

Cloning, recombinant expression and biochemical characterization of the murine CD83 molecule which is specifically upregulated during dendritic cell maturation

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Abstract Human CD83 (hCD83) is a glycoprotein expressed predominantly on the surface of dendritic cells (DC) and represents the best marker for mature DC. Here, we report the cloning of the cDNA encoding mouse CD83 (mCD83) from a murine bone marrow-derived DC (BM-DC) cDNA library. DNA sequence analysis revealed a 196 amino acid protein including a signal peptide of 21 amino acids which shares 63% amino acid identity with hCD83. Using Northern blot analyses, mCD83 mRNA was found to be strongly expressed in mouse BM-DC and its expression was upregulated following stimulation with LPS or TNF- α . Transfection experiments using COS-7 cells revealed that mCD83 is glycosylated. Furthermore, the extracellular CD83 domain was recombinantly expressed in *Escherichia coli* and one-dimensional NMR data strongly support that the protein is structurally folded.

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Key words: CD83; Dendritic cell; cDNA; Recombinant protein

1. Introduction

Human CD83 (hCD83) is a 45 kDa glycoprotein which belongs to the immunoglobulin superfamily. It is expressed predominantly on the surface of dendritic lineage cells, including skin Langerhans cells, circulating dendritic cells (DC) and interdigitating reticulum cells present in the T-cell areas of lymphoid organs [1–3]. hCD83 is also expressed on monocyte-derived DC after stimulation with inflammatory cytokines [4]. It consists of an extracellular Ig-like domain, a transmembrane domain and a 39 amino acid intracellular domain [2,5]. Although the function of hCD83 is still unknown, its selective expression and the upregulation of the expression of CD83 together with the co-stimulatory molecules CD80 and CD86 during DC maturation suggest an important role of CD83 in the immune response.

Furthermore, in the human system, we could show that inhibition of CD83 expression, by interfering with a specific RNA export pathway, leads to a dramatic reduction of the DC-mediated T-cell stimulation [6].

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Abbreviations: DC, dendritic cells; GM-CSF, granulocyte-macrophage colony-stimulating factor; LPS, lipopolysaccharide; PCR, polymerase chain reaction; TNF- α , tumor necrosis factor α ; NMR, nuclear magnetic resonance

DC are the most potent antigen presenting cells of the immune system. In their immature stage, they reside in peripheral tissues. Upon encounter with antigen, like pathogens or tumor antigens, they migrate to lymphoid organs where they trigger a specific T-cell response [7]. In vitro, immature DC can be generated from peripheral blood monocytes by culturing them in the presence of granulocyte-macrophage colony-stimulating factor (GM-CSF) and interleukin (IL-) 4 [8,9]. These cells show a high endocytic but low T-cell stimulatory capacity. Upon addition of monocyte-conditioned medium (MCM) [10] or a cytokine cocktail (IL-1 β , IL-6, tumor necrosis factor α (TNF- α) and PGE2) that mimicks MCM, these DC develop into fully mature immunostimulatory cells [11], which express high levels of CD83, CD80 and CD86.

Here, we report the molecular cloning of the cDNA encoding mouse CD83 (mCD83). We show that mCD83 mRNA is highly expressed in bone marrow-derived DC (BM-DC) and that in mature DC, its expression is upregulated after stimulation with lipopolysaccharide (LPS) or TNF- α . In addition, we demonstrate that mCD83 is glycosylated when expressed in COS-7 cells. Furthermore, one-dimensional (1-D) nuclear magnetic resonance (NMR) data strongly support that the recombinantly expressed extracellular CD83 domain is structurally folded.

2. Materials and methods

2.1. Generation of a mCD83 specific polymerase chain reaction (PCR) probe

In order to generate a mCD83 specific screening probe, PCR primers were deduced from an EST sequence (accession number AA120620), encoding a partial mCD83 sequence. The following primers were used to amplify a 152 bp fragment: mCD83 sense 5'-CTT GAC ACT CAT CAT TTT CAC CTG CAA-3', mCD83 antisense 5'-ATC CTA CTC ATA CCG TTT CTG TCT TAG-3'. PCR reactions included 0.5 μ M of each primer, 200 μ M dNTP, 1 U Taq polymerase (Amersham Pharmacia Biotech, Freiburg, Germany) in the supplied reaction buffer. The PCR cycling conditions were as follows: 45 s at 94°C, 45 s at 64°C, 90 s at 72°C. The PCR amplification was carried out for 35 cycles and included an initial 5 min denaturation step at 94°C and a final 10 min extension at 72°C. Using these conditions, a mCD83 specific fragment of 152 bp was amplified from a mouse BM-DC cDNA library.

2.2. Isolation of a mCD83 cDNA

The ³²P-labelled 152 bp PCR fragment was used to screen a mouse BM-DC cDNA library [12]. Plaques which were positive after hybridization were purified and in vivo excision was performed. Resulting plasmids were sequenced in both orientations (Sequiderv, Vaterstetten, Germany).

2.3. Generation of mouse DC from bone marrow

BM-DC were generated as described [13]. Briefly, bone marrow cells were plated onto bacteriological petri dishes (Falcon, Becton Dickinson, Heidelberg, Germany) at a density of 2×10^5 /ml in RPMI 1640 medium supplemented with 2 mM L-glutamine (Life Technologies, Karlsruhe, Germany), 100 IU/ml penicillin (Sigma, Deisenhofen, Germany), 100 µg/ml streptomycin (Sigma), 50 µM 2-mercaptoethanol (Sigma) and 10% heat-inactivated fetal calf serum (FCS) (PAA, Cölbe, Germany) (also termed complete medium). GM-CSF was added as culture supernatant from a cell line transfected with the murine GM-CSF gene [14] in a final concentration of 10% which was found to generate BM-DC equally well to 200 U/ml rmGM-CSF. On day 3, cells were fed with 10 ml of fresh medium containing 10% GM-CSF. On day 6, 10 ml of the culture supernatant was collected, centrifuged and the cell pellet resuspended in 10 ml complete medium containing 10% GM-CSF was reseeded into a new dish. On day 7 or 8, non-adherent cells were rinsed off the plates, centrifuged, resuspended in 10 ml complete medium supplemented with 10% GM-CSF and transferred to new dishes. Cells were stimulated with LPS (1 µg/ml, Sigma), TNF- α (500 U/ml, Peprotech/Tebo, Frankfurt, Germany) or α -CD40 (5 µg/ml) (Pharmingen, Hamburg, Germany) for 24–48 h.

2.4. FACS analysis

The maturation and differentiation status of DC was determined by staining for the surface markers CD86 (B7-2, FITC-conjugated, Pharmingen) and MHCII molecules I-A/I-E (2G9, PE-conjugated, Pharmingen). Staining and washing was performed in phosphate-buffered saline (PBS) (136 mM NaCl, 8 mM Na₂HPO₄, 1.5 mM KH₂PO₄, 2.7 mM KCl, pH 7.4) supplemented with 1% FCS. Cells were incubated for 30 min on ice with each antibody in the appropriate dilution. After washing once with PBS, samples were analyzed with a FACScan (Becton Dickinson, Heidelberg, Germany).

2.5. Northern blot analysis

Total cellular RNA was isolated using TriFast reagent (Pqrlab, Erlangen, Germany) according to the manufacturer's instructions. For Northern blots, 15 µg of total RNA was separated on formaldehyde denaturing gels and blotted onto nitrocellulose. Hybridization was performed at 42°C in a solution of 50% (v/v) formamide, 5×SSC (0.75 M NaCl, 0.075 M Na₃O₇C₂H₅), 20 mM Na₂HPO₄/NaH₂PO₄ pH 6.5, 0.025% Denhardt, 10 µg/ml herring sperm DNA, using a 1319 bp mCD83 cDNA fragment as probe. Labelling with [α -³²P]dATP was performed using the Random Prime DNA labelling kit (Boehringer Mannheim, Mannheim, Germany). The filter was washed three times for 20 min at 50°C with 1×SSC and exposed to X-ray film for autoradiography. In order to control for the amount of RNA bound, filters were stripped after the exposure and re-hybridized with a β -actin cDNA probe.

2.6. Antibodies and immunoblotting analysis

The RGS-6×HIS-tag specific antibody was obtained from Qiagen (Hilden, Germany). For immunoblotting, cells were lysed in lysis buffer (1% Nonidet P-40, 150 mM NaCl, 50 mM Tris-HCl pH 7.5, 5 mM EGTA, 5 mM Na₃VO₄, 10 µg/ml leupeptin, 2 µg/ml aprotinin, 1 mM Pefabloc (Boehringer Mannheim)) for 10 min on ice and the insoluble material was removed by centrifugation. Cleared lysates were separated on 15% sodium dodecyl sulfate (SDS)-polyacrylamide gels and blotted onto nitrocellulose filters. The membranes were blocked with 2% BSA in TBST (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.05% Tween 20) for 1 h or overnight. Incubation with specific antibodies was performed overnight at 4°C or for 1 h at room temperature. After washing five times with TBST, filters were incubated with an appropriate secondary antibody coupled to horseradish peroxidase. Immunoreactive bands were visualized using an epichemiluminescence Western blotting system (Amersham Pharmacia Biotech) according to the manufacturer's protocol.

2.7. Expression of in vitro transcribed/translated mCD83

Sequences encoding different domains of mCD83 were cloned in both orientations into the *EcoRI* site of the pcDNA3.1 vector (Invitrogen, San Diego, CA, USA) to generate the plasmids pmCD83s (sense) (amino acids 1–196), pmCD83as (antisense), pmCD83-SPs (amino acids 20–196), pmCD83-SPas, pmCD83 ext. s (amino acids 20–135), pmCD83 ext. as, for in vitro transcription with T7 polymerase. One µg of all plasmids was in vitro transcribed and translated

using the TNT system (Promega, Madison, WI, USA) with [³⁵S]cysteine (Amersham Pharmacia Biotech). The radioactively labelled mCD83 proteins were separated on 15% polyacrylamide gels and visualized by autoradiography.

2.8. Expression of mCD83 in COS-7 cells

COS-7 cells were maintained in DMEM medium supplemented with 10% heat-inactivated FCS, 2 mM glutamine, 100 IU/ml penicillin and 100 µg/ml streptomycin. Transfection experiments were performed using the calcium phosphate precipitation technique. Semi-confluent COS-7 cells were transfected with 15 µg of the expression plasmid encoding mCD83 provided with a C-terminal HIS-tag. After 48 h, the cells were lysed.

2.9. Deglycosylation assay

Lysates of transfected COS-7 cells were incubated with PNGase F (New England Biolabs, Schwalbach, Germany) for 1 h at 37°C in the supplied buffer according to the manufacturer's instructions. Proteins were separated by SDS-polyacrylamide gel electrophoresis (PAGE) and blotted onto nitrocellulose. The immunoreaction was performed with an antibody against the RGS-6×HIS epitope.

2.10. Recombinant expression of the extracellular CD83 domain in *Escherichia coli*

Using the full length mCD83 cDNA clone as a template, the extracellular domain (amino acids 20–135) was PCR-amplified using mCD83 pQE sense 5'-GGG GAT CCA TGG CGA TGC GGG AGG TGA CG-3' and mCD83 pQE antisense 5'-GGG GTA CCT CAT TCT GCC CTG TAC TTC CTG AAA GT-3' as primers. The amplified cDNA fragment was verified by sequencing and cloned into the *Bam*HI and *Kpn*I sites of the expression vector pQE30 (Qiagen, Hilden, Germany) resulting in the plasmid pQE30mCD83 ext. The fusion protein contained six histidine residues at the amino-terminus.

2.11. Purification of the recombinant protein mCD83 ext.

An overnight bacterial culture was diluted 1:60 in fresh LB medium (supplemented with 100 µg/ml ampicillin, 25 µg/ml kanamycin). At an optical density of 0.6, IPTG was added (final concentration 1 mM) and the culture proceeded for a further 4 h. The cells were pelleted and resuspended in 5 ml buffer B (8 M urea, 0.1 M NaH₂PO₄, 0.01 M Tris-HCl) pH 8.0 per g pellet. After 45 min at room temperature while stirring, the lysate was spun at 8000×g. The supernatant was added to Ni²⁺-NTA-agarose (Qiagen) (1.5 ml per g pellet) equilibrated in buffer B and mixed for 45 min at room temperature. The resin was packed into a column and washed with 10 volumes buffer B (pH 8.0) and 10 volumes buffer B (pH 6.3). The protein was eluted with buffer B (pH 4.5) in 1.5 ml fractions. Protein containing fractions were dialyzed against 10 mM Tris-HCl (pH 8.0) and loaded onto an anion exchange column (Mono Q, Amersham Pharmacia Biotech). Proteins were separated by a linear salt gradient (buffer A: 10 mM Tris-HCl, pH 8.0; buffer B: 10 mM Tris-HCl, pH 8.0, 0.5 M NaCl). The correct amino acid sequence of the purified protein was verified by N-terminal amino acid sequencing (Toplab, München, Germany).

2.12. 1-D NMR studies

1-D NMR spectra were recorded with a sample of the extracellular CD83 domain using a Bruker AM 400 spectrometer at a temperature of 300 K. Chemical shift values were determined using acetone as an internal reference at 2.214 parts per million (ppm). Prior to Fourier transformation, the free induction decays were zero-filled from 8192 to 16384 data points and a line broadening factor of −2 Hz was applied.

3. Results and discussion

3.1. Isolation and characterization of a mCD83 cDNA clone

PCR was used to generate a 152 bp mCD83 specific 'screening' fragment using a mouse BM-DC cDNA as template. This 152 bp fragment was then used to screen a mouse BM-DC cDNA library. One full length clone denominated pSB-1, encoding 2051 bp, was isolated and sequenced. It contained an open reading frame of 588 bp encoding a protein of 196 ami-

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gcgctccagccgc
ATGTCGCAAGGCTCCAGCTCCTGTTTCTAGGCTGCGCTGCAGCTGGCACCCGCGATG 60
M S Q G L Q L L F L G C A C S L A P A M
GCGATGCGGGAGGTGACGGTGGCTTGCTCCGAGACCGCCGACTTGCTTGCACAGCGCCC 120
A M R E V T V A C S E T A D L P C T A P
TGGGACCCGCGAGCTCTCCTATGCAGTGTCTGGGCCAAGGTCTCCGAGAGTGGCACTGAG 180
W D P Q L S Y A V S W A K V S E S G T E
AGTGTGGAGCTCCCGGAGAGCAAGCAAAACAGCTCCTTCGAGGCCCCAGGAGAAGGGCC 240
S V E L P E S K Q N S S F E A P R R R A
TATTCCTGACGATCCAAAACACTACCATCTGCAGCTCGGGCACCTACAGGTGTGCCCTG 300
Y S L T I Q N T T I C S S G T Y R C A L
CAGGAGCTCGGAGGGCAGCGCAACTTGAGCGGCACCGTGGTTCTGAAGGTGACAGGATGC 360
Q E L G G Q R N L S G T V V L K V T G C
CCCAAGGAAGCTACAGAGTCAACTTTTCAGGAAGTACAGGGCAGAAGCTGTGTGCTCTTC 420
P K E A T E S T F R K Y R A E A V L L F
TCTCTGGTTGTTTTCTACCTGACACTCATCATTTTCACCTGCAAATTTGCACGACTACAA 480
S L V V F Y L T L I I F T C K F A R L Q
AGCATTTTCCAGATATTTCTAAACCTGGTACGGAACAAGCTTTTCTTCCAGTCACCTCC 540
S I F P D I S K P G T E Q A F L P V T S
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P S K H L G P V T L P K T E T V *

gtaggatctccactggtttttacaaagccaagggcacatcagatcagtggtgcctgaatgc 651
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gtgagcagatacagcgtgcttacctctcagccatgactttcatgctattaaaagaatgc 2031
atgtgaa 2038

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Fig. 1. Nucleotide and predicted amino acid sequence of the mCD83 cDNA clone pSB-1. The deduced amino acid sequence is shown below the nucleotide sequence. Numbers along the right margin designate the nucleotide positions with the translation initiation codon demarking nucleotide 1.

no acids. The complete nucleotide and amino acid sequences are depicted in Fig. 1. The N-terminal 21 amino acids corresponded to a signal peptide resulting in a mature protein of 175 amino acids. The protein was composed of an extracellular domain consisting of a single Ig-like domain, a transmembrane region and a short cytoplasmic domain of 39 amino acids. The cDNA insert contained 13 bp of the 5'-untranslated region and 1447 bp of the 3'-untranslated region. mCD83 and hCD83 share an amino acid identity of 63%.

3.2. *In vitro* transcription/translation of mCD83

Different domains of mCD83 encoding full length mCD83, mCD83 lacking the signal peptide or only the extracellular part of mCD83 were cloned in both orientations into the expression vector pcDNA3.1. Coupled *in vitro* transcription/translation in the presence of [³⁵S]cysteine resulted in the synthesis of radiolabelled proteins of the expected molecular masses (Fig. 2, lanes 1, 3 and 5). In contrast, no signal was detectable when the corresponding control antisense sequen-

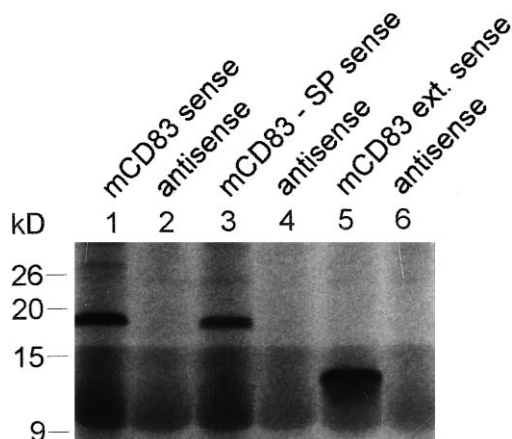


Fig. 2. Autoradiography of reticulocyte extracts expressing sense (lanes 1, 3 and 5) or antisense (lanes 2, 4 and 6) mCD83 constructs. The constructs included either the signal peptide (mCD83) or lacked it (mCD83-SP) or comprised only the extracellular domain (mCD83 ext.). Proteins were separated on 15% SDS-PAGE.

ces were transcribed in this in vitro reaction (Fig. 2, lanes 2, 4 and 6).

3.3. mCD83 is highly expressed in BM-DC and upregulated after stimulation

Since hCD83 was shown to be expressed predominantly on the surface of dendritic lineage cells, we analyzed the expression of mCD83 in mouse BM-DC. Cells were cultured in the presence of GM-CSF to obtain immature DC, under standard conditions or additionally stimulated for 24 h with LPS or TNF- α , to obtain mature DC reflected by increased expression of MHCII and CD86, as analyzed by FACS analyses. Northern blot analyses showed prominent expression of an approximately 2.4 kb specific mCD83 RNA in BM-DC (Fig. 3, lanes 1 and 2). Importantly, after stimulation with LPS (Fig. 3, lane 3) or TNF- α (data not shown), the expression of mCD83 was strongly upregulated. A20, a B-cell line, expressed low levels of mCD83, in the T-cell lines DO11 and 3DO, no mCD83 mRNA was detectable (Fig. 3, lanes 4–6). J774, a monocyte/macrophage-like cell line, showed weak expression of mCD83 mRNA, whereas in EL4, a different T-cell line, no expression could be detected by reverse transcription-PCR (data not shown).

3.4. mCD83 is glycosylated in COS-7 cells

Since mCD83 contains three potential N-linked glycosylation attachment sites in its extracellular domain, we investigated whether or not the protein is glycosylated. Therefore, COS-7 cells were transfected with vectors expressing either the full length mCD83 or the extracellular domain of mCD83 (mCD83 ext.) as C-terminal HIS-tag fusion proteins. After 48 h, lysates were prepared and digested with PNGase F (Fig. 4, lanes 4 and 6) or left untreated (Fig. 4, lanes 3 and 5). Lysates of COS-7 cells transfected with the vector alone served as controls (pcDNA3.1) (Fig. 4, lanes 1 and 2). Proteins were analyzed in Western blots using an antibody directed against the HIS-tag. Undigested proteins migrated at a position corresponding to a molecular weight of approximately 30 kDa (mCD83, Fig. 4, lane 3) and 20 kDa (mCD83 ext., Fig. 4, lane 5), respectively.

In sharp contrast, after digestion with PNGase F, both the

full length protein as well as the extracellular CD83 domain migrated at a lower molecular mass (Fig. 4, lane 4 and 6, respectively). This corresponds to the molecular weight of *E. coli*-expressed CD83 proteins, which are not glycosylated (data not shown). These data clearly show that in COS-7 cells, the extracellular domain of CD83 is glycosylated.

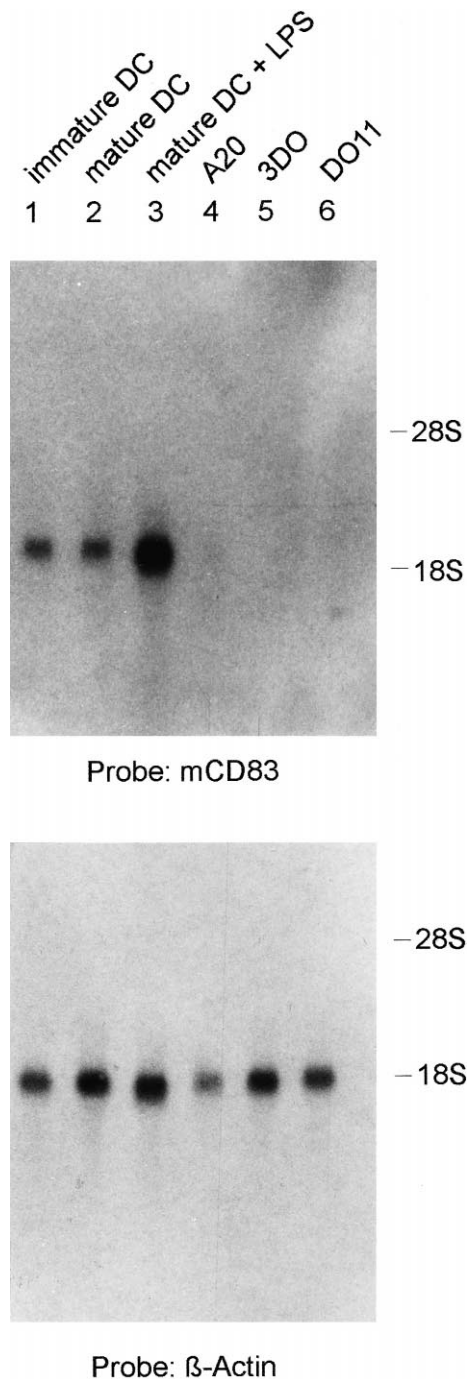


Fig. 3. Northern Blot analysis of total RNA. Fifteen μ g of total RNA was loaded as indicated on top of each lane. Hybridization was performed with a 1319 bp mCD83 cDNA fragment (upper panel). The blot was reprobbed with a β -actin cDNA probe to control for equal RNA loading (lower panel).

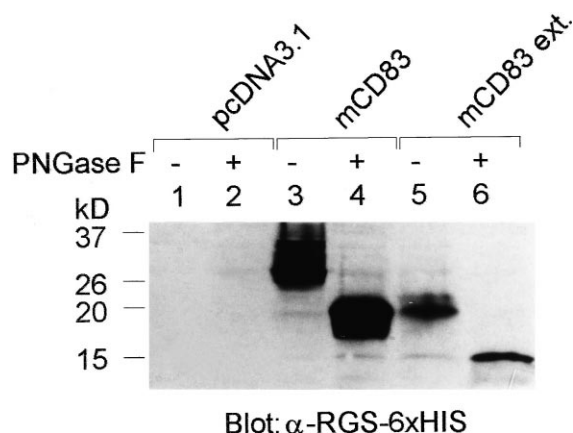


Fig. 4. mCD83 is glycosylated in COS-7 cells. COS-7 cells were transfected with pcDNA3.1, mCD83 or mCD83 ext. Lysates were prepared and treated with PNGase F or left untreated. Proteins were analyzed in Western blotting experiments using the anti-RGS-6 \times HIS antibody.

3.5. Recombinant expression of the extracellular mCD83 domain and 1-D NMR analyses

For NMR studies, the extracellular domain of mCD83 (mCD83 ext.) was expressed in *E. coli* as a 6 \times HIS fusion protein. Since the protein was mainly produced in inclusion bodies, the purification by Ni²⁺-NTA-agarose had to be performed under denaturing conditions. After an additional purification step using an anion exchange column, the protein was more than 95% pure.

The 1-D NMR spectrum of the extracellular CD83 domain at 300 K shows chemical dispersion, i.e. non-random coil peaks, typical of a structured protein (Fig. 5). In particular, the presence of slowly exchanging amide resonances (~ 7 – 9 ppm) indicates that certain parts of the protein back-bone are protected from solvent. Downfield-shifted α -CH resonances (~ 4.5 – 5.7 ppm) are indicative of β -structures. Upfield-shifted methyl resonances ($\sigma < 0.9$ ppm) provide further evidence of the protein being folded. In conclusion, these NMR data strongly support that the recombinantly expressed extracellular domain of CD83 is structurally folded. Therefore, this protein will be used for further functional and structural studies

in order to elucidate the mode of action of CD83 during DC maturation and to determine the three-dimensional structure of CD83.

Here, we report the molecular cloning and characterization of mCD83, a 175 amino acid glycoprotein which is strongly expressed in BM-DC. mCD83 shares 63% amino acid identity with hCD83 with the highest homology present within the transmembrane and cytoplasmic domain. Similar to human monocyte-derived DC, where the expression of hCD83 is up-regulated after cytokine stimulation, we found a strong increase in mCD83 mRNA expression after stimulation with TNF- α or LPS, indicating a function of mCD83 in mature DC. Deglycosylation experiments revealed that mCD83 undergoes prominent post-translational modifications. Future gene targeting experiments will help to elucidate the function of mCD83.

During the completion of this work, another group also reported the cDNA cloning of mCD83 showing the same amino acid sequence [15]. In contrast to the here reported cDNA sequence, which was derived from a full length cDNA clone, the sequence reported by Twist et al. was deduced from a PCR amplification product. Furthermore, the authors did not report any additional characterization regarding the induction of mCD83 in mature DC, nor on post-translational modifications or on recombinant and structural aspects.

Therefore, our data allow for further characterization of mCD83 and the cDNA cloning as well as the recombinant expression of CD83 provide important tools for the functional understanding of CD83 and DC biology.

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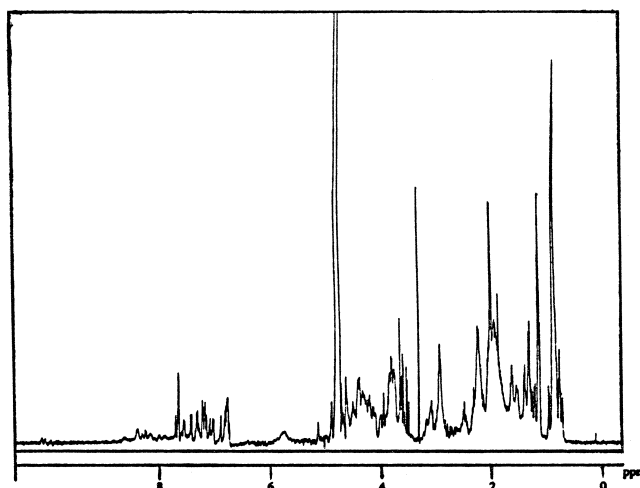


Fig. 5. 1-D NMR analyses of recombinant extracellular mCD83. The 1-D NMR spectrum, recorded at 300 K, shows chemical dispersion, i.e. non-random coil peaks, typical of a structured protein.

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