Overexpression, Purification, and Biochemical Characterization of the Extracellular Human CD83 Domain and Generation of Monoclonal Antibodies

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CD83 is a 45-kDa glycoprotein and member of the immunoglobulin (Ig) superfamily. It is the best known marker for mature dendritic cells. Although the precise function of CD83 is not known, its selective expression and upregulation together with the costimulators CD80 and CD86 suggests an important role of CD83 in the induction of immune responses. To perform functional studies and to elucidate its mode of action it is vital to obtain recombinant expressed and highly purified CD83 molecules. Therefore, the external Ig domain of human CD83 (hCD83ext) was expressed as a GST fusion protein (GST-hCD83ext) and the soluble protein was purified under native conditions. The fusion protein was purified using GSTrap columns followed by anion-exchange chromatography. GST-hCD83ext was then cleaved using thrombin and soluble hCD83ext was further purified using GSTrap columns and finally by a preparative gel filtration as a polishing step and used for further characterization. The purified GST-hCD83 fusion protein was also used to generate monoclonal anti-CD83 antibodies in a rat system. Two different monoclonal antibodies were generated. Using these antibodies, CD83 was specifically recognized in FACs and Western blot analyses. Furthermore, we showed that native CD83 is glycosylated and that this glycosylation influences the binding of the antibodies in Western blot analyses. Finally, the purified hCD83ext protein was analyzed by one-dimensional NMR and these analyses strongly indicate that hCD83ext is folded and could therefore be used for further structural and functional studies.

Key Words: CD83; dendritic cells; recombinant expression; monoclonal antibodies.

Dendritic cells (DC) are the best antigen presenting cells (APC) and since only DC are able to stimulate naive T cells they are also known as “nature’s adjuvant” (1–3). This fact has also led to the use of modified DC for the vaccination of tumor patients, and several encouraging clinical phase I studies have been reported (4, 5). In order to induce potent immune responses DC, which reside in an immature status in the periphery, have to mature. Upon antigen uptake and inflammation DC start to mature and migrate to the T cell areas of the peripheral lymph nodes, where they encounter and stimulate rare antigen-specific T cells. During this maturation process DC induce and express a whole variety of different proteins, including molecules for binding to and stimulation of T cells.

One of the molecules which is specifically and strongly upregulated on mature DC is CD83. In fact, CD83 represents the best known marker for mature human DC. Although the precise function of CD83 is

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2 Abbreviations used: DC, dendritic cells; APC, antigen presenting cells; Ig, immunoglobulin; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; RT, room temperature; rh, recombinant human; MLR, mixed leukocyte reactions.
not known, its selective expression and upregulation together with the costimulators CD80 and CD86 suggest an important role of CD83 in the induction of immune responses (6–9). In addition, we have recently shown that by interfering with the translocation of mRNA encoding CD83 and thus inhibiting CD83 protein synthesis, the T cell stimulatory capacity of DC was significantly reduced (10). Furthermore, we demonstrated a selective downregulation of the cell surface expression of CD83 after the infection of mature DC with herpes simplex virus type 1 and a reduced ability to stimulate allogeneic T cells in mixed leukocyte reactions (11). These data further suggest an important role for CD83. CD83 consist of an extracellular Ig-like domain, a short transmembrane region, and a 39-amino-acid intracellular domain. Recently, the murine CD83 has also been cloned and characterized (12, 13). Murine CD83 shares 63% amino acid identity with human CD83. The amino acids of its extracellular domain show a similarity of 71% to hCD83, while the highest similarity is present in the transmembrane and the cytoplasmic domain. These findings suggest a highly conserved function for human and murine CD83.

Nevertheless, in order to elucidate the function of CD83 it is vital to obtain recombinant expressed and highly purified CD83 molecules. Here we describe the overexpression, purification, and biochemical characterization of the human extracellular CD83 domain. Using native conditions and three purification steps we were able to generate large amounts of highly purified protein which is suitable for further functional and structural studies.

**EXPERIMENTAL**

Cloning of the Extracellular Domain of Human CD83 (hCD83ext)

The extracellular domain of human CD83 (amino acids 23–128) was PCR amplified using the following primers: sense, pGEX2ThCD83, 5'-TCCCCCGGGAA CGCCGAGGTTAGGTGCT-3'; and antisense, CD83extra, 5'-AATTAGAATTCTCAAATCTCCGCTC TGTATT-3'. The amplified fragment was subcloned into the Smal and EcoRI sites of the expression vector pGEX2T (Amersham Pharmacia Biotech, Freiburg, Germany), resulting in the plasmid pGEX2ThCD83ext and transformed into the Escherichia coli strain TOP10F' (Invitrogen, Groningen, The Netherlands). The correct insert was verified by sequencing.

Expression and Purification of hCD83ext

An overnight bacterial culture was diluted 1:10 in fresh LB medium (supplemented with 100 μg/ml of ampicillin). At an optical density (600 nm) of 0.9, 1 mM IPTG was added and the culture proceeded for a further 1 h at 37°C. Then the cells were pelleted and resuspended in 10 ml of native buffer (140 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4, 1.8 mM KH2PO4, 2.6 mM MnCl2, 26 mM MgCl2, 1 μg/ml of leupeptin, 1 μg/ml of aprotinin, 1 μg/ml DNaseI, pH 7.6) per 500-ml culture; 50 μg/ml of lysozyme was also added. After 15 min incubation on ice the lysate was sonicated at four times 10-s bursts and then spun at 20,000g. Protein purification was as follows. In the capture step, 40 ml of supernatant was added to a GSTrap 5-ml column on an AKTA Explorer 10 system (Amersham Pharmacia Biotech). The binding buffer was PBS (140 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4, 1.8 mM KH2PO4, pH 7.6). The elution buffer was 50 mM Tris·HCl, pH 8.0, with 5 mM reduced glutathione. The flow rate was 5 ml/min. The chromatographic procedure included 4 CV (column volumes) of binding buffer, 40 ml of supernatant, 12 CV of binding buffer, 5 CV of elution buffer, 5 CV of 2 M NaCl/PBS, pH 7.6, and 5 CV of binding buffer. For the intermediate purification steps, the GST-hCD83ext containing eluted fractions were pooled and dialyzed against 50 mM 1-methylpiperazine, 50 mM bis-Tris, 25 mM Tris, pH 9.5 (buffer A), and loaded on a Source 15Q PE 4.6/100 anion-exchange column (Amersham Pharmacia Biotech) on an AKTA Explorer 10 system. Proteins were separated by three different linear salt gradients: 16 CV to target concentration 10% buffer B (buffer A/1M NaCl); 20 CV to target concentration 50% buffer B; 10 CV to target concentration 100% buffer B. The GST-hCD83ext containing eluted fractions were dialyzed against PBS, pH 7.6. Then the GST-hCD83ext fusion protein was incubated with 20 U/ml of thrombin at 22°C for 16 h. To separate the hCD83ext protein from GST, the elution was loaded again on a GSTrap 5-ml column using the capture step buffer conditions. Under binding buffer conditions the flow through containing recombinant human CD83ext protein was collected. For the polishing step, finally a preparative gel filtration separation was performed loading the flow through onto a Superdex 75 (26/16) prep grade column (Amersham Pharmacia Biotech) on an AKTA Explorer 10 system, running buffer PBS, pH 7.6, flow rate 3 ml/min.

Production of Monoclonal Antibodies against Human CD83

Approximately 50 μg of the GST-hCD83ext fusion protein was injected intraperitoneal (ip) and subcutaneously (sc) into LOU/C rats. After a 2-month interval, a final boost with the antigen was given ip and sc 3 days before fusion. Fusion of the myeloma cell line P3X63-Ag8.653 with rat immune spleen cells was performed according to standard procedure. Hybridoma supernatants were tested in a solid-phase immunoassay using the GST-hCD83ext protein adsorbed to polystyrene microtiter plates. Following incubation with culture supernatants for 1 h, bound monoclonal antibodies were
detected with peroxidase-labeled goat antirat IgG + IgM antibodies (Dianova, Hamburg, Germany) and o-phenylenediamine as chromogen in the peroxidase reaction. An irrelevant GST fusion protein served as a negative control. The immunoglobulin isotype of the monoclonal antibodies was determined using biotinylated antirat immunoglobulin (IgG) subclass-specific monoclonal antibodies (ATCC, Rockville, MD). CD83-1G11 (rat IgG1) and CD83-4B5 (rat IgG2a) were used for Western blot and FACS analysis.

**Immunoblotting Analyses**

Cell extracts from mature DC or purified hCD83ext protein were resuspended in gel loading buffer (50 mM Tris–HCl (pH 6.8), 2% sodium dodecyl sulfate (SDS), 10% glycerol, 5% β-mercaptoethanol, 0.1% bromphenol blue) and separated by SDS–polyacrylamide gel electrophoresis (PAGE). The proteins were transferred onto nitrocellulose membranes (Amersham). The membranes were blocked with 5% dry milk in TBST (150 mM NaCl, 10 mM Tris–HCl, pH 8.0, 0.05% Tween 20) at 4°C overnight or 1 h at room temperature (RT), incubated for 1 h at RT with the monoclonal anti-CD83 antibodies (1:1000 CD83-1G11; CD83-4B5 both described in this paper; and 1:800 anti-CD83 from Immunotech, Marseilles, France), and washed three times with TBST. Subsequently, the membranes were incubated for 1 h at RT with the secondary antibody coupled to peroxidase. For CD83-1G11 and CD83-4B5 a 1:20,000 dilution of the rabbit antirat IgG antibody (Dianova) was used, while for the anti-CD83 antibody from Immunotech, a 1:10,000 dilution of the goat antimouse IgG antibody (Dianova) was used in TBST plus 5% dry milk. Specific bands were visualized using an enhanced chemiluminescence detection system (Amersham).

**N-Terminal Amino Acid Sequencing**

One portion of the purified extracellular CD83 domain was applied to PAGE, and the proteins were transferred to a PVDF membrane (Immobilon-P, Millipore, Eschborn, Germany). Proteins were visualized by Ponceau red staining, the only visible band was cut out, and N-terminal amino acid sequencing was performed by automated gas phase Edman degradation (Toplab, Martinsried, Germany).

**1D-NMR Studies**

One-dimensional (1-D) 1H-NMR spectra of a 0.7 mM sample of extracellular CD83 domain were recorded at 300 K, pH 6.5, in H2O/D2O (9.1, v/v) on a Bruker DRX600 spectrometer operating at a proton resonance frequency of 600 MHz with a spectral width of 9615.4 Hz and excitation sculpting (14) for water suppression.

**Generation of Dendritic Cells**

Cells were cultured using a standard medium (referred to as 1% human plasma medium), which consisted of RPMI 1640 (BioWhittaker, Verviers, Belgium) supplemented with 300 μg/ml of glutamine (BioWhittaker), 20 μg/ml of penicillin/streptomycin, 10 mM Hepes, pH 7.5 (Sigma-Aldrich, Deisenhofen, Germany), and 1% heat-inactivated (56°C; 30 min) human plasma from a single AB donor, obtained from the Department of Transfusion Medicine, Erlangen, Germany. PBMCs were isolated from buffy coats by sedimentation in Ficol–Hypaque (Amersham Pharmacia Biotech), seeded onto IgG-coated (10 μg/ml of γ-globulin from the Cohn fraction; Sigma-Aldrich) 100-mm culture dishes, and incubated at 37°C in 5% CO2. After 1 and 7 h of incubation, nonadherent cell fractions were harvested, and the remaining adherent cells were further cultured in 1% human plasma medium supplemented with the cytokines 800 U/ml rhGM-CSF (Leukomax; rh, recombinant human) and 1000 U/ml rhIL-4 (Amedak); 5 ml of fresh medium containing 4000 U of rhGM-CSF and 5000 U of IL-4 was added to the culture dish at day 3 of the incubation period. On day 4 or 5, nonadherent cells were collected, counted, and transferred into new dishes at a density of 0.3–0.5 × 105 cells/ml. For final DC maturation, 1% human plasma medium was supplemented with 40 U/ml of rhGM-CSF, 200 U/ml of IL-4, 1.25 ng/ml of rhTNF-α (Boehringer, Mannheim, Germany), 0.5 μg/ml of prostaglandin E2 (Sigma), and 1 ng/ml of IL-1β (Peprotech).

**FACS Analysis**

Phenotypic analyses of cells were performed by flow cytometry using saturating concentrations of CD83 mAbs (CD83-1G11 and CD83-4B5, both described in this paper, and anti-CD83 from Immunotech). The isotype control IgG2b (Becton–Dickinson, Heidelberg, Germany) or rat immune serum was run in parallel. Cells were analyzed on a FACSScan (Becton–Dickinson). Nonviable cells were gated out on the basis of their light scatter properties.

**Glycosylation Assay**

Lysates of mature DC were incubated with Peptide N-Glycosidase F (PNGaseF) (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 and blotted onto nitrocellulose membrane (Amersham). The specific immunodetection was performed with the three anti-CD83 monoclonal antibodies described above.
Allogenic T Cell Proliferation

Human PBMC were isolated from buffy coats and the T cell fraction was purified by rosetting with neuraminidase-treated sheep red blood cells as described by Bender et al. (15). The CD4- and CD8-positive T cells were stimulated at different ratios with mature allogenic DC in a final volume of 200 µl/well of RPMI 1640 supplemented with 5% human serum from a single AB donor in 96-well plates. DC were treated with different concentrations of the newly generated monoclonal anti-CD83 antibodies CD83-1G11 and CD83-4B5 and incubated for 4 days at 37°C. Rat immune serum was used as a negative control. Then the cells were pulsed with 1 µCi/well of [3H]thymidine (Amersham) for 16 h. The culture supernatants were harvested onto glass fiber filters (Printed Filtermat A; Wallac, Turku, Finland) by
RESULTS AND DISCUSSION

Recombinant Expression, Purification, and Characterization of hCD83ext

The coding region of the extracellular human CD83 domain (hCD83ext; amino acids 23–128) was amplified by PCR and cloned into the pGEX-2T vector, generating the plasmid pGEX2T-hCD83ext. This plasmid has an IPTG inducible tac promoter and a thrombin protease recognition site for cleaving the hCD83ext domain from the GST fusion partner. TOP10F' cells were transformed with plasmid pGEX2T-hCD83ext for the expression of the GST-hCD83ext fusion protein. Native purification conditions were used to obtain a correctly folded and purified protein without the need of refolding steps. Affinity chromatography with GST-tagged fusion proteins simplifies purification of large yields of recombinant proteins under mild conditions. Nevertheless, over 80% of the fusion product was expressed in inclusion bodies. To reduce the amount of recombinant protein contained within inclusion bodies, the IPTG controlled expression was induced at an OD of 0.9 for the short time period of 30 to 60 min. Using these conditions yields of 4–5 mg of soluble purified GST-hCD83ext per liter of E. coli culture were achieved.

The fusion protein was first purified using GSTrap columns and the collected fractions were analyzed by PAGE and Western blotting (Fig. 1A). Next, an anion-exchange chromatography step was performed using 50 mM 1-methylpiperazine, 50 mM bis-Tris, 25 mM Tris, pH 9.5, as a broad range buffer and a three-step linear NaCl gradient for elution (Fig. 1B). The collected protein fraction was dialyzed against PBS and concentrated by another affinity chromatography over a GSTrap column. This purified fusion protein was then used for the generation of monoclonal antibodies and for thrombin cleavage.

For functional studies hCD83ext had to be cleaved with thrombin from its fusion partner GST. After incubation with thrombin the cleavage products were loaded onto a GSTrap column and hCD83ext was collected in the flow through while GST was bound to the column (Fig. 1C). As a final polishing step HPLC gel filtration chromatography was applied using a Superdex 75 (26/16) prep grade column. The final yield of purified hCD83ext was 8 mg per 10 liters of culture. The purity was confirmed by SDS–PAGE analysis under reducing conditions and in addition by Western blot analyses using commercially available anti-CD83 antibodies (Fig. 1D). Comparable results were obtained with the two rat anti-CD83 monoclonal antibodies (CD83-1G11 and CD83-4B5) described in this paper (see Fig. 4). Amino-terminal amino acid sequencing analyses further confirmed the correct identity of the purified protein (data not shown).

In order to determine whether the recombinant protein was folded, one-dimensional NMR studies were performed. The 1-D NMR spectrum of hCD83ext at 300 K showed the chemical dispersion typical of a structured protein (Fig. 2). The large dispersion of the amide proton resonances (~7.7 to 9.7 ppm) and the presence of Cα proton resonances downfield from the water signal...
FIG. 3. FACS analyses of mature DC. Nonaivle cells were gated out on the basis of their light scatter properties (SSC-H, side scatter; FSC-H, forward scatter). (A) Mature DC at day 7. CD83-1G11 (7 μg/ml (rat IgG1)) (C) and CD83-4B5 (7 μg/ml (rat IgG2a)) (D) detected specifically CD83 on mature DC. A commercially available anti-CD83 antibody (12.5 μg/ml) (B) (Immunotech) was used as a positive control. The specific antibodies are shown in bold lines, and the individual isotype controls are shown as dotted lines.

\[ \text{(-4.8 to 5.7 ppm)} \]

indicate the presence of a \( \beta \)-sheet-type secondary structure consistent with the expected Ig-like structure. Upfield-shifted methyl resonances (\( \delta < 0.7 \) ppm) provide further evidence of the protein being folded. These NMR data strongly support that the recombinant expressed hCD83ext adopts a defined three-dimensional structure and relevant functional studies can be performed using this protein.

Generation and Testing of Anti-CD83 Antibodies

LOU/C rats were immunized with the fusion protein GST-hCD83ext. To produce monoclonal antibodies, spleen cells of the immunized rats were fused with the myeloma cell line P3X63-Ag8.653 after 2 months. The supernatants were tested in an immunoassay using the GST-hCD83ext fusion protein as a positive control and an irrelevant GST fusion protein as a negative control.

FIG. 4. CD83 expressed on mature human DC is glycosylated. (A) CD83-4B5 and CD83-1G11 detect the recombinant expressed hCD83ext in a Western blot. (B) Lysates of mature human DC (day 7) were prepared and treated with (+) PNGase F or left untreated (-). Proteins were analyzed in Western blot analyses using the CD83-1G11 antibody. When compared with the untreated samples (left) there was a clear shift to a lower molecular weight of the specific CD83 band associated with an increased affinity of this antibody.

The two specific clones CD83-1G11 (rat IgG1) and CD83-4B5 (rat IgG2a) were used for further investigations. Both antibodies could specifically detect CD83 on mature DC in FACS analysis, although CD83-4B5 showed a higher sensitivity when compared with CD83-1G11 and the commercially available anti-CD83 antibody (Fig. 3).

CD83 Expressed on Mature Human DC Is Glycosylated

The two new antibodies (CD83-4B5 and CD83-1G11) also specifically detected the recombinant hCD83ext protein in Western blot analyses when used at a dilution of 1:1000 (Fig. 4A). In contrast, when cellular lysates of mature DC were analyzed only weak signals, in multiple bands between 38 and 50 kDa, were observed when proteins were separated using PAGE under reducing conditions. Similar results were achieved with the commercially available antibody. These different bands, observed between 38 and 50 kDa, could be due to protein degradation or may represent different glycosylation products. Indeed, in a previous report we showed that murine CD83 is in fact glycosylated (13).

As shown in Fig. 4B human CD83 is also glycosylated since after digestion with PNGase F the CD83-specific multiple bands shifted down to a single lower mass band corresponding to the molecular weight of unglycosylated CD83. These data clearly prove that CD83 is glycosylated in mature DC. Interestingly, the binding affinity of CD83-1G11 strongly increased after PNGase treatment, indicating that this posttranslational modification interferes with the antibody detection in this type of immunoassay. Interestingly, in comparison to CD83-1G11, CD83-4B5 and the commercially available antibody did recognize the deglycosylated CD83 protein very weakly (data not shown). This indicates that CD83-1G11 probably recognizes a different epitope, which is only exposed once CD83 is deglycosylated.
In conclusion, we report here the expression, purification, and biochemical characterization of the extracellular domain of human CD83. The initial NMR data suggest that hCD83ext could be used for further functional and structural studies. In addition, two new monoclonal anti-CD83 antibodies, CD83-1G11 and CD83-4B5, were generated using the recombinantly expressed protein. CD83-4B5 is a clear alternative to FACS analysis, while CD83-1G11 could be used for immunoblot studies. Thus, these are very useful tools for further mechanistic studies. Experiments regarding this topic are ongoing in our laboratory. These antibody reagents, combined with recombinant hCD83ext, will help to define the functional role of CD83 and to better understand the development of DC and their role in the interaction with T cells.

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TABLE 1

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<td>—</td>
<td>—</td>
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<td>Wet weight cells</td>
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* Estimated by the method of Bradford.
* Yielding 180 ml of cell lysate after native extraction.
* Thrombin cleavage was performed before loading onto the column and the flow through was collected at this purification step.

FIG. 5. CD83 antibodies did not influence the ability of mature DC to induce allogenic T cell responses. DC were incubated with CD83-1G11, CD81-4B5, or isotype controls, cocultured with allogeneic T cells for 4 days, and pulsed with $[\text{H}]$thymidine for 16 h and then analyzed. Clearly these antibodies had no more effect on the proliferation of T cells than the controls, indicating that they are not inhibitory.

The New Generated Antibodies Are Not Inhibitory in Mixed Leukocyte Reactions

In order to analyze whether these two antibodies could inhibit DC-mediated T cell stimulation, so-called mixed leukocyte reactions (MLR) were performed. This is a very simple and straightforward assay in order to determine the T cell stimulatory capacity of DC. Thus the anti-CD83 antibodies were added in high concentrations to these MLR assays. However, no significant downmodulation of T cell proliferation could be observed (Fig. 5). This lack of inhibition is in agreement with results obtained with the commercially available anti-CD83 antibody. Also, this antibody has no inhibitory function. Nevertheless, these two new antibodies may be useful for the further biochemical characterization of CD83, since a clear difference between glycosylated and unglycosylated CD83 was observed in Western blot analyses.
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