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AN ALTERNATELY SPLICED mRNA ENCODING FUNCTIONAL DOMAINS OF MURINE MADCAM-1

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SUMMARY: cDNA clones representing a small (0.8 kb) form of murine mucosal addressin cell adhesion molecule-1 (MAdCAM-1) mRNA were obtained and sequenced. The sequence was identical to the published 1.6 kb murine MAdCAM-1 cDNA sequence, except that 432 nucleotides encoding the mucin-like and IgA-homologous portions were deleted. This cDNA most likely represents an alternately spliced mRNA. Substantial amounts of both the short and long MAdCAM-1 mRNAs are present in murine mesenteric lymph node. Ig fusion proteins displaying either the short or long forms of MAdCAM-1 can bind Mn^{++} -activated JY cells bearing human $\alpha 4\beta 7$ integrin, indicating that the two N-terminal Ig-like domains of MAdCAM-1 are sufficient to bind its integrin counter-receptor.

Mucosal addressin cell adhesion molecule-1 (MAdCAM-1) is a murine glycoprotein of approximately 58-66 kD. It was originally identified as a homing receptor on endothelial cells in gut lymphoid tissue. MAdCAM-1 binds the integrin receptor $\alpha 4\beta 7$ on lymphocytes and presumably assists their normal recirculation between lymphoid tissue and the bloodstream (1-3). The nucleotide sequence of a 1.6 kb murine MAdCAM-1 cDNA predicts a cell-surface protein of 384 amino acids (aa) consisting of two N-terminal Ig-like domains with homology to VCAM-1 and ICAM-1, a serine/threonine-rich mucin-like region, a membrane-proximal Ig-like domain with homology to IgA1, a hydrophobic transmembrane region, and a short cytoplasmic tail (4). We will refer to this protein as MAdCAM-384aa or long MAdCAM-1. In the same report a smaller mRNA of 0.8 kb was visible, in amounts approximately equal

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Abbreviations: aa, amino acid(s); bp, base pair(s); ICAM-1, intercellular adhesion molecule-1; Ig, immunoglobulin; kb, kilobase(s) or 1000 bp; MAdCAM-1, mucosal addressin cell adhesion molecule-1; MLN, mesenteric lymph node; PCR, polymerase chain reaction; RT, reverse transcription; VCAM-1, vascular cell adhesion molecule-1.

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to the 1.6 kb form, in Northern blots of murine Peyer's patch and mesenteric lymph node (MLN) RNA probed with MAdCAM-1 cDNA at high stringency. Here we report the cloning of a 0.8 kb cDNA whose sequence is identical to the 1.6 kb cDNA except for the absence of 432 nucleotides encoding the mucin-like and third Ig-homologous domains. This sequence predicts a cell-surface protein of 240 aa, which we call MAdCAM-240aa or short MAdCAM-1. Immunoglobulin fusion constructs displaying either short or long MAdCAM-1 extracellular regions are capable of binding the counter-receptor integrin $\alpha4\beta7$ on JY cells in the presence of Mn^{++}, indicating that the two N-terminal Ig-like domains are sufficient for integrin-mediated adhesion.

MATERIALS AND METHODS

RNA purification and RT-PCR Poly A^+ RNA was prepared from pooled Balb/c mouse mesenteric lymph nodes with QuickPrep Micro mRNA Purification Kit (Pharmacia, Piscataway, NJ). Reverse transcription (RT) was carried out with 100 ng of poly A^+ RNA and 15 pm of 3' antisense primer, MAD-16, as described (5). Polymerase chain reaction (PCR) was accomplished in 100 µl final volume with half of the RT reaction, 15 pm each of the same 3' primer and a 5' sense primer, MAD-5, 0.2 mM dATP, dCTP, dGTP and dTTP, *Pfu* polymerase and *Pfu* buffer (Statagene, La Jolla, CA), and incubated in a Perkin-Elmer Cetus thermal cycler for 30 cycles at 94°C 1 min, 55°C 1 min, and 72°C 3 min. MAD-5 and MAD-16 each contain a *Not* I site.

Cloning and nucleic acid sequencing To amplify fragments, secondary PCR was performed with gel purified (GENECLEAN, Bio101, La Jolla, CA) RT-PCR products, as above. The secondary products were gel purified, digested with *Not* I, and cloned into CDM8 vector (6) which had been modified with *Not* I/*Bst*XI adaptors. MAdCAM-1 sequences, from plasmids pSML101 (long), and pSML8 (short), were confirmed by cycle sequencing (fmol DNA Sequencing System, Promega, Madison, WI). Sequence analysis was carried out using the GCG Package (Genetic Computing Group, Madison, WI).

MAdCAM-Ig MAdCAM-Ig fusion constructs were made by combining the N-terminal extracellular portions of long or short MAdCAM-1 with a C-terminal Fc portion of human IgG1 including the hinge plus C_H2 and C_H3 regions, as described for another fusion protein (7). The MAdCAM-1 sequences were obtained by PCR using template plasmids pSM101 (long) or pSML8 (short), with primers 362-15 and 362-16, a 3' antisense primer which added a stop codon and Sal 1 site 5' to the transmembrane codons. The short and long MAdCAM-Ig cDNAs were cloned into plasmid CDM8, sequenced, and expressed in COS7 cells as described (8).

RT-PCR and Southern analysis PCR with internal primers, MAD-2 and 370-607, was performed to demonstrate the presence of long and short MAdCAM-1 mRNAs in murine mesenteric lymph nodes. RT and PCR were carried out as above, except *Taq* polymerase, *Taq* buffer (Perkin-Elmer, Norwalk, CT), and 2 mM $MgCl_2$ in 50 µl volumes were utilized. Positive control reactions employed 5 ng of plasmids SML101 or SML8 as template. A 1% agarose gel in TAE buffer containing 0.25 ug/ml ethidium bromide was loaded with 25 µl of each RT-PCR reaction and no-template negative control and 2.5 µl of positive controls. The gel was photographed using an Eagle Eye II Still Video System (Stratagene), and equilibrated in 0.5 M NaOH, 1.5 M NaCl. The

DNA was transferred to BIOTRANS nylon membrane (ICN, Costa Mesa, CA) by capillary action. The membrane was UV cross-linked and hybridized at 45°C in PSB (50 mM Tris pH 7.5, 1 M NaCl, 0.1% NaPyrophosphate, 1% SDS, 10x Denhardt's solution (9) plus 10% dextran sulfate and 100 ug/ml tRNA. The membrane was hybridized sequentially with ^{32}P-labeled internal oligomer probes, MAD-21 which specifically hybridizes to the short form, and 370-606 which recognizes both long and short forms. The membrane was then washed with PSB at 50°C and subjected to autoradiography. The membrane was stripped for re-hybridization by submerging in boiling water and allowing to cool to room temperature.

Oligomers Following are sequences of the oligomers described above:

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MAD-2    5'-TAG GTG TGT ACA TGA GCT AGT GTC TGG GCG-3'
MAD-5    5'-AGA GGC AGG CGC GGC CGC ATG GAA TCC ATC CTG GCC CT-3'
MAD-16   5'-TAA TGG AGC TGC GGC CGC TCA TAG GTG TGT ACA TGA GCT-3'
MAD-21   5'-GAC GGT GGA GGC TGG AAT CTC C-3'
362-15   5'-AAT TGT CGA CGC GGC CGC ATG GAA TCC ATC CTG GCC-3'
362-16   5'-TTT TGT CGA CTG GAA TCT CCT TTC TAT GGG T-3'
370-607  5'-AGA AGA GGA GAT ACA AGA GG-3'
370-606  5'-CAG CGT TGT GTC ATT CGG AA-3'
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Adhesion assay COS7 supernatants were harvested 72 hours after electroporation with plasmids encoding short and long MAdCAM-Ig constructs, and MAdCAM-Ig protein was quantitated by Fc ELISA as described (8). JY, a human B lymphocytic line, was used to assay adhesion; the subline used expresses integrin $\alpha4\beta7$ but not $\alpha4\beta1$ (10, and data not shown). MAdCAM-Ig supernatants were titered from 3 μg/ml to 0.0078 μg/ml in Blotto (5% milk, 0.1% Tween-20 in PBS) on plates coated at 5 μg/ml with goat anti-human IgG (Fc). Plates were then blocked for 30 minutes with 0.5% human serum in Blotto. JY cells were labeled with BCECF-AM (Molecular Probes, Junction City, OR) at 2 μM for 30 min at 37°C in TBS (24 mM TrisCl, 137 mM NaCl, 2.7 mM KCl, 10 mM HEPES, 0.1% BSA, 2 mM glucose, pH 7.4), and washed 1x in TBS. Cells were then resuspended at 1 x 10^6 cells/ml, and activated with 1 mM $MnCl_2$ (11) in the presence or absence of the blocking $\alpha4$ integrin monoclonal antibody (mAb) HP1/2 (12) for 10 minutes. 1 x 10^5 cells/well were added to MAdCAM-Ig coated plates in triplicate and incubated for 30 min at room temperature. Following 3 washes with TBS, fluorescence was read at 485/530 nm using a Cytofluor 2350 plate reader (Millipore Corp., Bedford, MA).

RESULTS AND DISCUSSION

By performing RT-PCR using murine mesenteric lymph node mRNA as template and synthetic DNA oligomers as primers, we have identified and cloned an 0.8 kb MAdCAM-1 cDNA which presumably represents the 0.8 kb mRNA species previously observed by northern blot (4). Sequence analysis of 2 independent clones indicate that this mRNA is identical to the published 1.6 kb species (4), except that 432 nucleotides encoding the mucin-like and IgA-homologous domains have been deleted. The deletion leaves intact a reading frame predicted to encode a cell-surface protein of 240 aa (MAdCAM-240aa or short MAdCAM-1), which comprises the two N-terminal Ig-like domains, transmembrane region and cytoplasmic tail of previously published MAdCAM-1 (MAdCAM-384aa or long MAdCAM) (Fig. 1). There is 100% nucleotide sequence identity within the retained domains, and the deletion occurs at

A

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ATGGAATCCATCCTGGCCCTCCTGCTGGCCCTGGCCCTAGTACCCTACCAGCTCAGCAGA    60
M  E  S  I  L  A  L  L  L  A  L  A  L  V  P  Y  Q  L  S  R     20

GGACAGTCCTTCCAGGTGAACCCCCCTGAGTCTGAGGTAGCTGTGGCCATGGGCACATCC   120
G  Q  S  F  Q  V  N  P  P  E  S  E  V  A  V  A  M  G  T  S     40

CTCCAGATCACCTGCAGCATGTCCTGTGACGAGGGTGTAGCCCGGGTGCACTGGCGTGGT   180
L  Q  I  T  C  S  M  S  C  D  E  G  V  A  R  V  H  W  R  G     60

CTGGACACCAGCTTGGGCAGTGTACAGACCCTCCCAGGCAGCAGTATCCTCTCTGTACGG   240
L  D  T  S  L  G  S  V  Q  T  L  P  G  S  S  I  L  S  V  R     80

GGCATGCTGTCAGACACAGGCACTCCTGTGTGTGTGGGCTCCTGCGGGAGTCGAAGCTTC   300
G  M  L  S  D  T  G  T  P  V  C  V  G  S  C  G  S  R  S  F    100

CAGCACTCCGTGAAGATCCTTGTGTATGCCTTCCCAGACCAGCTGGTGGTGTCCCCGGAG   360
Q  H  S  V  K  I  L  V  Y  A  F  P  D  Q  L  V  V  S  P  E    120

TTCCTTGTACCTGGACAGGACCAGGTGGTGTCCTGCACGGCCCACAACATCTGGCCTGCA   420
F  L  V  P  G  Q  D  Q  V  V  S  C  T  A  H  N  I  W  P  A    140

GACCCGAACAGTCTCTCCTTTGCCCTGCTACTGGGAGAGCAGAGACTGGAGGGTGCCCAA   480
D  P  N  S  L  S  F  A  L  L  L  G  E  Q  R  L  E  G  A  Q    160

GCCCTGGAACCAGAGCAAGAAGAGGAGATACAAGAGGCTGAGGGCACACCACTGTTCCGA   540
A  L  E  P  E  Q  E  E  E  I  Q  E  A  E  G  T  P  L  F  R    180

ATGACACAACGCTGGCGGTTACCCTCCCTGGGGACCCCTGCCCCTCCTGCCCTTCACTGC   600
M  T  Q  R  W  R  L  P  S  L  G  T  P  A  P  P  A  L  H  C    200

CAGGTCACCATGCAGCTGCCCAAACTGGTGCTGACCCATAGAAAGGAGATTCCAGCCTCC   660
Q  V  T  M  Q  L  P  K  L  V  L  T  H  R  K  E  I  P  A  S    220

ACCGTCGTCCTATGGATTGGCAGCTTGGTGCTGGGGCTGCTTGCACTGGTCTTCCTTGCC   720
T  V  V  L  W  I  G  S  L  V  L  G  L  L  A  L  V  F  L  A    240

TACCGCCTGTGGAAATGCTACCGGCCAGGTCCTCGCCCAGACACTAGCTCATGTACACAC   780
Y  R  L  W  K  C  Y  R  P  G  P  R  P  D  T  S  S  C  T  H    260

CTATGA  786
L  *    262
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B

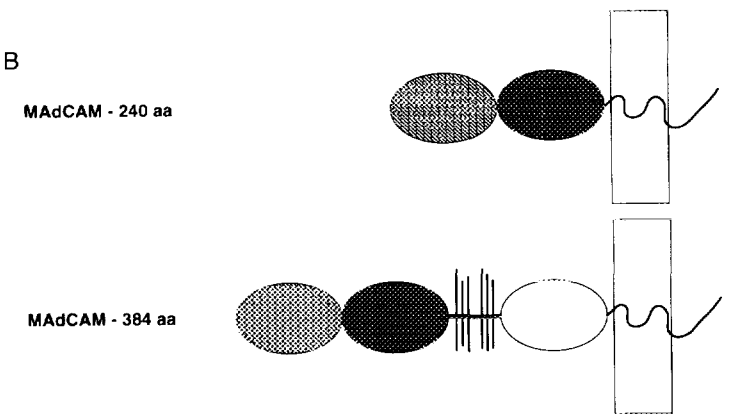

Figure 1. Sequence and schematic diagram of the 0.8-kb MAdCAM cDNA. **A.** Sequence of cDNA and predicted protein sequence encoded by the 0.8-kb RT-PCR product from murine mesenteric lymph node RNA, MAdCAM-240aa or short MAdCAM-1. Predicted signal peptide and transmembrane sequences are underlined (after ref. 4). Cysteines expected to be involved in disulfide bonds by analogy to VCAM-1 are boxed (17,18). The putative site of alternate splicing, compared to the 1.6-kb cDNA previously reported (4) is indicated by an arrowhead after nucleotide 655. **B.** Schematic diagram of short (MAdCAM-240aa) and long (MAdCAM-384aa) forms of MAdCAM-1 showing Ig-like domains as ovals and the mucin-like region as a horizontal line, with its carbohydrate moieties (probably including the ligand for L-selectin, ref. 16) shown as vertical lines.

the borders of functional domains (which are generally encoded by separate exons). This strongly suggests that the 0.8 kb mRNA is a product of alternate splicing from the same gene transcript that gives rise to the longer 1.6 kb form .

Murine mesenteric lymph node (mMLN) mRNA was analyzed by RT-PCR using internal primers designed to generate a 717 bp fragment from the 1.6 kb template, and a 285 bp fragment from the 0.8 kb template (oligomers MAD-2 and 370-607). Agarose gel electrophoresis revealed fragments of the appropriate size, plus the expected inter-fragment hybrid of intermediate mobility (resulting from annealing of one strand from the long fragment with the complementary strand from the short fragment) (Fig. 2a).

To confirm the identity of these RT-PCR fragments, a 22 nucleotide oligomer probe straddling the deletion, MAD-21, was synthesized. This probe does not hybridize with plasmid encoding the long form (Fig 2b, lane 2), indicating that the probe is specific for the short 0.8 kb alternately spliced form, as expected. Southern blot analysis of the internal RT-PCR fragments using MAD-21 confirms that the 285 bp RT-PCR product represents an mRNA spliced as indicated by the sequence of the 0.8 kb cDNA (Fig. 2b, lane 1). MAD-21 also recognizes the inter-fragment hybrid, as expected (Fig. 2b, lane 1). A probe recognizing sequences common to the long and short forms, 370-606, hybridized to all three fragments, as expected (Fig. 2c).

By analogy to the related integrin-binding Ig superfamily molecules ICAM-1 and VCAM-1, it seemed likely that the two N-terminal Ig-like domains contain the functional integrin binding sites of MAdCAM-1 (13-15). Two fusion proteins were constructed, including the extracellular domains of short or long MAdCAM-1 fused to a human IgG1 hinge and constant (Fc) region. Each of these proteins is capable of

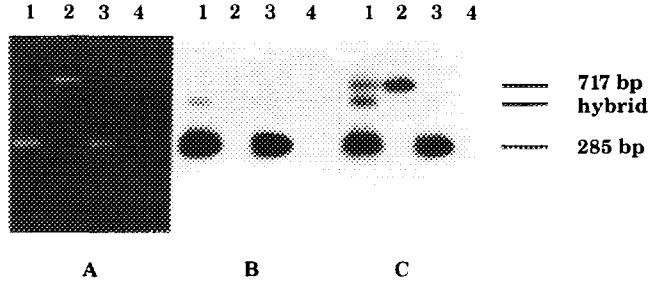

Figure 2. Southern analysis of RT-PCR confirming presence of long and short MAdCAM-1 mRNAs in murine MLN. **A.** Agarose gel of RT-PCR using internal primers 370-607 and MAD-2 and the following templates, starting from the left lane: 1) murine MLN poly A^+ RNA, 2) pSML101, 3) pSML8, 4) no template. **B.** Southern blot of gel from A, probed with MAD-21 oligo, sequence straddling the splice site. **C.** Southern blot in (B) stripped and re-probed with 370-606 oligomer, from second Ig-like domain.

binding to Mn^{++}-activated JY cells (Fig. 3). We used a subline of this B lymphocytic line which bears $\alpha4\beta7$ but not $\alpha4\beta1$ integrin (10, and data not shown). Binding to short or long MAdCAM-Ig is completely inhibited by anti-$\alpha4$ integrin monoclonal antibody HP1/2 (Fig.3). A control Ig fusion protein, LFA3-Ig, does not bind to these cells (Fig.3). Therefore, the two N-terminal domains of MAdCAM-1 are necessary and sufficient for binding to $\alpha4\beta7$ integrin.

In contrast to the integrin binding site, which is present on both long and short forms of MAdCAM-1, it is thought that the carbohydrate portion of the molecule which binds to L-selectin is presented on the mucin-like segment (16), whose coding sequence is not present in the short 0.8 kb mRNA. These differences in counter-receptor recognition imply that the two forms may function somewhat differently in leukocyte recruitment. It remains to be seen if the 0.8 kb mRNA species reported here gives rise to functional protein in vivo, and if so to identify differences in physiological synthesis and function of the two MAdCAM-1 forms.

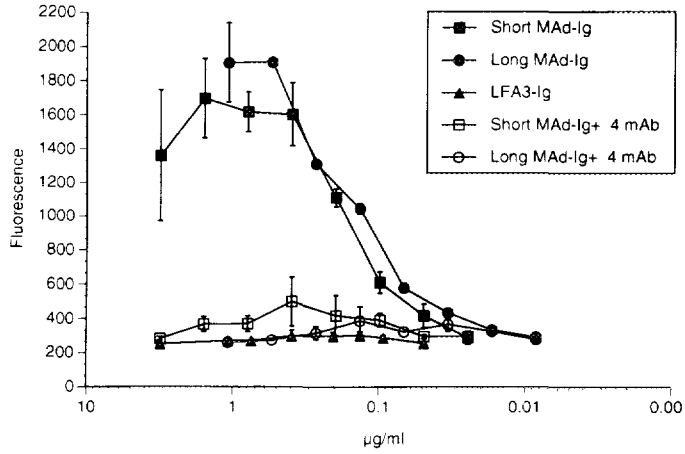

Figure 3. Adhesion of JY cells to the MAdCAM-Ig fusion proteins. Extracellular domains of the two alternately spliced forms of MAdCAM-1 fused to human IgG1 Fc made in COS7 cells were captured onto 96 well plates, and adhesion of fluorescently labeled JY cells bearing counter-receptor integrin $\alpha4\beta7$ was determined (see Materials and Methods for details). X-axis: µg/ml of fusion protein added to wells during coating. Y-axis: relative number of labeled JY cells which adhered to each well, in arbitrary units of fluorescence. Short MAd-Ig, extracellular domains of MAdCAM-240aa fused to IgG1 Fc; Long MAd-Ig, extracellular domains of MAdCAM-384aa fused to IgG1 Fc; LFA3-Ig, N-terminal domain of LFA-3 (CD58) fused to IgG1 Fc; $\alpha4$ mAb, 10 µg/ml of blocking anti-human $\alpha4$ integrin mAb HP1/2 was added to JY cells prior to and during adhesion assay. 1 mM $MnCl_2$ was present in all assays. Each point represents the mean $\pm$ standard deviation of triplicate determinations in a representative experiment.

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