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LFA-3 ΔD2: A NOVEL *IN VIVO* ISOFORM OF LYMPHOCYTE FUNCTION-ASSOCIATED ANTIGEN 3

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Lymphocyte function-associated antigen 3 (LFA-3) has previously been described as occurring as two isomers *in vivo*, a transmembrane (TM) form and a glycosyl phosphatidylinositol (GPI)-linked form, differing only in their membrane anchoring mechanism. A third isoform, LFA-3 ΔD2, which has the cytoplasmic tail of TM LFA-3 but a truncated extracellular domain, has been identified *in vitro*. We report that the LFA-3 ΔD2 isoform, identified by RT-PCR analysis and DNA sequencing, is also present *in vivo* and appears to share a signal sequence with the TM and GPI isoforms. Expression of LFA-3 ΔD2 was observed in both normal and diseased human buccal mucosa and gingiva. Thus, while specific functional differences between isoforms remain to be established, our results show that LFA-3 ΔD2 is constitutively expressed *in vivo*, along with the other, previously described, isoforms of LFA-3. © 1995 Academic Press, Inc.

Lymphocyte function-associated antigen 3 (LFA-3) is the major naturally occurring ligand for the T lymphocyte receptor CD2 (1). Both of these molecules are members of the immunoglobulin gene superfamily (2) and the genes for both are located on chromosome 1 (3). Two isoforms of LFA-3, generated by alternate splicing of RNA transcripts, have been described previously. These isoforms differ in their membrane anchoring mechanism, but are identical in their extracellular domains. Transmembrane (TM) LFA-3 contains a membrane-spanning domain and short cytoplasmic tail (4), whereas glycosyl phosphatidylinositol-linked (GPI) LFA-3 is anchored within the membrane (5).The cDNA sequence for a third isoform of LFA-3, termed LFA-3 ΔD2, has recently been identified *in vitro* in the human T cell line MOLT-4, (6). Sequence data indicates that this isoform encodes the TM-LFA-3 anchoring region and a truncated extracellular region consisting of only the membrane distal domain, domain 1, of the other isoforms. Both TM and GPI sequences indicate the presence of a signal peptide which is apparently cleaved from the mature protein. The presence of this signal sequence has not previously been reported for the ΔD2 isoform transcript.

Immunohistochemical studies have identified the presence of a number of possibly novel isoforms of LFA-3 in chronically inflamed oral tissues (7). In particular, we have

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observed cytoplasmic LFA-3 expression, without accompanying surface antigen, in a macrophage population within lesional tissue of patients with oral lichen planus (OLP). In addition, substantial levels of an apparently 'soluble' LFA-3, which may be associated with components of the extracellular matrix, are also present in OLP samples. In the present study we have utilized reverse transcriptase polymerase chain reaction (RT-PCR) to examine the LFA-3 isoforms expressed in normal and inflamed oral tissues. DNA sequencing of cloned PCR products has been used to establish unequivocally that transcripts corresponding to LFA-3 ΔD2 are present in these tissues *in vivo*.

MATERIALS AND METHODS

Tissue Samples

Lesional buccal mucosa was obtained from 6 patients with OLP, and samples of gingival tissue were obtained from 4 patients undergoing minor therapeutic surgery for chronic inflammatory periodontal disease (CIPD). Healthy mucosa (n=6) and gingiva (n=4) were obtained from patients undergoing surgical removal of lower third molars.

RNA Extraction and RT-PCR Analysis

Total RNA from tissue samples was extracted using a commercial kit (Stratagene). Reverse transcription of mRNA was performed with Moloney Murine Leukaemia Virus reverse transcriptase using oligo dT primers (Stratagene) and the resulting cDNA used as template for PCR analysis. Primers for PCR were designed, using the published sequences for the three known LFA-3 mRNA transcripts, to give products which could be identified by size as specific for each of the individual isoforms. PCR was carried out as previously described (8) using RedHot *Taq* polymerase (Advanced Biotechnologies) in an Eppendorf Thermocycler, with samples being amplified through 30 cycles. All reaction products were polyA-tailed by a final extension step (72°C for 10min) to facilitate cloning, if necessary. Analysis of products was carried out by agarose and acrylamide gel electrophoresis, followed by ethidium bromide staining which was visualized under UV light.

cDNA Cloning and Sequencing

PolyA-tailed PCR products, using primer pair LFA-3B/3E, from a gingival cDNA sample observed to contain all three LFA-3 isoforms were ligated into the TA pCRII cloning vector (Invitrogen) according to the manufacturers instructions. The ligated vector was electroporated into Top10 F′ *E. coli* and the bacteria grown for 24h at 37°C on Luria Bertani (LB) agar (Oxoid). Colonies containing vector and insert were identified by ampicillin resistance and XGal blue/white selection, respectively. These were screened by PCR using the primers described above. Those colonies producing a product corresponding in size to the LFA-3 ΔD2 insert were grown up in 200ml LB broth, harvested by centrifugation and plasmid DNA isolated using a Quiagen extraction kit. To confirm the insert size 1μg of plasmid DNA was digested with 40U each of the restriction enzymes EcoRV and Hind III (New England Biolabs) at 37°C for 1h and analysed by agarose electrophoresis. The insert sequence was determined for both strands, on an ABI automatic sequencer, by a commercial sequencing company (Rayne Institute, London).

RESULTS

RT-PCR

The predicted PCR product sizes for each isoform, along with the corresponding primer pair, are shown in Table 1(a). Both primer pairs detect the transcripts for all three known LFA-3 isoforms, using the same 5′-3′ inverse oligonucleotide (LFA-3E), with two different 5′-3′ primers distinguishing between the signal sequence (LFA-3S) and the 5′ start of the extracellular domain sequence (LFA-3B). Using the two primer pairs described in Table 1(b),

Table 1. **(a)** PCR oligonucleotide primers and **(b)** predicted sizes of PCR products using each primer pair

(a)	Primer	Orientation	Sequence
	LFA-3E	5′-3′ Inverse	tggagttggttctgtctg
	LFA-3S	5′-3′	cgacgagccatggttgctgg
	LFA-3B	5′-3′	ggtgttgtgtatgggaatgt

(b)	Primer pair	Predicted product size (bp)		
		TM-LFA-3	GPI-LFA-3	LFA-3 ΔD2
	LFA-3B/3E	646	681	381
	LFA-3S/3E	757	792	492

PCR products corresponding in size to the TM and GPI isoforms were observed in all samples examined, as shown in Fig. 1. However, the relative proportion of each isoform, as assessed visually, varied between samples, and showed no correlation with the presence or absence of inflammatory disease.

ΔD2 transcripts of the predicted size were observed in a majority of samples tested (Fig. 1), distributed among both normal and affected gingival and mucosal tissues. Overall, the relative levels of ΔD2 transcript present, again estimated visually, were much lower than that of either of the other isoforms, as shown in Fig. 1. The proportion of ΔD2 relative to the TM and GPI isoforms also varied between samples, and in one sample the ΔD2 product was far more apparent than that of either TM or GPI (Fig. 1, lane ii). However, there was again no apparent relationship between the expression of the ΔD2 isoform and a particular tissue or disease state.

PCR using the primer pair LFA-3S/3E produced bands corresponding to the predicted sizes for the three isoforms (Fig. 1). This indicates, firstly, that the amplified LFA-3 ΔD2 transcripts contain a sequence homologous to the known LFA-3 signal sequence defined by primer LFA-3S and, secondly, that the size of the resulting PCR product is precisely that predicted for a ΔD2 transcript containing a signal sequence of equal length to that described for the other LFA-3 transcripts.

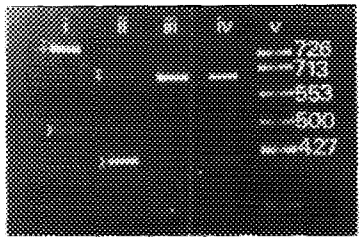

Figure 1. Acrylamide (6%) electrophoresis analysis of RT-PCR products stained with ethidium bromide and visualized under UV illumination. Isoforms are indicated as follows: ~, GPI; +, TM; >, ΔD2.
Lanes show: **(i)** RT-PCR products, corresponding to all three isoform transcripts, detected using primer pair LFA-3S/3E in normal buccal mucosa; **(ii)** RT-PCR products detected using primer pair LFA-3 B/E in a sample of normal buccal mucosa showing ΔD2 as the major isoform transcript; **(iii, iv)** RT-PCR products detected using primer pair LFA-3 B/E in samples of normal buccal mucosa and OLP lesional mucosa, respectively; **(v)** Phi X174/HinfI DNA size markers.

Cloning and Sequencing

PCR analysis of plasmid- and insert-containing bacterial colonies identified particular clones which were positive for the ΔD2 isoform. Restriction enzyme digestion analysis of the prepared plasmid DNA, as described in the materials and methods, confirmed that the insert was of the expected size (data not shown). As shown in Table 2, sequencing resulted in a 358bp sequence which, along with the 119 residue amino acid sequence derived from the cDNA, were completely homologous with the aligned region of the reported GenBank ΔD2 cDNA and amino acid sequences (6).

DISCUSSION

The present results show that all three known isoforms of LFA-3 are expressed in human tissue *in vivo*, and that the ΔD2 isoform identified in this study is homologous with the isoform previously reported only *in vitro*. Although a full length cDNA sequence has yet to be obtained for the isoform we have identified *in vivo*, its homology with the ΔD2 cDNA sequence previously reported in the human T cell line MOLT4 (6) is 100%, including the region of the transcript at which there is divergence from the sequences of the TM- and GPI-LFA-3 transcripts.

The ΔD2 isoform, despite lacking a large extracellular region, appears to be functionally complete. The sheep homologue of LFA-3 ΔD2 has been cloned into a bacterial expression system and has been shown to be functional in both inhibition of E-rosetting (9) and an immunosuppression assay (10). Immobilized sheep LFA-3 ΔD2 also specifically binds CD2+ve T cells and is capable of providing co-stimulatory activation signals to these cells (11). These findings implicate the distal domain (domain 1) of LFA-3 as the functionally active

Table 2

(a)

1	gtgtatggga	atgtaacttt	ccatgtacca	agcaatgtgc	ctttaaaaga
51	ggtcctatgg	aaaaaacaaa	aggataaagt	tgcagaactg	gaaaattctg
101	aattcagagc	tttctcatct	tttaaaaata	gggtttattt	agacactgtg
151	tcaggtagcc	tcactatcta	caacttaaca	tcatcagatg	aagatgagta
201	tgaaatggaa	tcgccaaata	ttactgatac	catgaagttc	tttctttatg
251	tgcttggtca	ttcaagacac	agatatgcac	ttattcccac	cccattagca
301	gtaatcacaa	catgtattgt	gctgtatatg	aatgggattc	tgaaatgtga
351	cagaaaac				

(b)

1 50
VYGNVTFHVPSNVPLKEVLWKKQKDKVGELENSEFRAFSSFKNRVYLDTV

51 100
SGSLTTYNLTSSDEDEYEMESPNITDTMKFFLYVLGHSRHRYALIPIPLA

101 119
VITTCIVLYMNGILKCDRK

(a) 358bp cDNA sequence corresponding to the internal region of human LFA-3 ΔD2 mRNA flanked by primers LFA-3B and LFA-3E; **(b)** Predicted internal 119 residue amino acid sequence derived by alignment of ΔD2 cDNA sequence with known cDNA sequences of both human TM-LFA-3 and MOLT-4 LFA-3 ΔD2.

region of the molecule, in agreement with epitope studies of human LFA-3 which have shown domain 1 to be essential for T cell activation via LFA-3 (12).

Although no differences in biological activity have yet been reported between the three isoforms, the membrane anchoring mechanism may be an important feature in determining functional specificities. This is based on the view that GPI-LFA-3, in common with other GPI-linked proteins such as Thy-1 (13) and alkaline phosphatase (14), has a far greater diffusion rate within the membrane than TM-LFA-3 (15, 16), which has been described as immobile (16). GPI-LFA-3 may, therefore, be more closely involved than are the other isoforms in the regulation of cell-cell adhesion reactions, since it can move more rapidly to areas of cell-cell contact. In contrast, because of its cytoplasmic tail, the TM form is far more likely to be involved in signal transduction pathways, both for T cell activation via CD2 and perhaps also for stimulation of apposing, non-T, cells such as B-cells, fibroblasts or keratinocytes via the LFA-3 molecule itself.

The present study is the first report of LFA-3 ΔD2 *in vivo*. Structurally, this isoform lacks the extracellular domain proximal to the membrane which is present in the TM and GPI forms. However, this domain has been shown, using recombinant human LFA-3 and monoclonal antibodies directed against specific epitopes, to be uninvolved in CD2/LFA-3 binding, although it may be involved in some T cell activation (12) and may have limited involvement in E-rosette formation (12). Thus, while the lack of the proximal domain would be expected to have only minimal effect on the function of LFA-3 ΔD2, the structure of LFA-3 ΔD2 places the major functional (distal) domain closer to the cell membrane. This change may allow this isoform to establish a closer, and perhaps more effective, region of cell-cell contact which may enhance signal transduction through adhesion-dependant pathways. The novel structure of this LFA-3 isoform might also result in an altered conformational structure during cell interactions, differing from that of the two other isoforms and possibly allowing 'alternative' activation pathways to be utilized.

In preliminary Southern blot analysis experiments, carried out using the cloned and sequenced ΔD2 PCR product as a probe, we have found that the ΔD2 isoform is expressed in all samples, including those in which no ΔD2 transcript was detected by PCR analysis alone. This observation strongly suggests that transcripts for all three currently known isoforms of LFA-3 are expressed constitutively in both normal and diseased tissue. However, these findings do not preclude the possibility that there could be an important relationship between the regulation of the relative levels of each type of LFA-3 transcript and inflammatory pathologies, particularly if different functional activities are indeed associated with each of the isoforms.

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