-PAGE and electroelution (Fig. 1*C*, lanes *1* and *2*). Deglycosylated LFA-3 ran as a relatively sharp band at 25,600 M_r, but still retained some heterogeneity in M_r (Fig. 1*B*, lane 2; Fig. 1C, lane *2*). The NH_2-terminal sequence of the glycosylated, SDS-PAGE-purified protein was in complete agreement with that for the LFA-3 purified by two cycles of affinity chromatography. The only difference in the sequence of deglycosylated LFA-3 (Table I) was an aspartic

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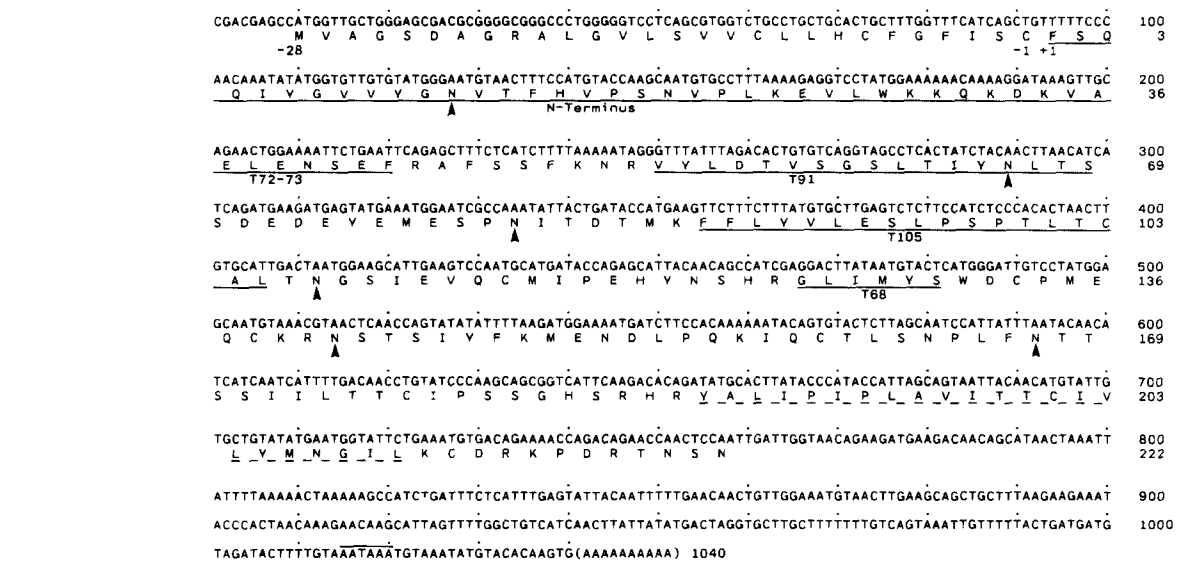

FIGURE 2. DNA sequence and deduced amino acid sequence of human LFA-3 cDNA. The signal sequence is indicated by the numbers −1 to −28. The amino acids of the mature LFA-3 are numbered starting with +1 and continued at the end of each line. Nucleotide numbering is given at the end of each line. Amino acid sequences that correspond to the sequences of the tryptic peptides are underlined with solid lines. Potential *N*-glycosylation sites are indicated by an arrow. The potential transmembrane domain is underlined with a broken line, and the poly(A) addition signal is indicated by a line above the sequence. These sequence data have been submitted to the EMBL/GenBank Data Libraries under accession number Y00636.

acid instead of a blank at position 12, suggesting an *N*-glycosylated asparagine at this residue, which was cleaved by *N*-glycanase to aspartic acid. We also determined the sequence of four tryptic peptides from deglycosylated LFA-3, which were purified by HPLC (Table I).

A series of antisense oligonucleotide probes were chemically synthesized based on the NH_2-terminal amino acid sequence of LFA-3 (Table I, underlined). Because of the high degeneracy of one probe pool, it was synthesized in four subpools (LF2 to LF5). The subpool containing the correct LFA-3 sequence was determined by hybridization of each individual subpool to human tonsil RNA in a Northern blot analysis, as described previously (27). One subpool (LF2) hybridized to the same-sized mRNA (1.3 kb) as did the independent oligonucleotide pool LF1, which suggested that these 2 oligonucleotide pools contained the correct sequence for LFA-3.

The two probe pools were used to screen two cDNA libraries: a size-selected human tonsil λgt11 cDNA library (19) and a λgt10 cDNA library prepared from human PBL. Initially, 26 positive phage were isolated from the PBL library and 12 positive phage from the tonsil library. Two independent isolates from each library were subcloned and their DNA sequences were determined (Fig. 2). Correspondence between the deduced amino acid sequence and LFA-3 NH_2-terminal and tryptic peptide sequences (underlined in Fig. 2) confirmed the identity of the clones.

The 1,040-bp-long sequence of LFA-3 cDNA contains a 5′ untranslated region of 9 bp, followed by an open reading frame of 750 bp (Fig. 2). The 281-bp 3′

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untranslated region contains a consensus polyadenylation signal (Fig. 2, overlined). The first ATG located at 84 bp upstream of the NH_2-terminus of the mature LFA-3 glycoprotein is the probable translational initiation site. This indicates that LFA-3 is synthesized as a 250-amino-acid preprotein containing a hydrophobic signal sequence of 28 residues. The mature protein of 222 amino acids is 24,400 M_r, which is in close agreement with the M_r determined by SDS-PAGE of the deglycosylated polypeptide chain of 25,500 M_r (Fig. 1) or 29,000 M_r (see below).

The cDNA sequence defines a typical membrane bound protein with an NH_2-terminal domain of average hydrophobicity, followed by a highly hydrophobic transmembrane region and a COOH-terminal hydrophilic domain. The NH_2-terminal domain of 188 amino acids contains all six potential *N*-glycosylation sites (Asn-Xaa-Ser/Thr) (Fig. 2). Sequencing of glycosylated and deglycosylated LFA-3 showed that this domain is glycosylated and hence is extracellular. Cleavage by *N*-glycanase of *N*-linked carbohydrates occurs within the amide bond of the asparaginyl moiety, converting the *N*-glycosylated asparagine residue to an aspartic acid residue. NH_2-terminal sequencing showed *N*-glycanase converted position 12 of LFA-3 from a blank (glycosylated asparagines are not identified) to an aspartic acid and the cDNA sequence shows asparagine at this residue. Thus, the LFA-3 protein is *N*-glycosylated at this position. Similarly, the cDNA predicts an asparagine in a potential *N*-glycosylation site at position 66, while the deglycosylated tryptic peptide T91 shows aspartic acid at this position (Table I, asterisk), indicating that this potential *N*-glycosylation site is also used.

Pulse-chase labeling of diverse LFA-3$^+$ cell types (Dustin, M. L., and T. A. Springer, unpublished observations) with [^{35}S]-methionine has revealed, in all cell lines thus far tested, two LFA-3 precursors of M_r 43,000 and 41,000 M_r. Digestion of these precursors, in which the carbohydrate moieties are in the high mannose form, with *N*-glycanase or Endo H reduces their apparent M_r to 29,000 and 25,500, respectively. This indicates a contribution of 14–15,000 M_r by high mannose carbohydrates. An estimate of 2,500 M_r per high mannose carbohydrate (30) predicts six *N*-linked carbohydrates, suggesting that all of the other potential *N*-glycosylation sites are used. The mature LFA-3 glycoprotein is heterogeneous in M_r on individual cell types and varies from 43,000 to 76,000 M_r depending on cell type (Dustin and Springer, unpublished observations). The polypeptide chain of M_r 24,400 defined by the cDNA sequence shows that the mature glycoprotein is 44–68% carbohydrate.

The predicted transmembrane domain consists of 23 uncharged amino acids, most of which are hydrophobic in character. The putative cytoplasmic domain is short, consisting of 12 hydrophilic residues. While this work was in progress we found, corresponding to the two LFA-3 precursors, two forms of mature LFA-3 that differ in membrane attachment as distinguished by their sensitivity to phosphatidylinositol-specific phospholipase C (PIPLC) (31; Dustin, M. L., et al., manuscript in preparation). The high molecular weight precursor remains membrane bound, while the lower molecular weight form is released upon PIPLC treatment, indicating membrane attachment through a PI-glycolipid membrane anchor (32). This suggests that the structural difference between these two proteins resides in the COOH-termini.

Phosphatidylinositol-glycan moiety is attached to the extracellular domain of

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membrane proteins by a transamidation reaction in which a COOH-terminal hydrophobic sequence in the protein is displaced (32). We have determined that the human genome contains a single LFA-3 gene (see below), suggesting generation of two forms at the protein or mRNA levels. Although most PI-anchored proteins have hydrophobic transmembrane precursor sequences with no or insignificant cytoplasmic domains, the "signal" for PI attachment remains undefined (32). It is thus possible that this LFA-3 sequence we have defined is expressed as the membrane-integrated form with the transmembrane domain intact and that it may also act as a precursor for PI attachment. This would generate both forms from a single protein and mRNA precursor. Since PI is added extremely rapidly (32), this is consistent with the detection of two protein precursors at the earliest time points. Alternatively, differential mRNA splicing could generate two distinct transmembrane domains, only one of which is exchanged for the PI anchor, as has been demonstrated recently for N-CAM (reviewed in reference 32).

We therefore examined a number of LFA-3 cDNA clones for differences in their 3′ sequences by Southern blot analysis, and determined the complete DNA sequence of four independent isolates. All clones examined so far had identical 3′ regions. More extensive investigation is required to determine whether the two forms of LFA-3 are coded for by a single or two distinct mRNAs.

LFA-3 is a cell surface molecule with a broad tissue distribution. It has been found in most tissues examined, including most blood cells, epithelial, and endothelial cells. It was of interest to determine the presence and size of LFA-3 transcripts in various tissues and cell lines. The LFA-3 cDNA hybridizes to an ~1.3-kb transcript in RNA extracted from human tonsil, kidney, placenta, and skin. LFA-3 transcripts are also present in HT1080 cells (a human fibrosarcoma cell line), A431 (a human carcinoma cell line), CEM (human acute lymphoblastic leukemia), THP1 (human monocyte leukemia), U937 (human histiocytic lymphoma, U266 (plasmacytoma, B lineage), JY (B lymphoblastoid), L428 (Reid-Sternberg, Hodgkin's), ROHA9 (human Burkitt lymphoma), K562 (erythroleukemia), and Jurkat (T lymphoma). No LFA-3 mRNA could be detected in this subline of HL60 cells (a promyelocytic leukemia cell line) (Fig. 3).

A Southern blot analysis on human genomic DNA indicated that LFA-3 is coded for by a single gene in the human genome. The 220-bp fragment from the 5′ end of the LFA-3 cDNA insert was used as a hybridization probe. This probe contains an internal Pvu II site, and hybridizes to only two fragments of the Pvu II–digested human DNA (Fig. 4), and to only one fragment of the Pst I digest, which indicates a single gene for human LFA-3. The two hybridizing bands of equal intensity in the Bam HI digest and the two bands of unequal intensity in the Eco RI digest predict that the 220-bp probe sequence is interrupted by an intron in the LFA-3 gene.

No significant homologies with LFA-3 were detected by a FASTP search of the National Biomedical Research Foundation Protein (release 12.0) and NEW (release 27.0) databases (33). CD2, the receptor for LFA-3, has recently been cloned in the human and rat (34–37). Homology of short CD2 segments with the Ig superfamily has been reported by some groups (34, 35, 37). It has been proposed that cell–cell adhesion may be mediated by interactions between Ig-

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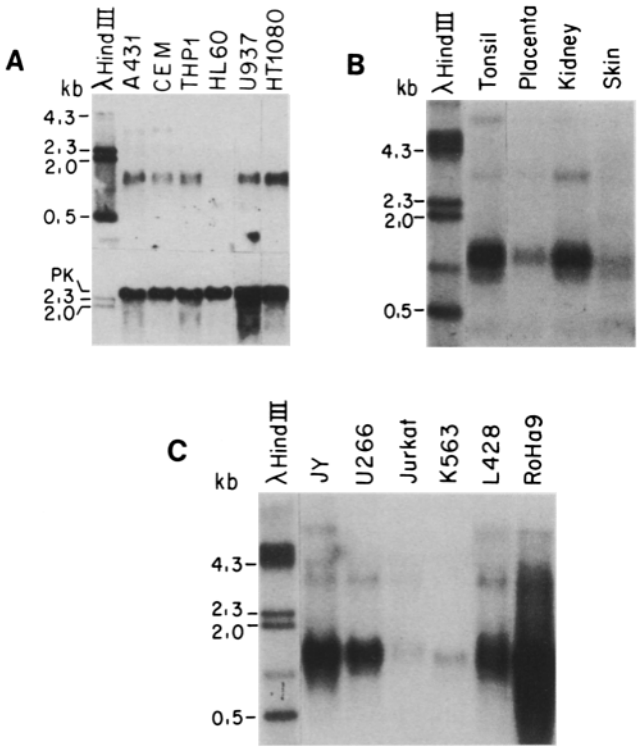

FIGURE 3. Northern blot analysis. RNA from indicated cell sources was separated on 1% formaldehyde-agarose gel and probed with ^{32}P-labeled LFA-3 cDNA. (*A* and *C*) 3 μg poly(A) RNA from HT1080 and ROHA-9 cells and 10 μg total RNA from all other cell lines was applied. (*B*) Human tonsil, kidney, and placenta: 3 μg poly(A) RNA; human skin: 10 μg total RNA. To assure equal loading of RNA in each sample, each Northern blot was also hybridized to ^{32}P-labeled bovine pyruvate kinase cDNA (shown for panel *A* only).

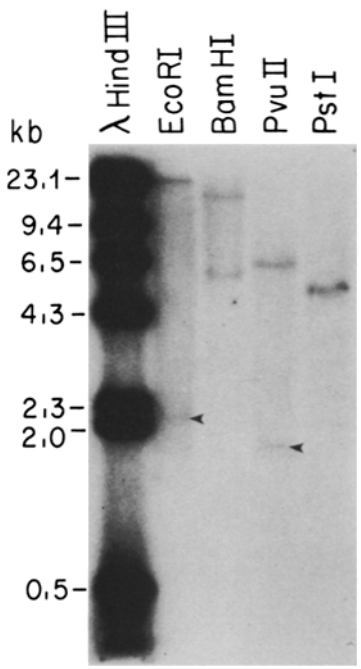

FIGURE 4. Southern blot analysis. Aliquots of 20 μg human genomic DNA was digested with the indicated restriction endonucleases, separated on 1% agarose gel, transferred to a nylon membrane and hybridized to ^{32}P-labeled 220-bp 5′ fragment of LFA-3 cDNA.

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like domains (38). Secondary structure predictions for LFA-3 show β strands containing cysteines that might assume an Ig-like structure.

As mentioned above, CD2 and LFA-3 are the first reported example of two distinct integral membrane glycoproteins that interact with one another to mediate cell adhesion. The isolated cDNA clones will facilitate study of the structural features of LFA-3 and CD2 that are important in this interaction, as well as in signal transduction.

Summary

We have isolated the cDNA for human lymphocyte function–associated antigen 3 (LFA-3), the ligand of the T lymphocyte CD2 molecule. The identity of the clones was established by comparison of the deduced amino acid sequence to the LFA-3 NH_2-terminal and tryptic peptide sequences. The cDNA defines a mature protein of 222 amino acids that structurally resembles typical membrane-anchored proteins. An extracellular domain with six *N*-linked glycosylation sites is followed by a hydrophobic putative transmembrane region and a short cytoplasmic domain. The mature glycoprotein is estimated to be 44–68% carbohydrate.

Southern blots of human genomic DNA indicate that only one gene codes for human LFA-3. Northern blot analysis demonstrates that the LFA-3 mRNA of 1.3 kb is widely distributed in human tissues and cell lines.

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