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A feline CD2 homologue interacts with human red blood cells

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SUMMARY

A cDNA encoding a feline homologue of CD2 (fCD2) was identified. Several amino acids (aa) important for ligand interaction, molecular folding or signal transduction, found in other mammalian CD2, were found to be highly conserved in the predicted fCD2 aa sequence. fCD2-expressing cells were able to form rosettes with human red blood cells (probably via human CD58), and the rosette formation was inhibited by an anti-fCD2 monoclonal antibody. These results are indicative of the similarity of feline and human CD2 structures. fCD2 was found to be expressed in feline peripheral blood T lymphocytes, monocytes and cultured lymphoid cells.

INTRODUCTION

T-lymphocyte surface CD2 antigen [also called leucocyte function-associated antigen-2 (LFA-2)] is a glycoprotein (of ≈50 000 molecular weight) that is expressed on T cells, natural killer (NK) cells, monocyte lineage cells and thymocytes. On T cells, CD2 functions as an adhesion molecule to bind to target or antigen-presenting cells.[1] In addition to this function, CD2 can also transduce several types of signals in T cells, namely activation[2–5] and negative[6,7] or apoptotic signals.[8,9] In NK cells, anti-CD2 monoclonal antibodies (mAbs) can induce up-regulation of interleukin (IL)-2 receptors, leading to the enhancement of cytotoxic activity,[10] and such effect via CD2 requires co-expression of CD16,[11] whereas CD2-mediated activation of T cells requires CD3 co-expression for its signal transduction.[12] CD2 expression levels on monocytes are lower than on T- or NK cells, and circulating $CD2^+$ and $CD2^-$ monocytes are thought to be dendritic cells and precursors of macrophages, respectively.[13] In the thymus, CD2 plays a role in pre-T-cell antigen receptor (TCR) function in $CD4^-$ $CD8^-$ double-negative thymocytes and TCR selection events during thymocyte development.[14] CD2 expression on murine B cells[15] and human fetal thymic B cells[16] has also been reported, while its function on such cells is unclear.[17]

The main ligand for CD2 is CD58,[1,18] which is broadly distributed, being found on non-haematopoietic as well as haematopoietic cells. Erythrocyte (E)-rosette formation of sheep red blood cells (RBCs) by human T cells,[19] a process widely used to identify human T cells prior to the advent of suitable antibodies, is mainly dependent on binding between CD2 on T cells and CD58 on sheep RBCs.[20–22] No rodent homologue of CD58 has been identified; instead, the structurally related molecule CD48 has been identified as a CD2 ligand in both mice and rats.[1]

CD2 belongs to the immunoglobulin superfamily.[23] An extracellular region of CD2 contains two domains that are flexibly linked, and the GFCC'C'' β-sheet of the first domain (domain 1) is a binding site for its ligands.[1,18] A cytoplasmic region contains proline-rich sequences.[24–28]

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Abbreviations: aa, amino acid; E, erythrocyte; fCD2, feline CD2; FCM, flow cytometry; fPBMCs, feline peripheral blood mononuclear cells; IL, interleukin; mAb, monoclonal antibody; PCR, polymerase chain reaction; RBC, red blood cell; TCR, T-cell receptor.

The nucleotide sequence data reported here have been submitted to the DNA Data Bank of Japan (DDBJ) and are available under the accession number AB062551.

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Several cytoplasmic proteins (p56^{lck}, CD2AP, CD2BP1 and CD2BP2) have been shown to bind to the specific sequences of the CD2 cytoplasmic region, and they are considered to be involved in the signal transduction via CD2.[29–32]

To investigate the feline immune system, especially related to feline immunodeficiency virus infection,[33,34] we have generated mAbs specific for feline immunological molecules.[35–37] In this study, we cloned a cDNA encoding a feline homologue of CD2 (fCD2) and used it to generate mAbs reactive to fCD2. Furthermore, we compared the fCD2 amino acid (aa) sequence with other mammalian homologues to predict its function in the feline immune system. In addition, we analysed fCD2 distribution in feline lymphoid cells.

MATERIALS AND METHODS

Cells

Feline peripheral blood mononuclear cells (fPBMCs) were separated from heparinized peripheral blood of specific pathogen-free cats by Ficoll–Paque™ (Amersham Pharmacia Biotech, Uppsala, Sweden). The fPBMCs were used for E-rosette formation and flow cytometric (FCM) analysis, or for extraction of RNA after 3 days of culture.[38] Human peripheral blood was mixed with the same volume of Alsever's solution and preserved at 4° until used for E-rosette formation.

Identification of fCD2 cDNA

The homologue cloning method[39] by polymerase chain reaction (PCR), using a fPBMC cDNA library, was performed. Briefly, a partial open reading frame (ORF) of *fCD2* cDNA (≈0·4 kb) was first amplified with a primer pair that was designed based on the highly conserved sequences between human[24] and murine[25] *CD2* cDNAs. Next, to analyse regions upstream and downstream of the partial *fCD2* cDNA, PCR was performed with a circularized cDNA library and another primer pair based on the partial *fCD2* sequence. The amplified fragments were cloned into vector pCR2.1 (Invitrogen, Groningen, the Netherlands) and sequenced using the ABI PRIZM® 377 auto sequencer (Perkin-Elmer, Branchburg, NJ). For confirmation of the *fCD2* cDNA sequence identified, the PCR and sequencing were performed three times independently.

Sequence analysis

The nucleotide and predicted aa sequences of the *fCD2* cDNA were analysed using the default settings of the genetic information processing software, GENETYX-MAC version 9.0 (Software Development, Tokyo, Japan). Signal peptides and transmembrane regions of aa sequences were determined by the methods of Nielsen *et al.*[40] and Sonnhammer *et al.*[41], respectively. Human[24] (accession no. M14362), rat[26] (NM 012830), murine[25] (NM 013486) and equine[28] (X69884) *CD2* sequences were used for comparisons with the feline sequence identified.

Expression of fCD2

For expression of fCD2 protein, a primer pair was designed based on the *fCD2* cDNA sequence; fCD2exF (5′-AACTAATCCCAAAGATG-3′) is upstream of the start codon, and fCD2exR (5′-CGATTTCTATCGCTTTTA-3′) is downstream of the stop codon. The PCR product amplified by the primer pair was cloned into pCR2.1, then digested with *Eco*RI, and the resultant *Eco*RI fragment was cloned into the *Eco*RI site of pcDNA3.1/Myc-His(+) A (Invitrogen) to produce the expression plasmid pcfCD2. COS-7 cells were transfected with pcfCD2 by electroporation and cultured for 3 days. An irrelevant expression plasmid, pcfCD80,[42] was used as a negative control.

E-rosette formation

Transfected COS-7 cells or fresh fPBMCs were suspended in phosphate-buffered saline supplemented with 50% heat-inactivated fetal calf serum, mixed with human RBCs[43,44] and incubated for 20 min at 37°. The mixture was then centrifuged and incubated on ice for 2 hr. The pellet was then gently resuspended and observed by microscopy. To analyse the effect of anti-fCD2 mAb on E-rosette formation, cells were incubated with mAb before mixing with human RBCs.

MAb to fCD2

Mouse hybridomas producing anti-fPBMC antibodies were screened using an indirect immunofluorescence assay against pcfCD2-transfected COS-7 cells.[37]

FCM

Single-colour FCM analysis was performed as described previously.[37] For two-colour analysis, anti-feline CD4 mAb 45B4 (IgG2a),[36] anti-feline CD8α mAb 2D7 (IgG2a),[37] fluorescein isothiocyanate-labelled anti-feline CD3ε mAb NZM1 (IgG3) (Y. Nishimura *et al.*, unpublished), phycoerythrin (PE)-labelled anti-feline CD5 mAb f43 (Southern Biotechnology Associates, Birmingham, AL), PE-labelled anti-mouse immunoglobulin G1 (IgG1) antibody (Exalpha, Boston, MA) and PE-labelled anti-mouse IgG2a antibody (Zymed Laboratories, San Francisco, CA) were used.

Northern blot analysis

Total RNA was isolated from a feline renal cell line CRFK,[45] cultured fPBMCs and a feline T-lymphoblastoid cell line MYA-1[46] using ISOGEN-LS (NIPPON GENE, Tokyo, Japan), and analysed by Northern blot analysis using the [α-^{32}P]dCTP-labelled *Hin*fI fragment of *fCD2* cDNA as a probe, as described previously.[47]

RESULTS

Cloning and sequence analysis of fCD2

A cDNA containing a *fCD2* ORF was cloned (see the Materials and methods) and sequenced (≈1·5 kb, DDBJ accession number AB062551). Sequence analysis of the *fCD2* cDNA revealed that it contains an ORF of 1008

nucleotides encoding 336 aa residues. The nucleotide sequence of this *fCD2* ORF has 64–72% identity with human, rat, murine and equine *CD2* sequences. The predicted aa sequence (Fig. 1) consists of a signal peptide of 19 residues, an extracellular region of 203 residues, a transmembrane region of 23 residues and a cytoplasmic region of 110 residues. Six potential *N*-linked glycosylation sites in the extracellular region and nine cysteine residues are present (Fig. 1). The predicted aa sequence shares 57%, 48%, 46% and 57% identity with human, rat, murine and equine CD2 sequences, respectively, and the highest degree of identity (62–71%) is found in the cytoplasmic region.

Interspecies comparison of CD2 sequences

The CD2 aa sequences of five species (cats, humans, rats, mice and horses) were aligned (Fig. 1). One-hundred and fifteen aa are identical among the five species. Six regions are probably important for ligand interaction, molecular folding or signal transduction,[28–31,48–52] which are completely or highly conserved in the feline sequence (Fig. 1). They are:

- L37[h19] (h corresponds to the aa residue of mature human CD2), W52[h35], A60[h45], L83[h68], I85[h70] and Y96[h81], which are the residues important for contact between two β-sheets (GFCC'C'' and BED) of CD2 domain 1 [these residues are shown as (1) in Fig. 1];

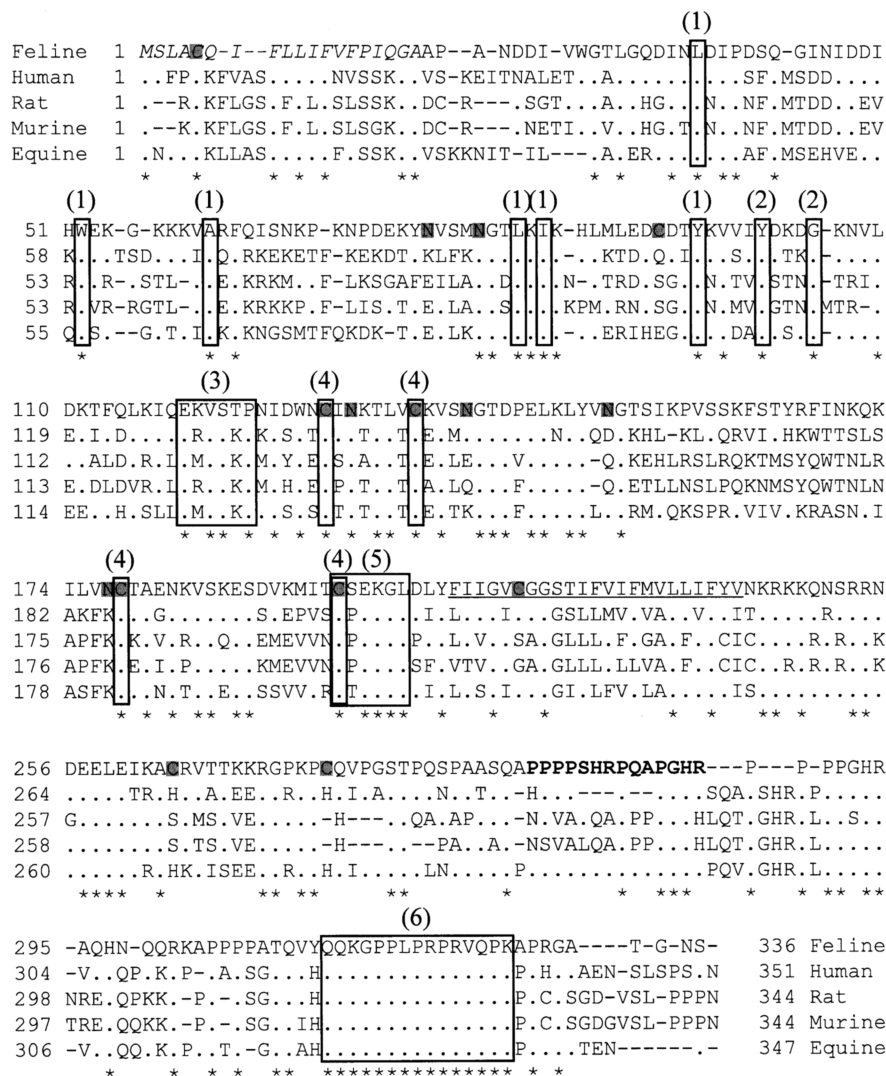

Figure 1. The predicted amino acid (aa) sequence of the feline homologue of CD2 (fCD2) and an interspecies comparison of CD2 molecules. The signal peptide and transmembrane region of feline sequence are in italics and underlined, respectively. Six potential *N*-linked glycosylation sites and nine cysteine residues are shaded. Dots are residues that are identical to fCD2, and the residues completely conserved among five species are indicated by asterisks (*). Completely or highly conserved regions/residues that are boxed have been shown to be important for molecular folding [labelled (1) and (3)–(5)], ligand interaction [labelled (2)], or signal transduction [labelled (6)].[28–31,48–52] The fCD2 sequence in bold is discussed in the text.

- Y101[h86] and G105[h90], which are two residues located within the GFCC'C'' β-sheet of the binding site to CD58 [these residues are shown as (2) in Fig. 1];
- the linker region which gives flexibility between domains 1 and 2 [this region is shown as (3) in Fig. 1];
- four (the third to the sixth) of nine cysteine residues present in the extracellular domain 2-corresponding region (the first cysteine residue is also conserved but positioned in the signal peptide), that would form two disulphide bonds [these residues are shown as (4) in Fig. 1];
- the C-terminal stalk that links the extracellular region to the transmembrane region [this region is shown as (5) in Fig. 1]; and
- 15 contiguous aa residues QQKGPPLPRPRVQPK towards the C-terminus of cytoplasmic domain [this region is shown as (6) in Fig. 1]. The three-dimensional positions of residues 1, 2 and 4 were confirmed by computer modelling (data not shown).

Human and rodent CD2 cytoplasmic regions have been extensively investigated and several regions responsible for signal transduction or binding of cytoplasmic proteins have been determined.[29–32,53] Two PPPGHR repeats are important for human CD2 signal transduction[53] and are the binding sites for the cytoplasmic CD2-binding protein, CD2BP2.[32] Two peptide sequences called 2 (PGHRPLPPSHR) and 4 (QQKGPPLPRPRV) from rat CD2 sequence have the potential to bind to $p56^{lck}$,[29] a protein involved in signal transduction. The peptide sequence 4 is part of the contiguous 15 aa residues described above (Fig. 1) and overlaps the binding sites (PPLPRPR and KGPPLPRPRV) for the CD2-associated protein (CD2AP) and CD2BP1, respectively.[30,31] The cytoplasmic region of fCD2 was aligned along with those of human and rodent CD2, and it was revealed that the cytoplasmic sequences described above are highly conserved in the feline sequence (Fig. 2).

Expression of fCD2 and generation of an anti-fCD2 mAb

Twenty-seven per cent and 22% of COS-7 cells transfected with the fCD2 expression plasmid pcfCD2 (see the Materials and methods) formed rosettes with human RBCs in two independent experiments (Fig. 3b). Using the transfected COS-7 cells for screening antigens, one mAb (SKR2, IgG1)-producing hybridoma was generated. This mAb blocked rosette formation completely between human RBCs and fCD2-transfected COS-7 cells (Fig. 3c) or fPBMCs (data not shown).

fCD2 distribution

To investigate fCD2 distribution on freshly isolated fPBMCs in single-colour FCM analysis, $fCD2^+$ PBMCs were gated (Fig. 4b) and then light scatters (forward- and side scatters) of the gated cells were shown (Fig. 4c, 4d). Two populations of different fluorescence intensities, $fCD2^{high}$ and $fCD2^{low}$, were observed; most of the $fCD2^{high}$ PBMCs were positioned in the lymphocyte area (Fig. 4c), whereas most of the $fCD2^{low}$ PBMCs were in a different area (Fig. 4d). In addition, most of the feline $CD3\varepsilon^+$ or $CD5^+$ PBMCs were positioned in the lymphocyte area (data not shown).

Two-colour FCM analysis of fresh peripheral blood lymphocytes (PBLs), which were gated based on light scatters, revealed that most of the feline $CD5^+$ or $CD3\varepsilon^+$ PBLs corresponded with $fCD2^+$ PBLs (data not shown). Almost all feline $CD4^+$ or feline $CD8\alpha^+$ PBLs were positive for fCD2 (data not shown).

In Northern blot analysis, a single band at ≈1·5 kb was detected from fPBMCs cultured for 3 days and the

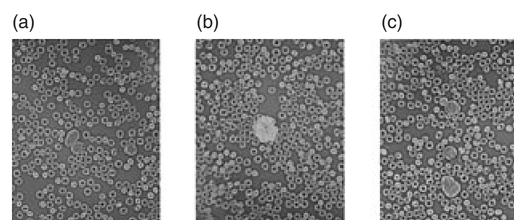

Figure 3. Erythrocyte (E)-rosette formation of COS-7 cells transfected with the expression vector pcfCD80 [negative control: (a)] or pcfCD2 [feline homologue of CD2 (fCD2): (b) and (c)]. The transfected COS-7 cells were reacted with anti-fCD2 monoclonal antibody (mAb) SKR2 before mixing with human red blood cells (RBCs) (c). COS-7 cells and human RBCs can be readily distinguished, the former being larger than the latter.

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Fel 227 NKRKKQNSRRNDEELEIKACRVTTKKRGPKP-CQVPGSTPQSPAASQ-APPPPSHRPQA
Hum 235 T.....R.........TR.H..A.EE..R..-H.I.A....N..T..HP....G..S..
Rat 229 -....R.R..KG.......S.MS.VE.....HSTQASAPASQNPVASQ.....G.HL.T
Mur 229 C..R.R.R..K........S.TS.VE.....HST--PAAAAQNSVALQ.....G.HL.T

Fel 284 PGHRPPPPGHRAQHNQQRKAPPPPATQVYQQKGPPLPRPRVQPKAPRGATGN-S------
Hum 293 .S.........V..QP.KRP.A.SG...H.............P.H..AE.-.LSPSSN
Rat 287 .....L..S..NREH.PK.R...SG...H...............P.C.SGD-V.LPPPN-
Mur 286 .....L.....TREH..K.R...SG..IH...............P.C.SGDGV.LPPPN-
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Figure 2. Alignment of feline, human and rodent CD2 cytoplasmic regions. Dots are residues identical to the feline homologue of CD2 (fCD2). Two repeats of the human PPPGHR motif[32,53] are boxed. Two peptide sequences that are involved in rat $p56^{lck}$ are underlined.[29] Binding sites for CD2AP in the murine sequence[30] and CD2BP1 in the human sequence[31] are shaded. The fCD2 sequence in bold is discussed in the text.

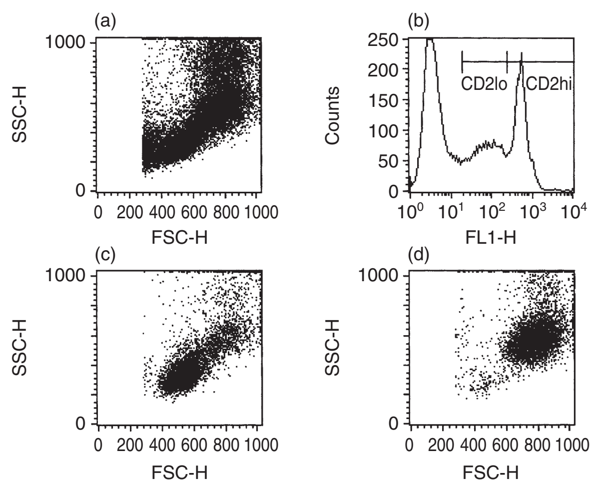

Figure 4. Flow cytometry (FCM) analysis of feline peripheral blood mononuclear cells (fPBMCs). fPBMCs expressing the feline homologue of CD2 (fCD2) (b) were gated and their light scatters are shown: (a) all fPBMCs isolated by Ficoll-Paque™; (c) fPBMCs strongly positive for fCD2; and (d) fPBMCs weakly positive for fCD2.

T-lymphoid cell line MYA-1, but not from the renal fibroblast cell line CRFK (data not shown). The detected size of 1·5 kb was in agreement with the nucleotide length of cloned *fCD2* cDNA (AB062551).

DISCUSSION

Crystallographic, mutational and nuclear magnetic resonance analyses[23,48–52] have revealed several sequences within the extracellular region of CD2 that are responsible for binding to its ligand or its intramolecular bonds. Several regions important for signal transduction present within the CD2 cytoplasmic domain have also been determined.[29–32,53] We found that such regions are highly conserved in fCD2 (Fig. 1, Fig. 2). Identity of fCD2 with human CD2 (57% aa sequence identity) is higher than with rodent CD2 (46–48%); furthermore, fCD2 has an *N*-linked glycosylation site at N80[h65][54] and lacks two membrane-proximal cysteine residues, as does human CD2[55] (Fig. 1). Moreover, fCD2 can bind to human RBCs, probably via human CD58 (Fig. 3), and conserves 10 (D49[h32], K56[h41], K58[h43], K97[h82], Y101[h86], D102[h87] and G105[h90]–V108[h93]; Fig. 1) out of 12 aa residues critical for human CD2 binding to human CD58.[51,52] Taken together, these findings indicate that fCD2 would behave like other mammalian CD2, especially like human CD2, both in receptor–ligand interaction and in signal transduction.

The feline sequence PPPPSHRPQAPGHR in the cytoplasmic region is identical to its equine counterpart (Fig. 1). Tavernor *et al.* discussed this region and suggested that equine CD2 may contain a six aa-insertion (SHRPQA) in the first PPPGHR motif.[28] However, this region of fCD2 is very similar to those of humans and rats (Fig. 2). We think that the inconsistent alignments (Fig. 1, Fig. 2) are caused by the existence of several repeats of similar sequence. In addition, we suggest that a different interpretation may be possible and that the feline and human, and equine sequences have three and four tandem repeats of PPPPGHR motif in this region, respectively.

Cross-species interaction between fCD2 and human CD58 (Fig. 3) may occur via the 10 conserved aa residues of CD2 described above, and the epitope recognized by anti-fCD2 mAb SKR2, which can block interaction between fCD2 and human CD58 (Fig. 3), might include one or more of the 10 residues. However, no cross-reactivity of the SKR2 mAb to human PBL was observed (data not shown), indicating that other residues in the feline original sequence are also involved in the SKR2 epitope.

In FCM analysis using the mAb SKR2, the distribution of fCD2 in feline PBLs was very similar to that of other species, as would be expected (data not shown). Therefore, this mAb will be useful for analysing fCD2 distribution and function on T cells in various viral infections in cats.[33,34] A subpopulation of fPBMCs, which is different from lymphocytes and has a low level of fCD2 (Fig. 4b, 4d), is probably monocytes, judging from the expression level and the position on light scatter plots.[13] Therefore, the mAb SKR2 will also be a useful tool for investigating feline monocytes. Although several forms of human and murine *CD2* mRNA have been reported,[24,25] only one size (of 1·5 kb) was detected in cats. This discrepancy may be a result of sensitivity of the assays used or the origins of the cells from which RNA was prepared.

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