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The bovine (*Bos taurus*) CD11a-encoding cDNA: molecular cloning, characterisation and comparison with the human and murine glycoproteins

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Abstract

The bovine cDNA encoding CD11a, a cell-surface glycoprotein involved in multiple leukocyte functions, was sequenced and compared with the human and murine sequences. Despite some focal differences, it shares all the main characteristics of its known mammalian homologs. Along with the bovine CD18-encoding cDNA, which is available for a long time, the sequence data provided here will allow the successful expression of bovine CD11a, thus giving the first opportunity to express the *Bos taurus* β_2-integrin CD11a/CD18 (LFA-1, $\alpha_L\beta_2$) *in vitro* as a tool to examine the specificities of inflammation in the bovine species.

Keywords: Integrins; LFA-1; ICAM; Adhesion; Cattle

1. Introduction

CD11a/CD18 (LFA-1, $\alpha_L\beta_2$) is a member of the integrin family of cell surface receptors. Integrins consist of a 120- to 180-kDa α subunit (CD11a in this case) and a 90- to 110-kDa β subunit (CD18 or β_2) that are noncovalently associated single-pass transmembrane proteins (Springer, 1990). The bulk of each integrin subunit is extracellular, where it typically functions as a receptor for extracellular matrix molecules or as a counterreceptor for surface proteins of apposed cells (Hynes, 1992). The heterodimer CD11a/CD18 is expressed on all leucocytes and mediates high affinity adhesion to a variety of cell types that express one or more of the β_2-integrins ligands, intercellular adhesion molecules (ICAM-1 to -5) (Bailly et al., 1995; Gahmberg, 1997; Tian et al., 1997). The adhesion process mediated is a critical step of a wide range of immunological activities, including cytolysis of target cells, cross-interaction and cross-stimulation between lymphocytes, phagocytosis of complement-coated targets, and the regulation of leukocyte traffic between the bloodstream and tissues. In this context, increasing our knowledge about bovine β_2 integrins is of great importance to offer new possibilities for research in the field of the pathogenesis of pneumonia and mastitis, i.e., the two leading causes of production losses to the cattle industry throughout the world.

The *Bos taurus* CD18 (β_2) subunit has been well characterised (Shuster et al., 1992), which is not the case of its partner into the LFA-1 heterodimer, CD11a. The purpose of this paper is to report the cloning and sequencing of a cDNA encoding bovine CD11a, along with a comparative sequence analysis with its human and murine homologs.

2. Materials and methods

2.1. RNA isolation

Total RNA from phorbol myristate acetate (PMA)-stimulated (25 ng/ml for 15 min) BL-3 cell line (ATCC No. CRL-8037) was extracted with TRIzol (Invitrogen) as described by the manufacturer. The BL-3 cell line was maintained in Dulbecco's modified Eagle's medium supplemented with nonessential aminoacids (aa), 20% fetal bovine

Abbreviations: BL-3, bovine lymphoblast; bo, bovine; hu, human; ICAM, intercellular adhesion molecule; kan, kanamycin; LFA, lymphocyte function-associated antigen; mu, murine; PMA, phorbol myristate acetate; RACE, rapid amplification of cDNA ends.

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serum (Gibco BRL), penicillin (100 U/ml) and streptomycin (100 μg/ml) at 37°C in a 5% CO_2 incubator.

2.2. Amplification of cDNA ends

We used SMART RACE technology (Clontech Laboratories) to clone bovine CD11a (BoCD11a) 5′ - and 3′ - ends and RT-PCR to clone full-length BoCD11a CDS. For first-strand cDNA synthesis, and according to the partial sequence of BoCD11a available (GenBank No. AF440778), gene-specific primers were designed, which were expected to give nonoverlapping ~ 1 kb RACE products: a sense primer for the 3′ -RACE PCR: 5′ -GGCTGAGTCTTGCTCGTTTG-GAACC-3′ (corresponding to nt 3071–3095) and an antisense primer for the 5′ -RACE PCR: 5′ -CAGGTCTGGCCG-GAACACTTCTTTC-3′ (nt 794–770). Reverse transcription and polymerase chain reactions (PCR) were carried out according to the instruction manual of the SMART RACE cDNA Amplification Kit. Two 5′ - and two 3′ -RACE products were obtained and gel-purified using the Qiaquick Gel Extraction Kit (Qiagen). After the four fragments were TA-cloned into pCRII-TOPO (Invitrogen), seeded on kanamycin IPTG plates, and miniprep were obtained from colonies grown in 5 ml LB-Kan broth, the clones were sequenced on the ABI-3100 Genetic Analyzer using the Big Dye terminator chemistry.

2.3. Molecular cloning of full-length cDNA

Total RNA from PMA-stimulated BL-3 cell line was reverse transcribed using Improm II (Promega). The full-length cDNA was then generated by long distance PCR using Elongase amplification technology (Invitrogen) with primers designed from the distal ends of both 5′ - and 3′ -RACE products: CCCAAGAGCCCTCTGAGACT (sense, within the 5′ -UTR) and GCACCTCAATCTCCACCACT (antisense, 3′ -UTR). The procedures recommended by the manufacturer were followed, with the following cycling parameters: 2 min 30 s at 94 °C, then 35 cycles including: (i) 30 s at 94 °C, (ii) 30 s at 56 °C, and (iii) 3 min 30 s at 68 °C, followed by a final extension at 68 °C for 10 min. Resulting PCR products were then processed for sequencing as aforementioned for the RACE products. The CD11a cDNA sequence was deduced from sequences obtained from five independent clones. Sequence data have been deposited at GenBank under accession No. AY 267467. Homology analyses were carried out using FASTA and alignment of amino acids sequences was drawn by Genedoc (v. 2.6.002).

3. Results and discussion

3.1. Characterisation of BoCD11a-encoding cDNA and deduced aa sequence

The cDNA sequence for BoCD11a contains ~ 4560 bp with an ORF of ~3498 bp that codes for 1165 aa followed by ~900 bp in the 3′ -UTR (Fig. 1). The mature BoCD11a contains a 23-aa putative leader peptide, an extracellular domain of 1061 residues (24–1084), a single hydrophobic transmembrane region of 24 residues (1085–1108) and a cytoplasmic tail of 57 residues (Fig. 1). Eight *N*-linked putative glycosylation sites (Asn-X-Thr/Ser) are present in the extracellular domain (Fig. 2). The mature protein contains 22 cysteine residues, among which 2 are located into the cytoplasmic tail (Fig. 2). The extracellular domain also contains an inserted (I) domain of 172 amino acids (residues 153–324) quite similar to those found in all the leukocyte integrin α subunits sequenced to date. These I domains are homologous with repeated domains in von Willebrand factor and cartilage matrix protein (Springer, 1990). Three repeats with a divalent cation binding motif are found within the I domain at amino acid residues 465–473, 527–535 and 587–595 (Fig. 2). All the cysteines and all but one of *N*-glycosylation sites are found outside the I region and divalent cation binding motifs (Fig. 2), consistent with the hypothesis that these regions may undergo conformational changes important in ligand binding (Rothlein and Springer, 1986; Larson et al., 1989).

3.2. Comparison among species

Overall, the general organization of bovine, human (Larson et al., 1989) and murine (Kaufman et al., 1991) CD11a proteins is quite similar (Fig. 2). Comparison of sequence homology between BoCD11a and its human and murine counterparts shows overall 77% and 68% identity, respectively, with the highest identity for the cation binding motifs and the lowest identity for the cytoplasmic domains (Table 1). The high conservation of the putative cation binding motifs is consistent with an involvement of these regions in the functional activity of LFA-1 α subunit, as suggested by the requirement of Mg^{2+} and Ca^{2+} for CD11a/CD18-dependent cellular interactions (Rothlein and Springer, 1986) or binding to purified ICAM-1 (Dustin and Springer, 1989). By contrast, the low conservation of the cytoplasmic tail suggests that it is

Table 1
Between-species percent identities of CD11a constitutive blocks

Block	Bo vs. Hu	Bo vs. Mu	Hu vs. Mu
Overall	77	68	71
Putative signal peptide	56	50	32
Extracellular domain	78	70	73
Transmembrane region	91	83	75
Cytoplasmic tail	56	50	62
I-domain	84	72	74
Putative cation binding motif 1	88	66	77
Putative cation binding motif 2	75	87	75
Putative cation binding motif 3	88	77	66

Bo, Hu and Mu: bovine, human and murine CD11a, respectively.

Fig. 1. The nucleotide and deduced amino acid sequences of *B. taurus* CD11a cDNA. The putative leader peptide and transmembrane region are underlined. Five independent clones were sequenced in both directions. Sequence data have been deposited at GenBank under accession No. AY 267467.

not required to guarantee adequate functioning of LFA-1. This is in agreement with the observation that truncation of the LFA-1 α subunit cytoplasmic domain has no effect on binding to ICAM-1, whereas binding is markedly diminished by β subunit cytoplasmic domain truncation (Hibbs et al., 1991).

Every cysteine residue in the bovine extracellular portion of mature CD11a is present at the same location in

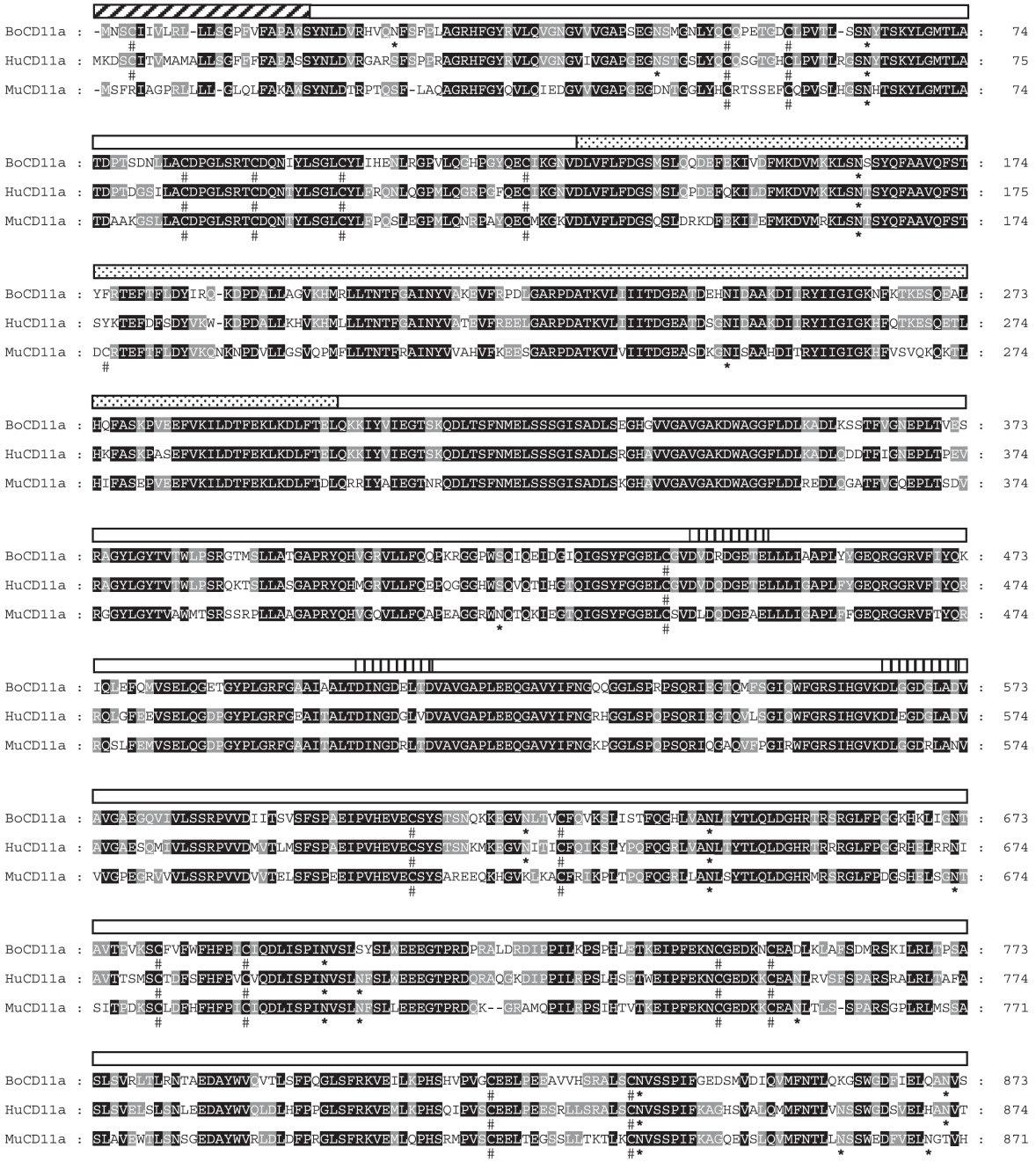

Fig. 2. Comparison of the bovine (Bo-), human (Hu-) and murine α subunits (MuCD11a) amino acid sequences. Cystein residues (#) and potential N-glycosylation sites (*) are marked. White letters in black and gray boxes indicate conservation among the three or only two species, respectively. The stripe above the sequences represents the deduced different constitutive parts of the protein: signal peptide (▨), extracellular domain (▭), transmembrane

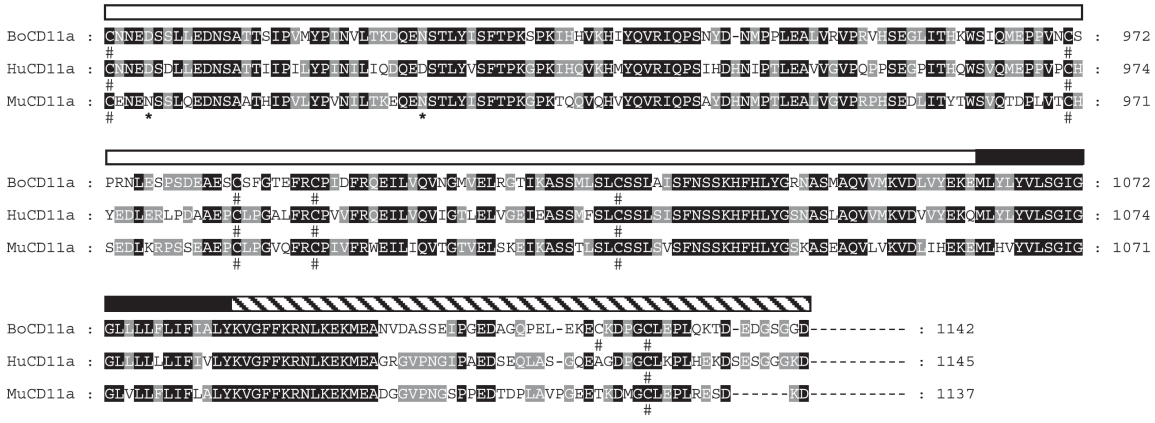

Fig. 2 (*continued*).

human and murine CD11a, which is consistent with a role in maintaining the global structure of the protein. The mouse version distinguishes by an additional cysteine residue at position 176 within the extracellular portion, whereas the bovine CD11a possesses an additional cysteine at position 1122 into the cytoplasmic tail. Whether this additional cysteine causes enough structural changes to underlie functional modifications remains unknown. Of 14 potential Asn-glycosylation sites in murine CD11a, the ones present at amino acids 247, 421, 673, 753, 857, 876 and 904 are absent from both human and bovine sequences, once more without predictable consequences on a functional point of view.

This study reports for the first time the isolation and sequencing of the bovine LFA-1 α subunit (CD11a) cDNA, and demonstrates that, despite some focal differences, it shares all the main characteristics of its known mammalian homologs. Along with the bovine CD18-encoding cDNA which is available (Shuster et al., 1992), the sequence data provided here allow the successful cloning of BoCD11a, thus giving the first opportunity to express bovine LFA-1 in vitro as a tool to examine the specificities of inflammation in the bovine species.

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Cloning and characterisation of the primary structure of the sheep lymphocyte function-associated antigen-1 α subunit

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Abstract

The leukocyte integrins play a critical role in a number of cellular adhesive interactions during the immune response. The ovine cDNA encoding CD11a, the predominant α subunit of the β_2-integrin family, was sequenced and compared with the human, bovine and murine sequences. Despite some focal differences, it shares all the main characteristics of its known mammalian homologues. Along with the ovine CD18-encoding cDNA, which is available for a few months, the sequence data provided here will allow the *Ovis aries* β_2-integrin CD11a/CD18 (LFA-1, $\alpha_L\beta 2$) expression in vitro as a tool to examine the specificities of inflammation in the ovine species.

Keywords: Integrin; LFA-1; ICAM; Adhesion; Ovine

1. Introduction

Lymphocyte function-associated antigen-1 (LFA-1, $\alpha_L\beta_2$, CD11a/CD18) is a member of the β_2-integrin subfamily of cell surface receptors. Integrins consist of a 120- to 180-kDa α subunit (CD11a in this case) and a 90- to 110-kDa β subunit that are noncovalently associated single-pass transmembrane proteins (Springer, 1990). The bulk of each integrin subunit is extracellular, where it typically functions as a receptor for extracellular matrix molecules or as a counterreceptor for surface proteins of apposed cells (Hynes, 1992). The heterodimer CD11a/CD18 is expressed on all leukocytes and mediates high affinity adhesion to a variety of cell types that express one or more of the β_2-integrins ligands, intercellular adhesion molecules (ICAM-1 to -5) (Bailly et al., 1995; Gahmberg, 1997; Tian et al., 1997). The adhesion process mediated is a critical step of a wide range of immunological activities, including cytolysis of target cells, cross-interaction and cross-stimulation between lymphocytes, phagocytosis of complement-coated targets, neutrophils clearance from inflamed tissue, and the regulation of leukocyte traffic between the bloodstream and tissues (Dunne et al., 2003; Hogg et al., 2004; Salas et al., 2004; Yan et al., 2004). As the relevance of the ovine model has been well established in such diverse areas as immunology (Mitazaka and Trnka, 1985), haematology (Zanjani et al., 1997), genomic cloning (Fulka et al., 1998) or asthma (Abraham et al., 2000; Bischof et al., 2003; Collie, 2003), increasing our knowledge about ovine β_2 integrins is of great importance to offer new possibilities for research and to provide additional insights into those fields.

The *Ovis aries* CD18 (β_2) subunit has been well characterised (Zecchinon et al., 2004), which is not the case of its partner into the LFA-1 heterodimer, CD11a. The purpose of this paper is to report the cloning and sequencing of a cDNA encoding ovine CD11a, along with a comparative sequence analysis with its human, bovine and murine homologues.

Abbreviations: Bo, bovine; hu, human; ICAM, intercellular adhesion molecule; IDAS, I-domain allosteric site; kan, kanamycin; LFA, lymphocyte function-associated antigen; MIDAS, metal-ion dependent adhesion site; mu, murine; ov, ovine; PBMC, peripheral blood mononuclear cell; PMA, phorbol myristate acetate; RACE, rapid amplification of cDNA ends; RT-PCR, reverse transcription-polymerase chain reaction

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2. Materials and methods

2.1. RNA isolation

Total RNA from phorbol myristate acetate (PMA)-stimulated (25 ng/ml for 15 min) ovine peripheral blood mononuclear cells (PBMC) was extracted with TRIzol (Invitrogen) as described by the manufacturer. The PBMCs were obtained by density gradient centrifugation with Ficoll-Paque Plus (Amersham) and maintained in RPMI 1640 supplemented with 10% foetal bovine serum (Gibco BRL), penicillin (100 U/ml) and streptomycin (100 μg/ml) at 37 °C in a 5% CO_2 atmosphere.

2.2. Amplification of cDNA ends

We used SMART rapid amplification of cDNA ends (RACE) technology (Clontech Laboratories Inc.) to obtain ovine CD11a (OvCD11a) 5′- and 3′-ends and reverse transcription-polymerase chain reaction (RT-PCR) to amplify full-length OvCD11a CDS. For first-strand cDNA synthesis, and according to the sequence of BoCD11a available (GenBank no. AY267467), gene-specific primers were designed which were expected to give non overlapping ∼1 kb RACE products: a sense primer for the 3′-RACE PCR: 5′-TGCAATGTRAGCTCTCCCATCTTC-3′ (corresponding to nt 2572–2595) and an antisense primer for the 5′-RACE PCR: 5′-CCGGCCTCCTCTCTGCTCCCCATAG-3′ (nt 1470–1446). Reverse transcription and polymerase chain reactions (PCR) were carried out according to the instruction manual of the SMART RACE cDNA Amplification Kit. The 5′- and 3′-RACE products were gel-purified using the S.N.A.P.™ Gel Purification Kit (Invitrogen), TA-cloned into pCRII-TOPO (Invitrogen) and seeded on kanamycin IPTG plates. Miniprep were obtained from colonies grown in 5 ml LB-Kan broth and the clones were sequenced on the ABI-3730 Genetic Analyzer using the Big Dye terminator chemistry (Applied Biosystems).

2.3. Molecular cloning of full-length cDNA

Total RNA from PMA-stimulated PBMCs was reverse transcribed using Improm II (Promega). The full-length cDNA was then generated by long distance PCR using Advantage 2 polymerase (Clontech Laboratories Inc.) with primers designed from the distal ends of both 5′- and 3′-RACE products: 5′-GTCGCCAGTAAATCCCAAGA-3′ (sense, within the 5′-UTR) and 5′-GCACCTCAATCTCCACCACT-3′ (antisense, 3′UTR). The procedures recommended by the manufacturer were followed, with these cycling parameters: 5 min at 94 °C, then 35 cycles including (i) 30 s at 94 °C, (ii) 30 s at 60.5 °C and (iii) 3 min 30 s at 68 °C, followed by a final extension at 68 °C for 5 min. Resulting PCR products were then processed for sequencing as aforementioned for the RACE products. The CD11a cDNA sequence was deduced from sequences obtained from nine independent clones. Sequence data have been deposited at GenBank under accession no. AY731091 and AY731092.

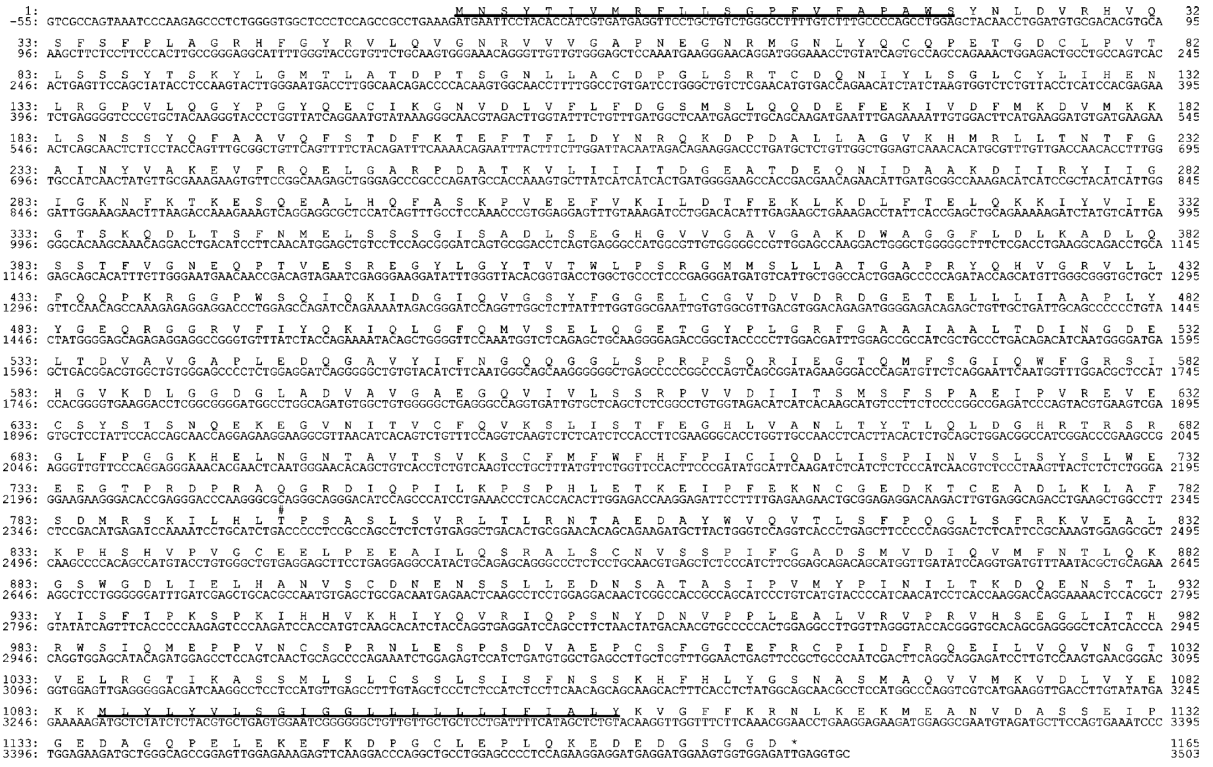
2.4. Bioinformatics

Primers design was performed with Netprimer (http://www.premierbiosoft.com/netprimer) and Primer 3 (Rozen and Skaletsky, 2000). Nucleotidic sequence and similarity analyses were carried out using, respectively, Chromas v.2.21 (http://www.technelysium.com.au) and BLAST programs (Altschul et al., 1990). Alignment of amino acids sequences were drawn by GeneDoc v.2.6.002 (Nicholas et al., 1997) following the BLOSUM 62 matrix. SignalP v.2.0.b2 (Nielsen et al., 1997) and NetNGlyc v.1.0 (Jensen et al., 2002) provided peptide signal and *N*-glycosylation sites prediction, respectively.

3. Results and discussion

3.1. Characterisation of OvCD11a-encoding cDNA and deduced aa sequence

Two alleles have been identified for the OvCD11a cDNA. The sequence contains ∼4200 bp with an ORF of 3498 (Genbank no. AY731092) or 3495 bp (Genbank no. AY731091) depending on the allele that codes for 1165 or 1164 aa followed by ∼600 bp in the 3′-UTR (Fig. 1). The mature OvCD11a contains a 23-aa putative leader peptide, an extracellular domain of 1061 or 1062 residues (24–1084/1085), a single hydrophobic transmembrane region of 24 residues (1085/1086–1108/1109) and a cytoplasmic tail of 57 residues (Fig. 1). Seven N-linked putative glycosylation sites (Asn-X-Thr/Ser) are present in the extracellular domain (Fig. 2). The mature protein contains 21 cysteine residues, among which one is located into the cytoplasmic tail (Fig. 2). The extracellular domain also contains an inserted (I) domain of 172 amino acids (residues 153–324) quite similar to those found in all the leukocyte integrin α subunits sequenced to date and located between the β sheets 2 and 3 of a seven bladed β-propeller region (Huang et al., 1997). The I domain is homologous with repeated domains found in von Willebrand factor and cartilage matrix protein (Springer, 1990) and can be expressed as an isolated domain. Its three-dimensional structure consists of a five-stranded parallel β-sheet core surrounded on both faces by α-helices, with a short antiparallel strand occurring on one edge of this sheet (Qu and Leahy, 1995). The I domain contains a metal ion dependent adhesion site (MIDAS) (Lee et al., 1995) (residues 159–163, 228, 261) (Fig. 2) and an I-domain allosteric site (IDAS) that plays a functional role in ICAM-1 binding (Huth et al., 2000; Lupher et al., 2001; Lum et al., 2002). Three repeats with a divalent cation binding motif are found at amino acid residues 465–473, 527–535 and 587–595 (Fig. 2). All the conserved cysteines and all but one *N*-glycosylation sites are found out-



ig. 1. The nucleotide and deduced amino acid sequences of *Ovis aries* CD11a cDNA. The putative leader peptide and transmembrane region are underlined. ine independent clones were sequenced in both directions. Sequence data have been deposited at GenBank under accession no. AY731091 and AY731092 shown here). Both sequences differ by a glutamine deletion in position 743 (#) in sequence AY731091.

ide the I region and divalent cation binding motifs (Fig. 2), consistent with the hypothesis that these regions may undergo conformational changes important in ligand binding Rothlein and Springer, 1986; Larson et al., 1989).

3.2. *Comparison among species*

Overall, the general organization of ovine, bovine (Fett et al., 2004), human (Larson et al., 1989) and murine (Kaufman et al., 1991) CD11a proteins is quite similar (Fig. 2). Comparison between OvCD11a sequence and its bovine, human and murine counterparts shows overall 94%, 77% and 68% identity, respectively, with the highest identity for the MIDAS and the cation binding motifs and the lowest identity for the cytoplasmic domain (Table 1). The high conservation of the MIDAS and the putative cation binding motifs is consistent with an involvement of these regions in the functional activity of LFA-1 α subunit, as suggested by the requirement of Mg^{2+} and Ca^{2+} for CD11a/CD18-dependent cellular interactions Rothlein and Springer, 1986) or binding to purified ICAM-1 Dustin and Springer, 1989; Vitte et al., 2004). By contrast, he low conservation of the cytoplasmic tail suggests that it s not required to guarantee adequate functioning of LFA-. This is in agreement with the observation that truncation of the LFA-1 α subunit cytoplasmic domain has no effect on binding to ICAM-1, whereas binding is markedly diminished by β subunit cytoplasmic domain truncation (Hibbs et al., 1991). Residue Glu-332 that is located in the linker following the I domain and that is known to be critical for communication to the β_2 I-like domain, rolling, integrin extension and activation by Mn^{2+} of firm adhesion (Salas et al., 2004) is strictly conserved.

Every cysteine residue in the ovine extracellular portion of mature CD11a is present at the same location in bovine, human and murine CD11a, which is consistent with a role in maintaining the global structure of the protein. The mouse

Table 1
Between-species percent identities of CD11a constitutive blocks

Block	Ov vs. Bo	Ov vs. Hu	Ov vs. Mu
Overall	94	77	68
Putative signal peptide	82	52	41
Extracellular domain	94	78	70
Transmembrane region	95	95	79
Cytoplasmic tail	96	56	50
I-domain	96	86	73
MIDAS	100	100	85
Putative cation binding motif 1	100	88	66
Putative cation binding motif 2	100	75	87
Putative cation binding motif 3	100	88	77

Ov, Bo, Hu and Mu: ovine, bovine, human and murine CD11a, respectively; MIDAS: metal-ion dependent adhesion site.

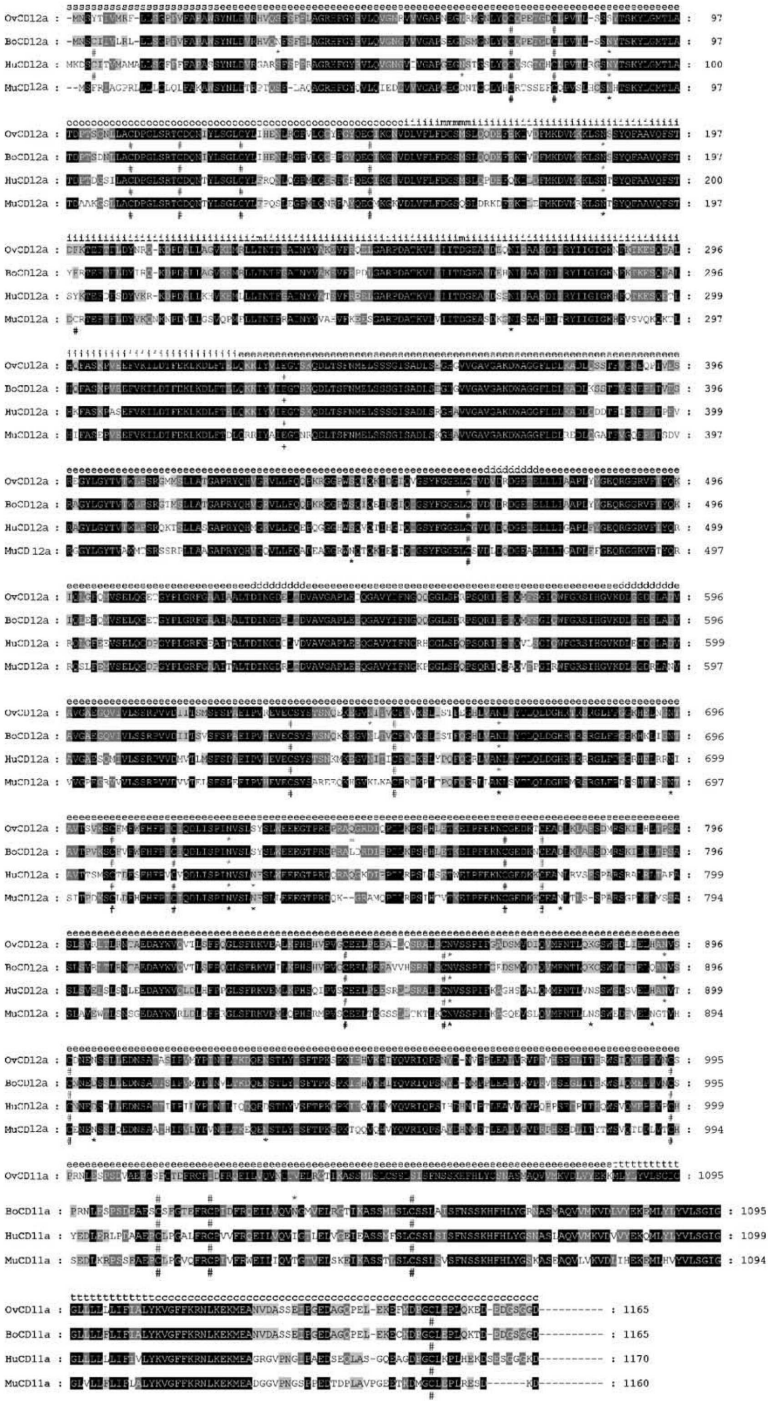


Fig. 2. Comparison of the ovine (Ov-), bovine (Bo-), human (Hu-) and murine (Mu-) α subunits amino acid sequences. The letters in the top row identify the constitutive blocks: putative signal peptide (s), extracellular domain (e), transmembrane region (t), cytoplasmic tail (c), I-domain (i), MIDAS motif (m) and divalent cation binding motifs (d). Black, dark and light gray columns represent identity among 4, 3 and 2 species, respectively. Cysteine residues (#) and potential *N*-glycosylation sites (*) are marked at the bottom of the sequences. The important Glu-332 residue (+) and the Gln-743 residue, which is absent in the smaller allele (=), are identified.

version distinguishes by an additional cysteine residue at position 199 (mouse numbering) within the extracellular portion. Of seven potential Asn-glycosylation sites in ovine CD11a, the ones present at amino acids 185, 667, 723 and 859 are strictly conserved, one is only absent from murine CD11a (residue 894) and two are only present in ovine CD11a (residues 646 and 1030), once more without predictable consequences on a functional point of view.

This study reports for the first time the isolation and sequencing of the ovine LFA-1 α subunit (CD11a) cDNA, and demonstrates that, despite some focal differences, it shares all the main characteristics of its known mammalian homologues. Along with the ovine CD18-encoding cDNA which is now available (Zecchinon et al., 2004), the sequence data provided here allow the successful cloning of OvCD11a, thus giving the first opportunity to express ovine LFA-1 in vitro as a tool to examine the specificities of inflammation in the ovine species.

Acknowledgements

This study is supported by the Belgian federal services for public health and security of the food chain and environment, grant S-6107. The authors are grateful to Professor M. Georges for giving free access to all the facilities of the laboratory of molecular genetics and to Professor P. Leroy for giving access to the PBMCs.

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Molecular characterisation of the caprine (*Capra hircus*) lymphocyte function-associated antigen-1 alpha subunit-encoding cDNA

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Abstract

Background: Lymphocyte function-associated antigen-1 (LFA-1, CD11a/CD18, alpha L beta 2) is required for many cellular adhesive interactions during the immune response.

Methods: We used SMART RACE technology to obtain caprine CD11a 5'- and 3'-ends and RT-PCR to amplify the full-length CDS.

Results: The *Capra hircus* CD11a-encoding cDNA was sequenced and compared with its human, murine, rat, bovine and ovine counterparts. Despite some focal differences, it shares all the main characteristics of its known mammalian homologues.

Conclusion: Therefore, along with the caprine CD18-encoding cDNA, which has been available for a few months, the sequence data revealed here will allow the *Capra hircus* LFA-1 expression *in vitro* as a tool to explore the specificities of inflammation in the caprine species.

Background

Lymphocyte function-associated antigen-1 (LFA-1, $\alpha_L\beta_2$, CD11a/CD18) is a member of the β_2-integrin subfamily of cell surface receptors. Integrins consist of a 120 to 180 kDa α subunit (CD11a in this case) and a 90 to 110 kDa β subunit that are noncovalently associated single-pass transmembrane proteins [1]. The bulk of each integrin subunit is extracellular, where it typically functions as a receptor for extracellular matrix molecules or as a counter-receptor for surface proteins of apposed cells [2]. The heterodimer CD11a/CD18 is expressed on all leukocytes and mediates high affinity adhesion to a variety of cell types that express one or more of the β_2-integrins ligands, intercellular adhesion molecules (ICAM-1 to -5) [3-5]. The adhesion process mediated is a critical step of a wide range of immunological activities, including cytolysis of target cells, cross-interaction and cross-stimulation between lymphocytes, phagocytosis of complement-coated targets, neutrophils clearance from inflamed tissue, and the regulation of leukocyte traffic between the bloodstream and tissues [6-9]. As the relevance of the goat model for studying leukocyte traffic, diapedesis and pathologic tissue infiltration is well established in such important areas as mastitis [10-13] or lentivirus-associated diseases [14-16], increasing our knowledge about caprine β_2 integrins is of great importance to offer new possibilities for research and to provide additional insights into those fields. Along with the caprine CD18-encoding cDNA, which is available for a few months [17], the sequence data provided here will allow the *Capra hircus* β_2-integrin CD11a/CD18

expression *in vitro* as a tool to examine the specificities of inflammation in the caprine species.

Methods

RNA isolation

Total RNA from phorbol myristate acetate (PMA)-stimulated (25 ng/ml for 15 min) caprine (*Boer* breed) peripheral blood mononuclear cells (PBMC) was extracted with TRIzol (Invitrogen, USA) as described by the manufacturer. The PBMCs were obtained by density gradient centrifugation with Ficoll-Paque Plus (Amersham, USA) and maintained in RPMI 1640 supplemented with 10% foetal bovine serum (Gibco BRL, USA), penicillin (100 U/ml) and streptomycin (100 µg/ml) at 37°C in a 5% CO_2 atmosphere.

Amplification of cDNA ends

We used SMART RACE technology (Clontech Laboratories Inc., USA) to obtain caprine CD11a (CaCD11a) 5'- and 3'-ends and RT-PCR to amplify full-length CaCD11a CDS. For first-strand cDNA synthesis, and according to the sequence of bovine CD11a available [GenBank: AY267467], gene-specific primers were designed which were expected to give non overlapping ~1 kb RACE products: a sense primer for the 3'-RACE PCR : 5'-TGCAATGTRAGCTCTCCCATCTTC-3' (corresponding to nt 2572 to nt 2595) and an antisense primer for the 5'-RACE PCR : 5'-CCGGCCTCCTCTCTGCTCCCCATAG-3' (nt 1470 to nt 1446). Reverse transcription and polymerase chain reactions (PCR) were carried out according to the instruction manual of the SMART RACE cDNA Amplification Kit. The 5'- and 3'-RACE products were gel-purified using the S.N.A.P.™ Gel Purification Kit (Invitrogen, USA), TA-cloned into pCRII-TOPO (Invitrogen, USA) and seeded on kanamycin IPTG plates. Miniprep were obtained from colonies grown in 5 ml LB-kanamycin broth and the clones were sequenced on the ABI-3730 Genetic Analyzer using the Big Dye terminator chemistry (Applied Biosystems, USA).

Molecular cloning of full-length cDNA

Total RNA from PMA-stimulated PBMCs was reverse transcribed using Improm II (Promega, USA). The full-length cDNA was then generated by long distance PCR using Platinum Taq DNA polymerase High Fidelity (Invitrogen, USA) with primers designed from the distal ends of both 5'- and 3'-RACE products : 5'-GTCGCCAGTAAATCCCAAGA-3' (sense, within the 5'-UTR) and 5'-GCACCTCAATCTCCACCACT-3' (antisense, 3'UTR). The procedures recommended by the manufacturer were followed, with these cycling parameters : 5 min at 94°C, then 35 cycles including (*i*) 30 s at 94°C, (*ii*) 30 s at 58°C and (*iii*) 3 min 30 s at 68°C, followed by a final extension at 68°C for 5 min. Resulting PCR products were then processed for sequencing as aforementioned for the RACE products. The CD11a cDNA sequence was deduced from sequences obtained from nine independent clones. Sequence data have been deposited at GenBank under accession No. AY773018 and AY773019.

Bioinformatics

Primers design was performed with Netprimer [18] and Primer 3 [19]. Nucleotidic sequence and similarity analyses were carried out using respectively Chromas v.2.21 [20] and BLAST programs [21]. Alignment of amino acids sequences were drawn by GeneDoc v.2.6.002 [22] following the BLOSUM62 matrix. SignalP v.2.0.b2 [23] and NetNGlyc v.1.0 [24] provided peptide signal and N-glycosylation sites prediction, respectively.

Results & discussion

Characterisation of CaCD11a-encoding cDNA and deduced aa sequence

Two alleles have been identified for the CaCD11a cDNA. The sequence contains ~4200 bp with an ORF of 3498 [Genbank: AY773019] or 3495 bp [Genbank: AY773018] depending on the allele that codes for 1165 or 1164 aa followed by ~600 bp in the 3'-UTR (Fig. 1). The mature CaCD11a contains a 23-aa putative leader peptide, an extracellular domain of 1061 or 1062 residues (24-1084/1085), a single hydrophobic transmembrane region of 24 residues (1085/1086–1108/1109) and a cytoplasmic tail of 57 residues (Fig. 1). Nine N-linked putative glycosylation sites (Asn-X-Thr/Ser) are present in the extracellular domain (Fig. 2). The mature protein contains 19 cysteine residues among which one is located into the cytoplasmic tail (Fig. 2). The extracellular domain also contains an inserted (I) domain of 172 amino acids (residues 153–324) quite similar to those found in all the leukocyte integrin α subunits sequenced to date and located between the β sheets 2 and 3 of a seven bladed β-propeller region [25]. The I-domain is homologous with repeated domains found in von Willebrand factor and cartilage matrix protein [1] and can be expressed as an isolated domain. Its three-dimensional structure consists of a five-stranded parallel β-sheet core surrounded on both faces by α-helices, with a short antiparallel strand occurring on one edge of this sheet [26]. The I-domain contains a metal ion-dependent adhesion site (MIDAS) [27] (residues 159–163, 228, 261) (Fig. 2) and an I-domain allosteric site (IDAS) that plays a functional role in ICAM-1 binding [28-30]. Three repeats with a divalent cation binding motif are found at amino acid residues 465–473, 527–535 and 587–595 (Fig. 2). All the conserved cysteines and all but one N-glycosylation sites are found outside the I region and divalent cation binding motifs (Fig. 2), consistent with the hypothesis that these regions may undergo conformational changes important in ligand binding [31,32].

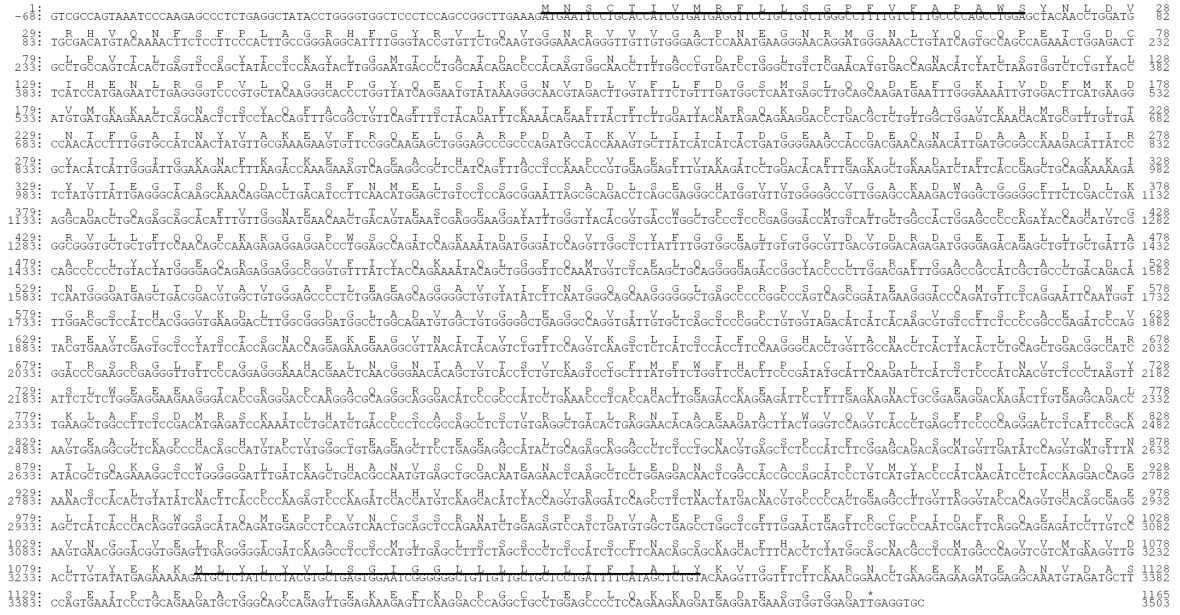

Figure 1
The nucleotide and deduced amino acid sequences of *Capra hircus* CD11a cDNA. The putative leader peptide and transmembrane region are underlined. Nine independent clones were sequenced in both directions. Sequence data have been deposited at GenBank under accession No. AY773018 and AY773019 (shown here), respectively without and with Gln-743 (#).

General comparison among species

Overall, the general organization of caprine, bovine [33], ovine [34], human [32], murine [35] and rat [GenBank: NW_047562] CD11a proteins is quite similar (Fig. 2). Comparison between mature CaCD11a sequence and its bovine, ovine, human, murine and rat counterparts shows overall 94%, 98%, 77%, 68% and 55% identity, respectively, with the highest identity for the MIDAS, the cation binding motifs and the transmembrane region and the lowest identity for the cytoplasmic tail (Table 1). The high conservation of the MIDAS and the putative cation binding motifs is consistent with an involvement of these regions in the functional activity of LFA-1 α subunit, as suggested by the requirement of Mg^{2+} and Ca^{2+} for CD11a/CD18-dependent cellular interactions [31] or binding to purified ICAM-1 [36,37]. The transmembrane region shows also a high degree of conservation that could be explained by (i) physicochemical, and (ii) functional constraints. Indeed, (i) residues lying in the membrane have to possess rather hydrophobic character to allow liposolubility, which is confirmed by the presence of many leucine residues (figure 2) and (ii) bi-directional integrin signalling (inside-out and outside-in) is accomplished by transmission of information across the plasma membrane [38]. By contrast, the low conservation of the cytoplasmic tail suggests that it is not required to guarantee adequate functioning of LFA-1. This is in agreement with the observation that truncation of the LFA-1 α subunit cytoplasmic domain has no effect on binding to ICAM-1, whereas binding is markedly diminished by β subunit cytoplasmic domain truncation [39]. Residue Glu-332 that is located in the linker following the I-domain and that is known to be critical for communication to the β_2 I-like domain, rolling, integrin extension and activation by Mn^{2+} of firm adhesion [8] is strictly conserved.

Every cysteine residue in the caprine extracellular portion of mature CD11a is present at the same location in bovine, ovine, human, murine and rat CD11a, which is consistent with a role in maintaining the global structure of the protein whereas two cysteine residues (positions 1009 and 1048) are absent from caprine CD11a and therefore do not seem to be indispensable. The mouse version distinguishes by an additional cysteine residue at

Figure 2
Comparison of the caprine (Ca-), bovine (Bo-), human (Hu-), murine (Mu-) and rat (Ra-) α subunits amino acid sequences. The letters in the top row identify the constitutive blocks: putative signal peptide (s), extracellular domain (e), transmembrane region (t), cytoplasmic tail (c), I-domain (i), MIDAS motif (m) and divalent cation binding motifs (d). Black, dark grey and light grey columns represent identity among 6, 5 and 4 species, respectively. Cysteine residues (#) and potential N-glycosylation sites (*) are marked at the bottom of the sequences. The important Glu-332 residue (+) and the Gln-743 residue which is absent in the smaller allele (=) are identified.

position 199 (mouse numbering) within the extracellular portion. Of nine potential Asn-glycosylation sites in caprine CD11a, the ones present at amino acids 185, 667, 723 and 859 are strictly conserved, one is only absent from murine and rat CD11a (residue 894), without predictable consequences on a functional point of view.

Interestingly, as in sheep [34] and human [GenBank: NM_002209 and AY892236], an allelic variant with a triplet insertion resulting in an additional Glu744 in the extracellular domain was consistently identified, which suggests an allelic polymorphism that might be biologically relevant. Studies of genomic sequences will permit to know if this addition represents two alleles or not.

Finally, one has to note that the lowest between-species percent identities are observed with the rat CD11a sequence which has been derived from an annotated genomic sequence. Cloning and characterisation of rat CD11a from rat PBMCs would probably give a higher identity.

Conclusion

This study reports for the first time the isolation and sequencing of the caprine LFA-1 α subunit (CD11a) cDNA, and demonstrates that, despite some focal differences, it shares all the main characteristics of its known mammalian homologues. Along with the caprine CD18-encoding cDNA which is now available [17], the sequence

Table 1: Between-species percent identities of CD11a constitutive blocks. Ca, Bo, Ov, Hu, Mu and Ra: caprine, bovine, ovine, human, murine and rat CD11a, respectively; MIDAS: metal-ion dependent adhesion site.

Block	Ca vs. Bo (%)	Ca vs. Ov (%)	Ca vs. Hu (%)	Ca vs. Mu (%)	Ca vs. Ra (%)
Overall	94	98	77	68	55
Putative signal peptide	86	95	60	41	4
Extracellular domain	94	98	78	69	60
Transmembrane region	95	100	95	79	87
Cytoplasmic tail	92	94	58	50	47
I-domain	95	99	86	72	75
MIDAS	100	100	100	85	85
Putative cation binding motif 1	100	100	88	66	55
Putative cation binding motif 2	100	100	77	88	88
Putative cation binding motif 3	100	100	88	77	88

data provided here will allow the successful expression of caprine LFA-1 *in vitro* as a tool to examine the specificities of inflammation in the caprine species.

Competing interests

The author(s) declare that they have no competing interests.

Authors' contributions

TF carried out cloning and sequencing, participated in the sequence alignment and to the draft of the manuscript. LZ participated in the sequence comparison and to the draft of the manuscript. EB participated in the design of the study. DD conceived the study, and participated in its design and coordination and helped to draft the manuscript. All authors read and approved the final manuscript.

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Molecular cloning and characterisation of the CD18 partner in ovine (*Ovis aries*) β_2-integrins

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Abstract

The leukocyte integrins play a critical role in a number of cellular adhesive interactions during the immune response. We describe here the isolation and characterization of the ovine β_2 (CD18) subunit, common to the leukocyte β_2-integrin family. The deduced 770-amino-acid sequence reveals a transmembrane protein with 81%, 83% and 95% identity with its murine, human and bovine homologues, respectively. Comparisons of CD18 sequences emphasize the functional importance of the β_2 subunit I-like domain and included metal ion-dependent adhesion site (MIDAS)-like motif and confirm that of the cytoplasmic tail. The data provided here will offer the possibility to explore new avenues in studies based on the ovine model.

Keywords: Leukocyte; Adhesion; Sheep; LFA-1

1. Introduction

Integrins consist of a 120- to 180-kDa α subunit and a 90- to 110-kDa β subunit that are noncovalently associated single-pass transmembrane proteins (Springer, 1990). The bulk of each integrin subunit is extracellular, where it typically functions as a receptor for extracellular matrix molecules or as a counterreceptor for surface proteins of apposed cells (Hynes, 1992). Mammalian integrins form several subfamilies sharing common β subunits that associate with different α subunits. β_2 integrins include four different heterodimers: CD11a/CD18 (lymphocyte function-associated antigen (LFA)-1), the predominant β_2-integrin, CD11b/CD18 exclusive to granulocytes and monocytes, CD11c/CD18, and CD11d/CD18.The CD11a–d/CD18 heterodimers are expressed on all leukocytes and mediate high affinity adhesion to a variety of cell types that express one or more of the β_2-integrins ligands, intercellular adhesion molecules (ICAM-1 to -5) (Bailly et al., 1995; Gahmberg, 1997; Tian et al., 1997). The adhesion process mediated is a critical step of a wide range of immunological activities, including cross-interaction and cross-stimulation between lymphocytes, phagocytosis of complement-coated targets, regulation of leukocyte traffic between the bloodstream and tissues, or cytolysis of target cells.

As the relevance of the ovine model has been well established in such diverse areas as immunology (Mitazaka and Trnka, 1985), hematology (Zanjani et al., 1997), genomic cloning (Fulka et al., 1998) or asthma (Abraham et al., 2000; Bischof et al., 2003; Collie, 2003), increasing our knowledge about ovine β_2-integrins is of critical importance to offer new possibilities for research and to provide additional insights into those fields. The *Ovis aries* CD18 (β_2) subunit has never been characterized so far. The purpose of this paper is to report the cloning and sequencing of a cDNA encoding ovine CD18, along with a comparative sequence analysis with its bovine, human and murine homologs.

Abbreviations: Bo, bovine; Hu, human; Mu, murine; Ov, ovine; ICAM, intercellular adhesion molecule; kan, kanamycin; LFA, lymphocyte function-associated antigen; PMA, phorbol myristate acetate; RACE, rapid amplification of cDNA ends; MIDAS, metal ion-dependent adhesion site; PBMC, peripheral blood mononuclear cell.

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2. Materials and methods

2.1. RNA isolation

Total RNA from phorbol myristate acetate (PMA)-stimulated (25 ng/ml for 15 min) ovine peripheral blood mononuclear cells (PBMC) was extracted with TRIzol (Invitrogen) as described by the manufacturer. The PBMCs were obtained by density gradient centrifugation with Ficoll-Paque Plus (Amersham) and maintained in RPMI 1640 supplemented with 10% fetal bovine serum (Gibco BRL), penicillin (100 U/ml) and streptomycin (100 μg/ml) at 37 °C in a 5% CO_2 incubator.

2.2. Amplification of cDNA ends

We used SMART RACE technology (Clontech Laboratories) to clone ovine CD18 (OvCD18) 5′ - and 3′ -ends and RT-PCR to clone full-length OvCD18 CDS. For first-strand cDNA synthesis, and according to the bovine CD18 sequence available (GenBank no. M81233), gene-specific primers were designed which were expected to give nonoverlapping ~ 1 kb rapid amplification of cDNA ends (RACE) products: a sense primer for the 3′-RACE PCR: 5′ -GAARGAGAARCTCAAGTCCCARTGGAACAA-3′ (corresponding to nt 2302 to nt 2331) and an antisense primer for the 5′ -RACE PCR: 5′ -ACCTGCAGACGCCG-CACTCCATC-3′ (nt 1496 to nt 1474). Reverse transcription and polymerase chain reactions (PCR) were carried out according to the instruction manual of the SMART RACE cDNA Amplification Kit. The 5′ - and 3′ -RACE products were gel-purified using the Qiaquick Gel Extraction Kit (Qiagen), TA-cloned into pCRII-TOPO (Invitrogen) and seeded on kanamycin IPTG plates. Miniprep were obtained from colonies grown in 5 ml LB-Kan broth and the clones were sequenced on the ABI-3100 Genetic Analyzer using the Big Dye terminator chemistry (Applied Biosystems).

2.3. Molecular cloning of full-length cDNA

Total RNA from PMA-stimulated PBMCs was reverse transcribed using Improm II (Promega). The full-length cDNA was then generated by long distance PCR using *Taq* polymerase (Promega) with primers designed from the distal ends of both 5′ - and 3′ -RACE products: 5′ -CAGCCTGGTGAAGAGCAGAG-3′ (sense, within the 5′ -UTR) and 5′ -GCAGAAGGCCTTGTCTTCAC-3′ (antisense, 3′ -UTR). The procedures recommended by the manufacturer were followed, with the following cycling parameters: 2 min 30 s at 94 °C, then 35 cycles including (i) 30 s at 94 °C, (ii) 30 s at 53 °C and (iii) 2 min 30 s at 68 °C, followed by a final extension at 68 °C for 10 min (*Taq* polymerase, Promega). Resulting PCR products were then processed for sequencing as aforementioned for the RACE products. The CD18 cDNA sequence was deduced from sequences obtained from nine independent clones. Sequence data have been deposited at GenBank under accession no. AY484425 and AY484426.

2.4. Bioinformatics

Primers design was performed with Netprimer (http://www.premierbiosoft.com/netprimer) and Primer 3 (Rozen and Skaletsky, 2000). Nucleotidic sequence and similarity analyses were carried out using respectively Chromas v.2.21 (http://www.technelysium.com.au) and BLAST programs (Altschul et al., 1990). Alignment of amino acids sequences was drawn by GeneDoc v.2.6.002 (Nicholas et al., 1997). SignalP v.2.0.b2 (Nielsen et al., 1997) and NetNGlyc v.1.0 (Jensen et al., 2002) provided peptide signal and *N*-glycosylation sites prediction, respectively.

3. Results and discussion

3.1. Characterisation of OvCD18-encoding cDNA and deduced aa sequence

The cDNA sequence for OvCD18 contains ~ 3300 bp with an ORF of 2310 bp that codes for 770 aa followed by ~ 600 bp in the 3′ -UTR (Fig. 1). Two alleles were identified, differing by eight silent substitutions (C57T, A87G, T138C, C261T, T312C, T429C, G489A and A525G). The region immediately preceding the ATG start codon is roughly similar to that in other species, containing the sequence $AG_{(n)}AC$, where $n = 5$ in the mouse (Wilson et al., 1989), $n = 4$ in the sheep (present study) and the bovine (Shuster et al., 1992), and $n = 3$ in the human (Kishimoto et al., 1987), which pleads for a regulatory role of this motif. The mature OvCD18 contains a 22-aa putative leader peptide, an extracellular domain of 679 residues (23–701), a single hydrophobic transmembrane region of 23 residues (702–724) and a short cytoplasmic tail of 46 residues (Fig. 1). Starting from the N-terminal end, the extracellular region successively contains a cysteine-rich repeats-containing analogue of the so-called (Bork et al., 1999) plexin semaphoring integrin domain (residues 23–74), an inserted I-like domain (21) of 240 amino acids (residues 124–363) and a C-terminal cysteine-rich region (445–696, Fig. 2). A putative metal ion-dependent adhesion site (MIDAS)-like D*X*S*X*S motif is predicted within the I-like domain (residues 134–138). Overall, the protein contains 56 cysteine residues and 5 N-linked putative glycosylation sites (Asn-X-Thr/Ser), all located within the extracellular region (Fig. 2).

3.2. Comparison among species

Overall, the general organization of ovine, human (Weitzman et al., 1991), murine (Wilson et al., 1989) and

```
   1:                                                                                 M  L  P  Q  R  P  Q  L  L  L  L  A  G  L  L  S  L  Q  S  V  L  S  Q     23
 -81: AGCCTGGTGAAGAGCAGAGCTGAAGCCCCTGCCAGTCCAGCTGGGACACCCCTGCCGTGGTCTCCAGGGCATCCAGGGGACATGCTGCCCCAGCGCCCCCAGCTGCTGCTCCTAGCGGGCCTGCTCTCCCTCCAGTCCGTCCTGTCCCAG   69

  24: E  C  T  K  Y  K  V  S  T  C  R  D  C  I  E  S  G  P  G  C  A  W  C  Q  K  L  N  F  T  G  Q  G  E  P  D  S  T  R  C  D  T  R  A  Q  L  L  S  K  G  C     73
  70: GAGTGCACCAAGTACAAAGTCAGCACCTGCCGGGACTGCATCGAGTCGGGCCCCGGCTGTGCCTGGTGTCAGAAACTGAACTTCACAGGGCAAGGGGAGCCCGACTCCACTCGCTGTGATACACGGGCGCAGCTGCTGTCGAAGGGCTGC  219

  74: P  A  D  D  I  M  E  P  K  S  L  A  E  T  R  Q  S  Q  A  G  K  Q  K  Q  L  S  P  E  E  V  T  L  Y  L  R  P  G  Q  A  A  A  F  N  V  T  F  Q  R  A  K    123
 220: CCAGCTGATGACATCATGGAACCCAAGAGCCTCGCTGAGACCCGGCAGAGCCAGGCGGGCAAACAGAAGCAGCTGTCCCCAGAGGAAGTGACTCTCTACCTGAGACCAGGTCAGGCAGCTGCGTTCAACGTGACCTTCCAGAGGGCCAAG  369

 124: G  Y  P  I  D  L  Y  Y  L  M  D  L  S  Y  S  M  V  D  D  L  A  N  V  K  K  L  G  G  D  L  L  R  A  L  N  D  I  T  E  S  G  R  I  G  F  G  S  F  V  D    173
 370: GGCTACCCCATCGACCTGTACTATCTGATGGATCTCTCCTACTCCATGGTGGACGACCTTGCCAACGTCAAGAAGCTGGGGGGTGACCTGCTCCGGGCCCTCAATGACATCACCGAGTCGGGCCGCATTGGTTTCGGGTCCTTCGTGGAC  519

 174: K  T  V  L  P  F  V  N  T  H  P  E  K  L  R  N  P  C  P  N  K  E  K  E  C  Q  P  P  F  A  F  R  H  V  L  K  L  T  D  N  S  K  Q  F  E  T  E  V  G  K    223
 520: AAGACAGTGCTCCCCTTCGTCAACACGCACCCCGAGAAGCTGAGGAACCCCTGCCCCAACAAGGAGAAGGAGTGCCAGCCGCCGTTCGCCTTCAGGCACGTGCTGAAGCTCACCGACAACTCCAAACAGTTCGAGACAGAAGTCGGGAAG  669

 224: Q  L  I  S  G  N  L  D  A  P  E  G  G  L  D  A  M  M  Q  V  A  A  C  P  E  E  I  G  W  R  N  V  T  R  L  L  V  F  A  T  D  D  G  F  H  F  A  G  D  G    273
 670: CAGCTGATCTCGGGGAACTTGGACGCCCCTGAGGGTGGGCTGGACGCCATGATGCAAGTGGCCGCGTGCCCGGAGGAAATTGGCTGGCGCAATGTCACCAGGCTGCTGGTGTTCGCCACAGACGATGGGTTCCACTTTGCGGGCGATGGA  819

 274: K  L  G  A  I  L  T  P  N  D  G  R  C  H  L  E  D  N  L  Y  K  S  S  N  E  F  D  Y  P  S  V  G  Q  L  A  H  K  L  A  E  S  N  I  Q  P  I  F  A  V  T    323
 820: AAGCTGGGTGCCATCCTCACCCCCAACGACGGCCGCTGCCACCTGGAAGACAACCTGTACAAAAGCAGCAACGAATTTGACTACCCATCGGTGGGCCAGCTGGCACACAAACTGGCAGAAAGCAACATCCAGCCCATCTTCGCGGTAACC  969

 324: K  K  M  V  K  T  Y  E  K  L  T  E  I  I  P  K  S  A  V  G  E  L  S  E  D  S  K  N  V  V  E  L  I  K  S  A  Y  N  K  L  S  S  R  V  F  L  D  H  N  T    373
 970: AAGAAGATGGTGAAAACGTACGAGAAGCTGACAGAAATCATCCCCAAGTCTGCAGTCGGGGAGCTGTCTGAAGATTCCAAGAACGTGGTGGAGCTTATCAAGAGTGCCTACAATAAACTGTCCTCCAGAGTATTCCTGGATCACAACACC 1119

 374: L  P  D  T  L  K  V  A  Y  D  S  F  C  S  N  G  V  S  Q  V  D  Q  P  R  G  D  C  D  G  V  Q  I  N  V  P  I  T  F  Q  V  K  V  T  A  T  E  C  I  Q  E    423
1120: CTCCCTGACACCCTGAAAGTCGCCTACGACTCCTTCTGCAGTAACGGGGTGTCGCAGGTGGACCAGCCCAGAGGGGACTGCGACGGCGTCCAGATCAACGTCCCGATCACCTTCCAGGTGAAGGTCACAGCCACCGAGTGCATCCAGGAG 1269

 424: Q  S  F  T  I  R  A  L  G  F  T  D  T  V  T  V  R  V  L  P  Q  C  E  C  Q  C  R  E  A  S  R  D  R  S  V  C  G  G  R  G  S  M  E  C  G  V  C  R  C  D    473
1270: CAGTCCTTCACCATCCGGGCGCTGGGCTTCACGGACACGGTGACCGTGCGGGTCCTTCCCCAGTGCGAGTGCCAATGCCGGGAAGCCAGCAGGGACCGCAGCGTCTGCGGTGGCAGAGGCTCGATGGAGTGCGGCGTCTGCAGGTGCGAC 1419

 474: A  G  Y  I  G  K  N  C  E  C  Q  T  H  G  R  S  S  Q  E  L  E  G  S  C  R  K  D  N  S  S  I  I  C  S  G  L  G  D  C  I  C  G  Q  C  V  C  H  T  S  D    523
1420  GCCGGCTACATCGGGAAGAACTGCGAGTGCCAGACGCACGGCCGGAGCAGCCAGGAGCTGGAGGGCAGCTGCCGCAAGGACAACAGCTCCATCATCTGCTCGGGGCTGGGGGACTGCATCTGCGGGCAGTGCGTGTGCCACACGAGCGAC 1569

 524: V  P  N  K  K  I  Y  G  Q  F  C  E  C  D  N  V  N  C  E  R  Y  D  G  Q  V  C  G  G  D  K  R  G  L  C  F  C  G  T  C  R  C  N  D  Q  H  E  G  S  A  C    573
1570: GTGCCCAACAAGAAGATCTACGGCCAGTTCTGCGAGTGCGACAACGTCAACTGCGAGCGCTACGACGGCCAAGTCTGCGGGGGCGACAAGAGGGGGCTCTGCTTCTGCGGCACCTGCAGGTGCAACGACCAGCATGAGGGCTCGGCGTGC 1719

 574: Q  C  L  K  S  T  Q  G  C  L  N  L  D  G  V  E  C  S  G  R  G  R  C  R  C  N  V  C  Q  C  D  P  G  Y  Q  P  P  L  C  I  D  C  P  G  C  P  V  P  C  A    623
1720: CAGTGCCTCAAGTCCACTCAGGGCTGCCTCAACCTGGACGGCGTCGAGTGCAGCGGCCGCGGCCGATGCCGCTGCAACGTGTGCCAGTGCGACCCCGGCTACCAGCCGCCCCTGTGCATCGACTGCCCGGGCTGCCCCGTGCCCTGCGCT 1869

 624: G  F  A  P  C  T  E  C  L  K  F  D  K  G  P  F  A  K  N  C  S  A  A  C  G  Q  T  K  L  L  S  S  P  V  P  G  G  R  K  C  K  E  R  D  S  E  G  C  W  M    673
1870: GGCTTCGCCCCCTGCACCGAGTGCCTGAAGTTCGACAAGGGTCCCTTCGCCAAGAACTGCAGCGCAGCGTGCGGGCAGACGAAGCTGCTGTCCAGCCCGGTGCCCGGCGGCCGCAAGTGCAAGGAGCGTGACTCCGAGGGCTGCTGGATG 2019

 674: T  Y  T  L  V  Q  R  D  G  R  N  R  Y  D  V  H  V  D  D  M  L  E  C  V  K  G  P  N  I  A  A  I  V  G  G  T  V  G  G  V  V  L  V  G  I  L  L  L  A  I    723
2020: ACCTACACCCTGGTGCAGCGCGACGGGCGGAACAGATACGACGTGCACGTGGACGACATGCTCGAGTGTGTGAAGGGCCCCAACATCGCTGCCATCGTGGGGGGCACCGTGGGGGGAGTTGTGCTCGTCGGCATCCTCCTGCTGGCCATC 2169

 724: W  K  A  L  T  H  L  S  D  L  R  E  Y  H  R  F  E  K  E  K  L  K  S  Q  W  N  N  D  N  P  L  F  K  S  A  T  T  T  V  M  N  P  K  F  A  E  S  *        770
2170: TGGAAGGCCCTGACACACCTGAGCGACCTCAGGGAGTACCATCGCTTCGAGAAGGAGAAGCTCAAGTCCCAGTGGAACAACGATAACCCTCTTTTCAAGAGTGCCACCACGACAGTCATGAACCCTAAGTTTGCCGAGAGTTAGGGGTGC 2319

2320: CTGGTGAAGACAAGGCCTTCTGCACCACCCAGACGGGAACATGCCCTCTCCTCGTCCCCTCCAGC  2384
```

Fig. 1. The nucleotide and deduced amino acid sequences of *O. aries* CD18 cDNA. The putative leader peptide and transmembrane region are underlined. Nine independent clones were sequenced in both directions. The AG$_{(n)}$AC motif is double-underlined. Sequence data have been deposited at GenBank under accession no. AY484425 and AY484426.

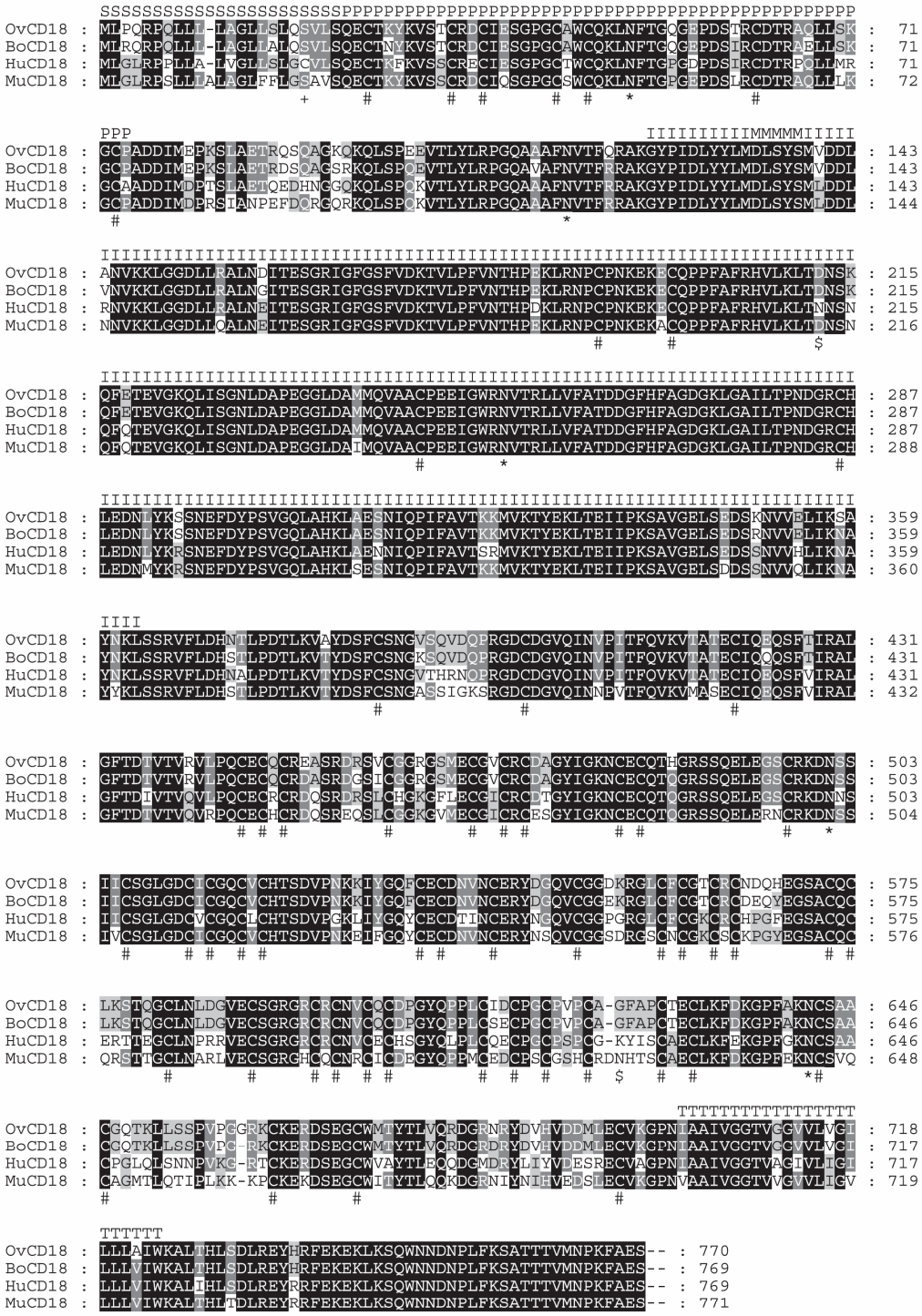

Fig. 2. Comparison of the ovine (Ov-), bovine (Bo-), human (Hu-) and murine (Mu-) β_2 subunits (CD18) amino acid sequences. The letters in the top row identify the constitutive blocks: putative signal peptide (S), plexin–semaphorin–integrin domain (P), I-like domain (I), MIDAS-like stretch (M) and transmembrane region (T). Black, dark and light gray columns represent identity among 4, 3 and 2 species, respectively. Conserved (#) and additional (+) cysteine residues, along with conserved (*) and additional ($) potential *N*-glycosylation sites are marked at the bottom of the alignment.

Table 1
Between-species percent identities of CD18 constitutive blocks

Block	Ov vs. Hu (%)	Ov vs. Mu (%)	Ov vs. Bo (%)
Overall	83	81	95
Putative signal peptide	68	60	90
Extracellular region/overall	82	80	95
Extracellular region/PSI domain	78	88	94
Extracellular region/I-like domain	94	93	98
I-like domain/MIDAS-like motif	100	100	100
Transmembrane region	86	82	95
Cytoplasmic tail	95	95	100

Ov, Hu, Mu and Bo: ovine, human, murine and bovine CD18, respectively.

bovine (Shuster et al., 1992) CD18 proteins is quite similar (Fig. 2). Sequence comparisons between OvCD18 and its human, murine and bovine counterparts shows overall 83%, 81% and 95% identity, respectively, with the highest identity for the MIDAS-like motif (100%), the cytoplasmic tail (>95%) and the I-like domain (>93%) and the lowest identity for the signal peptide and the plexin semaphorin integrin domain (Table 1). The very high interspecies conservation of the putative MIDAS-like and I-like domains and the cytoplasmic tail is consistent with an involvement of these regions in the functional activities of β_2-integrins. Overall, the high evolutionary conservation of the I-like domain confirms its importance in β_2-integrins functions, which is compatible with the observation that monoclonal antibodies the epitopes of which were mapped within this region inhibit binding of LFA-1 to ICAMs 1–3 (Huang et al., 2000). The maximum conservation being observed for the CD18 MIDAS-like motif, it is tempting, despite a lack of available experimental evidence, to speculate that it plays a fundamental role in β_2-integrin function. The high degree of conservation in the cytoplasmic tail of CD18, with many Ser, Thr, and Tyr residues, is compatible with the important role that phosphorylation of these residues plays in regulating adhesive activity (Chatila et al., 1989) and with the observation that cytoplasmic domain truncation of CD18 markedly diminishes binding of LFA-1 to ICAM-1 (Hibbs et al., 1991). Every cysteine residue in the ovine extracellular portion of mature CD18 is present at the same location in human, murine and bovine CD18, which is consistent with a role in maintaining the global structure of the protein. Similarly, all five potential Asn-glycosylation sites observed in sheep are present at the same location in other species.

This study reports for the first time the isolation and sequencing of the ovine β_2-integrins β subunit (CD18) cDNA, and demonstrates that, despite some focal differences, it shares all the main characteristics of its human, bovine and murine homologs. The functional importance of the MIDAS-like motif and of the I-like domain has been emphasized on the basis of an exceptional degree of sequence conservation between mammals. The sequence data provided here will allow successful cloning and expression of sheep CD18 *in vitro* as a tool to examine the specificities of inflammation in the ovine species.

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Characterization of the caprine (*Capra hircus*) beta-2 integrin CD18-encoding cDNA and identification of mutations potentially responsible for the ruminant-specific virulence of *Mannheimia haemolytica*

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Summary

The leukocyte integrins play a critical role in a great number of cellular adhesive interactions during the immune response. We describe here the isolation and characterization of the caprine β_2 (CD18) sub-unit, common to the leukocyte β_2-integrin family. The deduced 770-amino-acid sequence reveals a transmembrane protein with 80, 81, 83, 96 and 99% identity with its canine, murine, human, bovine and ovine homologues respectively. Analysis of CD18 sequences emphasizes the functional importance of the β_2 sub-unit I-like domain, and included metal ion-dependent adhesion site-like motif and confirms that of the cytoplasmic tail. Moreover, comparisons of ruminant versus non-ruminant CD18 sequences allowed the identification of 16 potential mutation sites that could be held responsible for the unique virulence of *Mannheimia haemolytica* for ruminants. Mannheimiosis is known to be the major respiratory disease among ruminants, whereas it is not pathogenic for other mammals, an observation that has been attributed to a specific interaction between *M. haemolytica* leukotoxin and ruminants' CD18. Therefore, the data provided here offer the possibility to explore new avenues in studies based on the caprine model and provide key information for future studies aimed at elucidating the molecular mechanisms underlying the ruminant-specific virulence of *M. haemolytica*.

Keywords: Goat, CD18, integrins, *Mannheimia haemolytica*.

Abbreviations: Bov, bovine; can, canine; cap, caprine; hum, human; mur, murine; ovi, ovine; EGF, epidermal growth factor; ICAM, intercellular adhesion molecule; kan, kanamycin; LFA, lymphocyte function-associated antigen; PMA, phorbol myristate acetate; RACE, rapid amplification of cDNA ends; MIDAS, metal ion-dependent adhesion site; PBMC, peripheral blood mononuclear cell.

Introduction

Integrins consist of a 120 to 180 kDa α sub-unit and a 90 to 110 kDa β sub-unit that are non-covalently associated single-pass transmembrane proteins (Springer 1990). The bulk of each integrin sub-unit is extracellular, where it typically functions as a receptor for extracellular matrix molecules or as a counter-receptor for surface proteins of apposed cells (Hynes 1992). Mammalian integrins form several sub-families sharing common β sub-units that associate with different α sub-units. β_2 integrins include four different heterodimers: CD11a/CD18 (Lymphocyte Function-associated Antigen-1), the predominant β_2-integrin; CD11b/CD18, exclusive to granulocytes and monocytes; CD11c/CD18; and CD11d/CD18. The CD11a-d/CD18 heterodimers are expressed on all leukocytes and mediate high affinity adhesion to a variety of cell types that express one or more of the β_2-integrins ligands, intercellular adhesion molecules (ICAM-1 to –5) (Bailly *et al*. 1995, Gahmberg 1997, Tian *et al*. 1997). The adhesion process involving β_2-integrins is a critical step of a wide range of immunological activities, including cross-interaction and cross-stimulation between lymphocytes, phagocytosis of complement-coated targets, regulation of leukocyte traffic between the bloodstream and tissues or cytolysis of target cells.

As the relevance of the goat model for studying leukocyte traffic, diapedesis and pathologic tissue infiltration is well established in such important areas as mastitis (Koul *et al*. 1993, Rana *et al*. 1993, Paape and Capuco 1997, Singh *et al*. 1997) or lentivirus-associated diseases (Jolly *et al*. 1997, Jan *et al*. 2000, Milhau *et al*. 2003), increasing our knowledge about caprine β_2 integrins is of critical importance to offer new possibilities for research and to provide additional insights into those fields. Moreover, the CD18 implication in mannheimiosis—a very important respiratory disease that only targets ruminants (Jensen *et al*. 1976, Edwards 1996, Vogel and Parrott 1994)—has been well established (Jeyaseelan *et al*. 2002, Deshpande *et al.* 2002). The current opinion is that ruminant CD18s, unlike other mammalian CD18s, are able to participate in a ligand–receptor interaction with *Mannheimia haemolytica* leukotoxin, an event that, consequently, leads to extensive necrosis of macrophages and neutrophils, the content of which percolates throughout the lungs and exacerbates the propagation and the severity of lesions. In this context, comparing ruminant and non-ruminant CD18 sequences could open new avenues to identify the key residue(s) that make the difference between mannheimiosis-sensitive and -resistant species.

The purpose of this paper is to report the cloning and sequencing of a cDNA encoding the caprine CD18 (β_2) sub-unit, which so far has never been characterized, along with a comparative sequence analysis with its bovine and ovine (ruminant) and human, murine and canine (non-ruminant) homologues.

Results and discussion

Characterization of CapCD18-encoding cDNA and deduced aa sequence

The cDNA sequence for CapCD18 contains ~3300 bp with an ORF of 2310 bp that codes for 770 aa followed by ~600 bp in the 3′-UTR (Figure 1). The region immediately preceding the ATG start codon is roughly similar to that in

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other species, containing the sequence $AG_{(n)}AC$, where $n=5$ in the mouse (Wilson *et al*. 1989), $n=3$ in the human (Kishimoto *et al*. 1987), $n=4$ in the goat (present study), the ovine (Zecchinon *et al*. 2004) and the bovine (Shuster *et al*. 1992), which pleads for a regulatory role of this motif. The mature CapCD18 contains a 22-aa putative leader peptide, an extracellular domain of 679 residues (23–701), a single hydrophobic transmembrane region of 23 residues (702–724) and a short cytoplasmic tail of 46 residues (Figure 1).

Starting from the N-terminal end, the extracellular region successively encloses a cysteine-rich repeats-containing analogue of the so-called plexin semaphorin integrin (PSI) domain (residues 23–74; Bork *et al*. 1999), an inserted I-like domain (Huang *et al*. 2000) of 240 amino acids (residues 124–363) and a C-terminal cysteine-rich region (Xiong *et al*. 2001, Beglova *et al*. 2002) containing four epidermal growth factor (EGF) domains (449–496, 497–540, 541–581 and 582–617). A putative metal ion-dependent adhesion site (MIDAS)-like D*X*S*X*S motif (Goodman and Bajt 1996) is predicted within the I-like domain (residues 134–138). Overall, the protein contains 56 Cys residues and five N-linked putative glycosylation sites (Asn-X-Thr/Ser), all located within the extracellular region (Figure 2).

General comparison among species

The general organization of CD18 proteins is quite similar (Figure 2). Sequence comparisons between CapCD18 and its bovine (Shuster *et al.* 1992), ovine (Zecchinon *et al*. 2004), human (Weitzman *et al*. 1991), murine (Wilson *et al*. 1989) and canine (Kijas *et al*. 1999) counterparts shows overall 96, 99, 83, 81 and 80% identity respectively, with the highest identity for the MIDAS-like motif (100%), the cytoplasmic tail ($>90\%$) and the I-like domain ($>90\%$) and the lowest identity for the signal peptide, the plexin semaphorin integrin domain and the EGF domains 1, 3 and 4 (Table 1). The very high interspecies conservation of the putative MIDAS-like and I-like domains and the cytoplasmic tail is consistent with an involvement of these regions in the functional activities of β_2-integrins. Overall, the high evolutionary conservation of the I-like domain confirms its importance in β_2-integrins functions, which is compatible with the observation that monoclonal antibodies, the epitopes of which were mapped within this region, inhibit binding of LFA-1 to ICAMs 1–3 (Huang *et al*. 2000). The maximum conservation being observed for the CD18 MIDAS-like motif is consistent with its importance in ligand recognition (Bajt *et al*. 1995, Hogg *et al*. 1999). The high degree of conservation in the cytoplasmic tail of CD18, with many Ser, Thr and Tyr residues, is compatible with the important role that phosphorylation of these residues plays in regulating adhesive activity (Chatila *et al*. 1989) and with the observation that cytoplasmic domain truncation of CD18 markedly diminishes binding of LFA-1 to ICAM-1 (Hibbs *et al*. 1991). Every cysteine residue in the caprine extracellular portion of mature CD18 is present at the same location in bovine, ovine, human, murine and canine CD18, which is consistent with a role in maintaining the global structure of the protein. Similarly, all five potential Asn-glycosylation sites observed in goat are present at the same location in other species.

Comparison between ruminant and non-ruminant species

Multiple comparisons of CD18 sequences have provided a list of 16 positions at which the amino acid residue is identical within each group (ruminant/non-ruminant) but differs between groups (Table 2). One site is located in the transmembrane region and the other 15 in the extracellular part, four of which in the very conserved I-like domain and two, one and one of which in the EGF domains 1, 2 and 3 respectively. These 16 positions were then classified (Table 2) on the basis of their putative impact on protein structure following the scoring matrix BLOSUM 62 (Henikoff and Henikoff 1992, 1993). This blocks substitution matrix is used for sequence alignments where it assigns, for each position, a probability score that is derived from observations of the frequencies with which substitutions are known to occur among consensus blocks within related proteins. Analysis of secondary structure prediction results in the vicinity of these sites did not reveal significant changes. Using published structures of (i) the extracellular segment of human integrin $\alpha V\beta 3$ (PDB access numbers 1M1X, 1JV2 and 1L5G; Xiong *et al*. 2001, 2002) and (ii) the cysteine-rich repeats from human β_2-integrins (PDB access number 1L3Y; Beglova *et al*. 2002), Swiss-PdbViewer enabled us to assign an accessibility pattern (exposed, half-exposed, half-buried and buried) to 12 of the 16 positions, the four remaining not being resoluted in the crystal structure available (Table 2). Four residues appear to be exposed and three to be semi-exposed, one of which (position 80) becoming only exposed in the activated (liganted) form. The exposition on the surface of position 561, located in the EGF 3 domain, could be of special interest since epitopes that become exposed upon integrin activation and residues that restrain activation are defined in EGF domains 2 and 3. More, the structure of this domain has led to the observation that release of contacts with EGF modules 2 and 3 triggers a switchblade-like opening motion extending the integrin into its active conformation (Beglova *et al*. 2002). In the light of the perfect identity between ruminants EGF domain 2, it is unfortunate that residue 533 has not been resolved in the crystal structure.

This study reports for the first time the isolation and sequencing of the caprine β_2-integrins CD18 sub-unit cDNA, and demonstrates that, despite some focal differences, it shares all the main characteristics of several mammalian homologues. The functional importance of the MIDAS-like motif and the I-like domain has been emphasized on the basis of an exceptional degree of sequence conservation between mammals. The sequence data provided here will allow successful cloning and expression of goat CD18 *in vitro* as a tool to examine the specificities of inflammation in the caprine species. In addition, contrasting ruminant and non-ruminant CD18 sequences allowed the identification of 16 potential residues that could be responsible for the ruminant-specific virulence of *M. haemolytica*. With these data in mind, targeted mutagenesis experiments can be designed in which a set of recombinant ruminant CD18 would be constructed and tested in transfection experiments aimed at identifying the precise residue(s) that underlie(s) the virulence of *M. haemolytica*.

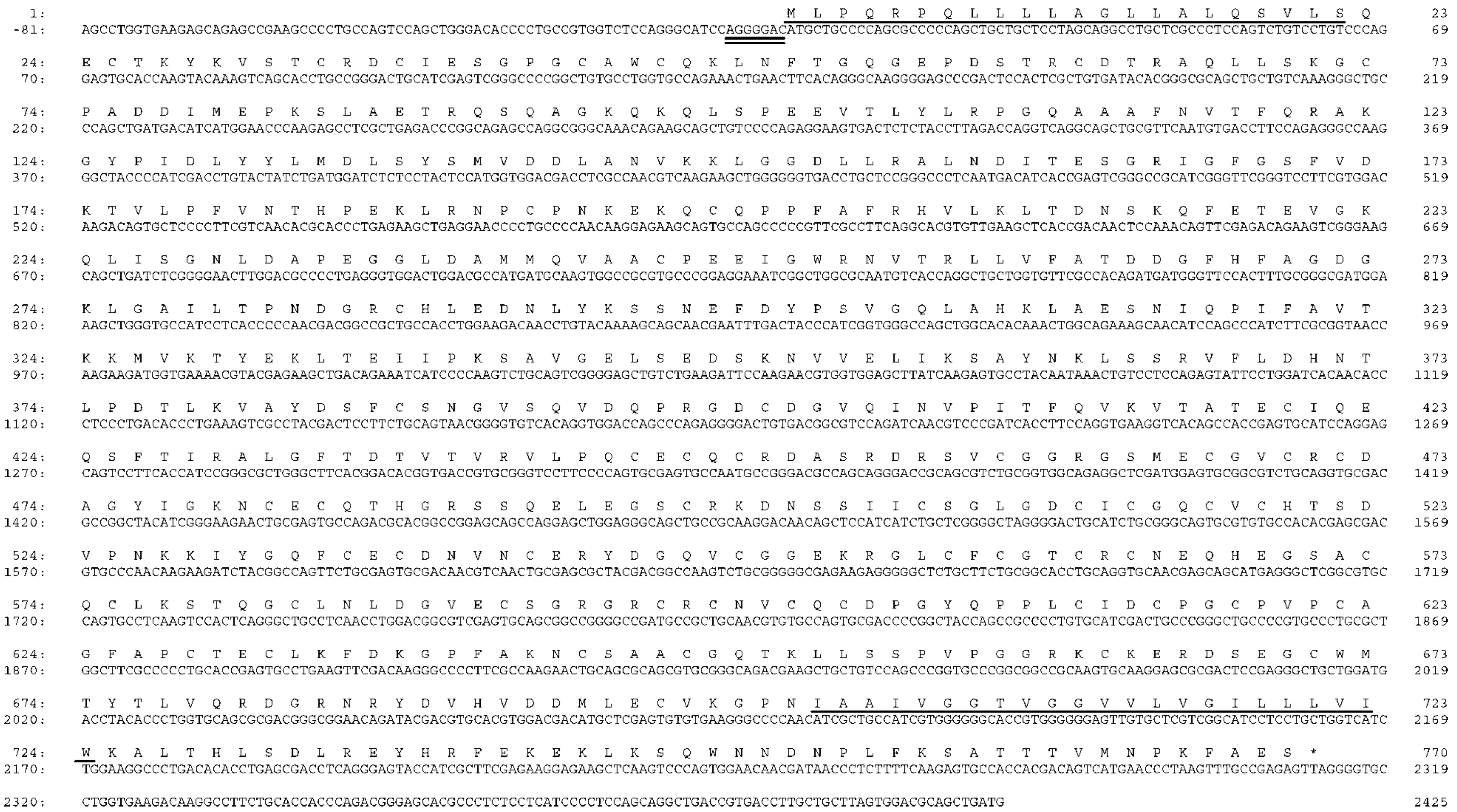

Figure 1. The nucleotide and deduced amino acid sequences of *Capra hircus* CD18 cDNA. The putative leader peptide and transmembrane region are underlined. Six independent clones were sequenced in both directions. The $AG_{(n)}AC$ motif is double-underlined. Sequence data have been deposited at GenBank under accession No. AY452481.

Table 1. Between-species percentage identities of CD18 constitutive blocks.

Block	Cap vs. Bov (%)	Cap vs. Ovi (%)	Cap vs. Hum (%)	Cap vs. Mur (%)	Cap vs. Can (%)
Overall	96	99	83	81	80
Putative signal peptide	95	95	63	60	34
Extracellular region/overall	96	99	82	81	81
Extracellular region/PSI domain	94	100	78	88	88
Extracellular region/I-like domain	97	99	93	93	91
I-like domain/MIDAS-like motif	100	100	100	100	100
Extracellular region/EGF domain 1	93	97	81	75	75
Extracellular region/EGF domain 2	100	100	81	90	81
Extracellular region/EGF domain 3	95	95	70	63	73
Extracellular region/EGF domain 4	94	100	75	69	75
Transmembrane region	100	95	86	82	82
Cytoplasmic tail	100	100	95	95	91

Cap, Bov, Ovi, Hum, Mur and Can: caprine, bovine, ovine, human, murine and canine CD18 respectively; EGF, epidermal growth factor; MIDAS, metal ion dependent adhesion site; PSI, plexin semaphorin integrin.

Experimental procedures

RNA isolation

Total RNA from phorbol myristate acetate (PMA)-stimulated (25 ng/ml for 15 min) caprine (*Boer* breed) peripheral blood mononuclear cells (PBMC) was extracted with TRIzol (Invitrogen) as described by the manufacturer. The PBMCs were obtained by density gradient centrifugation with Ficoll-Paque Plus (Amersham) and maintained in RPMI 1640 supplemented with 10% foetal bovine serum (Gibco BRL), penicillin (100 U/ml) and streptomycin (100 μg/ml) at 37°C in a 5% CO_2 atmosphere.

Amplification of cDNA ends

We used SMART RACE technology (Clontech Laboratories Inc.) to obtain caprine CD18 (CaCD18) 5′- and 3′-ends and RT-PCR to amplify full-length CapCD18 CDS. For first-strand cDNA synthesis, and according to the bovine CD18 sequence available (GenBank No. M81233), gene-specific primers were designed, which were expected to give non-overlapping ~1-kb RACE products: a sense primer for the 3′-RACE PCR: 5′-GAARGAGAARCTCAAGTCC-CARTGGAACAA-3′ (corresponding to nt 2302 to nt 2331) and an anti-sense primer for the 5′-RACE PCR: 5′-ACCTGCAGACGCCG-CACTCCATC-3′ (nt 1496 to nt 1474). Reverse transcription and polymerase chain reactions (PCR) were carried out according to the instruction manual of the SMART RACE cDNA Amplification Kit. The 5′- and 3′-RACE products were gel-purified using the Qiaquick Gel Extraction Kit (Qiagen), TA-cloned into pCRII-TOPO (Invitrogen) and seeded on kanamycin plates. Miniprep were obtained from colonies grown in 5 ml LB-Kan broth and the clones were sequenced on the ABI-3100 Genetic Analyzer using the Big Dye terminator chemistry (Applied Biosystems).

Molecular cloning of full-length cDNA

Total RNA from PMA-stimulated PBMCs was reverse transcribed using Improm II system (Promega). The full-length cDNA was then generated by long-distance PCR using *Taq* polymerase (Promega) with primers designed from the distal ends of both 5′- and 3′-RACE products: 5′-CAGCCTGGTGAAGAGCAGAG-3′ (sense, within the 5′-UTR) and 5′-GCAGAAGGCCTTGTCTTCAC-3′ (anti-sense, 3′-UTR). The procedures recommended by the manufacturer were applied, with these cycling parameters: 2 min 30 s at 94°C, then 35 cycles including (i) 30 s at 94°C, (ii) 30 s at 53°C and (iii) 2 min 30 s at 68°C, followed by a final extension at 68°C for 10 min (*Taq* polymerase, Promega). Resulting PCR products were then processed for sequencing as previously mentioned for the RACE products. The CD18 cDNA sequence was deduced from sequences obtained from six independent clones. Sequence data have been deposited at GenBank under accession No. AY452481.

Table 2. Ruminant- and non-ruminant-specific amino acid substitutions.

Score	Position	Domain	Accessibility	Ruminant AA	Non ruminant AA
−2	620	EC	Half-buried	V	S
−1	295	ID	Buried	S	R
−1	561	EGF 3	Exposed	T	K
−1	692	EC	Exposed	M	S
0	90	EC	Half-exposed	S	D
0	215	ID	Exposed	K	N
0	629	EC	Buried	T	A
1	102	EC	Half-exposed	E	K
1	140	ID	Exposed	V	L
2	80	EC	Half-exposed (NAF); Exposed (AF)	E	D
2	218	ID	Buried	E	Q
2	462	EGF 1	ND	R	K
3	469	EGF 1	ND	V	I
3	533	EGF 2	ND	F	Y
3	687	EC	Buried	V	I
3	715	TM	ND	V	I

Numbering of residue positions is according to bovine CD18. The scoring refers to the results yielded by the BLOSUM 62 matrix and the classification is done following the putative impact on the structure (the lower score, the biggest putative impact). AF, activated form; EC, extracellular region; EGF, EGF domain; ID, I-like domain; NAF, non activated form; ND, not determinable; TM, transmembrane region; AA, amino acid.

Bioinformatics

Primers design was performed with Netprimer (http://www.premier-biosoft.com/netprimer) and Primer 3 (Rozen and Skaletsky 2000). Nucleotidic sequence and similarity analyses were carried out using, respectively, Chromas v.2.21 (http://www.technelysium.com.au) and BLAST programs (Altschul *et al*. 1990). Alignment of amino acids sequences was drawn by GeneDoc v.2.6.002 (Nicholas *et al*. 1997) following the BLOSUM 62 matrix. SignalP v.2.0.b2 (Nielsen *et al*. 1997) and NetNGlyc v.1.0 (Jensen *et al*. 2002) provided peptide signal and N-glycosylation sites prediction respectively. Secondary structure predictions were obtained with GOR IV (Garnier *et al*. 1996, Combet *et al*. 2000) and tertiary structure analysis was done with Swiss-PdbViewer v.3.7 (http://www.expasy.org/spdbv/).

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