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Characterization of CDw92 as a Member of the Choline Transporter-Like Protein Family Regulated Specifically on Dendritic Cells

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CDw92 is a 70-kDa surface protein broadly expressed on leukocytes and endothelial cells. In this manuscript, we present the molecular cloning of the CDw92 molecule by using a highly efficient retroviral expression cloning system. Sequence analysis of the CDw92 cDNA revealed a length of 2679 bp. The 1595-bp open reading frame encodes a protein of 652 amino acids. Computational analysis of the CDw92 protein sequence indicates 10 transmembrane domains, three potential N-linked glycosylation sites, and an amino acid stretch in the C-terminal region that is related to the immunoreceptor tyrosine-based inhibitory motif. Comparison of the sequence of the CDw92 clone presented in this study with various database entries show that it is a C-terminal variant of human choline transporter-like protein 1, a member of a recently identified family of multitransmembrane surface proteins. Furthermore, we found that CDw92 is stably expressed on monocytes, PBLs, and endothelial cells, as we did not yet find modulation of expression by various stimuli on these cells. In contrast to this factor-independent expression of CDw92, we detected a specific regulation of CDw92 on monocyte-derived dendritic cells (Mo-DCs). Maturation of Mo-DCs by ionomycin or calcium ionophore resulted in down-regulation of CDw92 and incubation of these cells with IL-10 in a specific re-expression. Moreover, targeting of CDw92 on LPS-treated Mo-DCs by CDw92 mAb VIM15b augmented the LPS-induced IL-10 production 2.8-fold. Together, these data suggest a crucial role of the CDw92 protein in the biology and regulation of the function of leukocytes in particular DCs. The Journal of Immunology, 2001, 167: 5795–5804.

The cluster designation CDw92 was first assigned at the Fifth International Workshop on Human Leukocyte Differentiation Antigens (Boston, MA, 1993) to a pair of mAbs termed VIM15 and VIM15b. Both mAbs were produced after immunization of BALB/c mice with cells of the myeloid cell line MV4-11. Comodulation studies showed reciprocal reduction of binding of the respective other mAb, indicating that both are directed to the same molecular species, the CDw92 molecule, which has been identified by immunoprecipitation as a single chain protein of 70 kDa. Cross-inhibition studies revealed that the epitopes recognized by VIM15 and VIM15b are overlapping or are in close proximity. In respect to the staining of cells by the mAbs, the CDw92 molecule was described to be preferentially expressed on human peripheral blood monocytes and neutrophils, as well as several myeloid and T cell lines. Weak expression was reported on PBLs, fibroblasts, endothelial cells, and epithelial cells (1). Furthermore, CDw92 expression was detected on mast cells but not on basophilic cells (2).

In this study, we report the molecular cloning of the CDw92 molecule by using mAbs VIM15 and VIM15b and a retroviral vector-based expression cloning approach, which has been recently established in our laboratory. Furthermore, we show that the CDw92 protein is specifically regulated upon maturation and differentiation of dendritic cells (DCs), and we provide evidence for a role of CDw92 in regulation of expression of IL-10 by DCs.

Materials and Methods

Antibodies

Both CDw92 mAbs VIM15 (isotype IgG1) and VIM15b (isotype IgG2b) were established at our institute by immunization of BALB/c mice with cells of the myeloid cell line MV4-11 (1). The CD4 mAb VIT4, the CD8 mAb VIT8, the CD14 mAb VIM13, the CD15 mAb VIM16, the anti-MHC class II mAb VID1, as well as the negative control mAb VIAP (to calf intestine alkaline phosphatase) were also generated by us. The CD14 mAb MEM-18 was purchased from An der Grub/Scandiv (Vienna, Austria). The hybridomas producing the CD16 mAb 3G8 and the CD19 mAb BU-12, respectively, were obtained from the American Type Culture Collection (Manassas, VA). The negative control mAb APF-01 directed to human α-fetoprotein was a kind gift of V. Horejsi (Institute of Molecular Genetics, Academy of Sciences of the Czech Republic, Prague, Czech Republic).

For the functional assays, the mAbs were used at a concentration of 10 μg/ml. The working dilutions of the mAb preparations contained less than 10 pg/ml LPS. No biological effects of these very low LPS concentrations were detectable in our assay systems.
Cell lines and cell preparations

Cell lines, including human myeloid KG1a cells, Sp2/6 mouse myeloma cells, P815 mouse mastocytoma cells, EL-4 mouse lymphoma cells, CII mouse mast cells, and BW5147 mouse thymoma cells were cultured in RPMI 1640 medium supplemented with 10% FCS in a fully humidified atmosphere of 5% CO\textsubscript{2} at 37°C. Phoenix packaging cells were maintained in RPMI 1640 medium containing 10% FCS. Endothelial cells were cultured in DMEM containing 20% FCS, 1% basic fibroblast growth factor, and 10 µg/ml heparin.

PBMCs were isolated from heparinized whole blood of normal healthy donors by standard density gradient centrifugation using Ficoll-Paque (Pharmacia Biotech, Uppsala, Sweden). To examine CDw92 surface expression on activated PBMCs, PBMCs were seeded in 96-well cell culture plates in RPMI 1640 medium and cultured for 2 days with and without ionomycin (1 µM; Sigma-Aldrich, St. Louis, MO) or PHA (5 µg/ml; Abbott Laboratories, Vienna, Austria) in RPMI 1640 medium supplemented with 5% human AB serum (PAA Laboratories, Munich, Germany). Monocytes were separated from freshly isolated PBMC preparations by magnetic sorting using biotinylated CD14 mAb VIM13 in conjunction with the MACS magnetic bead technology of Miltenyi Biotech (Bergisch Gladbach, Germany). Monocyte-derived DCs (Mo-DCs) were generated by culturing purified blood monocytes for 7 days in RPMI 1640 medium supplemented with 10% FCS and a combination of recombinant human GM-CSF and recombinant human IL-4 (50 ng/ml and 100 U/ml, respectively; kindly provided by the Novartis Research Institute, Vienna, Austria). For some experiments, immature Mo-DCs as well as peripheral blood monocytes were further treated for 1 day with IFN-α (100 U/ml; kindly provided by Dr. G. Adolf, Boehringer Ingelheim, Vienna, Austria), IFN-γ (500 ng/ml; Boehringer Ingelheim), LPS (1 µg/ml, Escherichia coli serotype 0127:B8; Sigma-Aldrich), ionycin (1 µM; Sigma-Aldrich), or calcium ionophore (100 ng/ml; Sigma-Aldrich). For IL-10 (10 ng/ml; R&D Systems, Minneapolis, MN) stimulation, ionomycin- or calcium ionophore-treated Mo-DCs and peripheral blood monocytes were cultured for 1 day in the presence of this IL.

Immunofluorescence analysis

The direct and indirect immunofluorescence techniques were used to determine mAb binding. For indirect immunofluorescence, either Oregon Green-labeled goat (Molecular Probes, Eugene, OR) or FITC-labeled sheep (An der Grub/Scandic) F(ab’)\textsubscript{2} anti-mouse IgG and IgM (H plus L) Abs were used as secondary reagents. The cell staining was assessed by flow cytometry and fluorescence microscopy.

Retroviral cDNA library construction

Total RNA was extracted from human myeloid KG1a cells using the guanidine thiocyanate method as described by Chomczynski and Sacchi (3). Poly(A)\textsuperscript{+} mRNA was isolated using an Oligotex mRNA minikit (Qiagen, Chatsworth, CA) according to the manufacturer’s instructions. Double-stranded cDNA was synthesized from 5 µg of poly(A)\textsuperscript{+} mRNA by using a Pharmacia Biotech cDNA synthesis kit. 5-Phosphorylated adapters were ligated to the 5’ and 3’ ends of the cDNA. After removal of excess adapters by chromatography, the cDNA library was ligated into the retroviral expression vector pBabeMN-lacZ, kindly provided by G. Nolan (Stanford University, Stanford, CA). Then, the cDNA library was used to transform E. coli DH5\textalpha, which were plated on Luria Bertani agar plates containing 100 µg/ml ampicillin (Sigma-Aldrich). Bacteria were harvested from the plates by scraping into Luria Bertani medium, and the plasmid cDNA was extracted by using a Maxi Plasmid Kit (Qiagen, Dassel, Germany). This retroviral library was characterized by −2 × 10\textsuperscript{6} independent bacterial clones and cDNA inserts ranging from 0.8 to 4 kb.

Transfection and infection procedures

One day before transfection, 3 × 10\textsuperscript{5} cells of the ecotropic retroviral packaging cell line Phoenix (kindly provided by G. Nolan) were seeded in 6-cm tissue culture dishes (Nunc, Roskilde, Denmark). Cells were grown overnight and were transfected with Superfect transfection reagent (Qiagen). After 24 h, the culture supernatant containing secreted viruses representing the library was filtered and polybrene (Sigma-Aldrich) was added to a concentration of 10 µg/ml. Five hundred microliters of this viral supernatant was used to infect 5 × 10\textsuperscript{5} mouse target cells (including Sp2/6 mouse myeloma, P815 mouse mastocytoma, EL-4 mouse lymphoma, and BW5147 mouse thymoma cells) in 100 µl of RPMI 1640 medium and 10% FCS in 6-well culture dishes (Nunc). After cultivation for 6 h at 32°C in a 5% CO\textsubscript{2} atmosphere, 2 ml of fresh RPMI 1640 medium containing 10% FCS was added and the cells were grown overnight at 37°C. Afterward, the infected cells were washed and grown in fresh medium for another 2 days at 37°C.

Screening procedure

Infected mouse target cells (2 × 10\textsuperscript{5}) were washed twice with PBS/1% BSA and were incubated with the CDw92 mAbs VIM15 and VIM15b (each 10 µg/ml) for 20 min on ice. Then, the cells were incubated with MACS goat anti-mouse IgG microbeads (Miltenyi Biotec) according to the manufacturer’s instructions. After washing with PBS/1% BSA, cells were resuspended in 500 µl of MACS-sorting buffer (PBS/0.5% BSA/2 mM EDTA) and were loaded onto MACS RS+ selection columns (Miltenyi Biotec) for positive selection of CDw92-transduced cells. Selected cells were maintained in RPMI 1640 medium and 10% FCS and were expanded to a cell number of 2 × 10\textsuperscript{5} and again subjected to MACS sorting. After four cycles of sorting, 60% of the cells (depending on the mouse cell line used) reacted with the CDw92 mAbs VIM15 and VIM15b. One positive single-cell clone was isolated by limiting dilution and was used to recover the CDw92 cDNA.

RT-PCR recovery of the CDw92 cDNA

The CDw92 single-cell clone was expanded and total RNA was extracted. The CDw92 cDNA insert was rescued using RT-PCR (Superscript II RT; Life Technologies, Rockville, MD) and the Advantage-GC polymerase system (Clontech Laboratories, Palo Alto, CA) and primers flanking the multiple cloning site of the retroviral vector pBabeMN-lacZ. The PCR was run for 32 cycles (1 min at 94°C, 1 min at 58°C, and 1 min at 68°C) on a DNA Thermal Cycler (PerkinElmer/Cetus, Norwalk, CT). The amplified 3.4 kb PCR fragment was gel purified and subcloned into pBabeMN-lacZ. The resulting vector (termed CDw92A6) was transfected into Phoenix packaging cells using the DEAE-Dextran transfection method described before (4), with some modifications. Phoenix packaging cells (1.5 × 10\textsuperscript{6}) were suspended in 4 ml of DMEM containing 1% NuSerum (Genome Therapeutics, Waltham, MA), 200 µg/ml DEAE-Dextran, 100 µM chloramphenicol, 100 µM dextrose, and 4 µg of CDw92A6 plasmid DNA. After 2 h of incubation at 37°C, the Phoenix cells were washed with DMEM containing 10% FCS and were transferred into 6-well culture plates (Nunc). After overnight cultivation at 37°C, the medium was exchanged and 1 ml of fresh DMEM containing 10% FCS was added. The cells were then cultured for 2 days and the culture supernatant was used to infect mouse target cells. Reaction of both CDw92 mAbs with these cells was examined at day 3 postfection by immunofluorescence analysis.

Sequence analysis

Sequencing of the cDNA insert of the CDw92A6 plasmid was performed by using an automatic sequencing facility based on a 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA). The putative transmembrane domains (TMDs) of CDw92 and the sequence topography were estimated using TopPred II 1.3 (5). This program was used with the settings for eukaryotic proteins and the Kyte-Doolittle (6) hydrophobicity scale with an upper cutoff of 1.5 for certain TMDs and a lower cutoff of 1.1 for putative TMDs. Also, the Goldman, Engelman, and Steitz (7) hydrophobicity scale with the default setting was applied. In addition, we analyzed the sequence topology by the PHDtopology system (8) (http://www.EMBL-Heidelberg.DE/predictprotein).

Surface labeling of cells and immunoprecipitation analysis

Cells were surface biotinylated using 0.5 mg/ml sulfo-NHS-LC-biotin (Pierce, Rockford, IL) in PBS (pH 8.5). After 50 min of incubation at 4°C, the reaction was stopped by adding 20 mM Tris-HCL (pH 8.2). Cells (1 × 10\textsuperscript{5}) were lysed for 40 min at 4°C in lysis buffer (20 mM Tris-HCL (pH 8.2), 140 mM NaCl) containing 1% NP40 (Pierce) and 0.2% deoxycholic acid (Sigma-Aldrich) as detergents and a mixture of protease inhibitors (1 mM PMFS, 10 µg/ml aprotinin, and 10 µg/ml leupeptin). The cell lysates were centrifuged at 12,000 rpm for 20 min at 4°C and afterward were probed by using an irrelevant mAb coupled to CNBr-Sepharose for 1 h. Then, the lysates were subjected to immunoprecipitation using the Solid Phase Immunoisolation Technique as described (9). Immunoprecipitates were separated by SDS-PAGE using 10% gels under nonreducing conditions followed by transfer to Immobilon P polyvinylidene difluoride membranes (Millipore, Bedford, MA). Membranes were blocked with 5% BSA (100 g/ml; Sigma) for 1 h at room temperature and then incubated with a streptavidin-peroxidase conjugate (Amersham, Aylesbury, U.K.) and the chemiluminescence detection system from Roche Molecular Biochemicals (Mannheim, Germany).

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Determination of cytokine production

Mo-DCs (1 × 10⁶/ml) were cultured with or without LPS (1 µg/ml; Sigma-Aldrich) in the presence of absence of the CDw92 mAbs VIM15 or VIM15b, the anti-MHC-class II mAb VID1, or the control mAb VIAP (to calf intestine alkaline phosphatase) in 24-well plates (Costar, Cambridge, MA). After 48 h, the supernatants were harvested and cytokines were measured by a sandwich ELISA using matched-pair Abs. Capture as well as detection Abs for human IL-1β were obtained from Genzyme (Cambridge, MA), for IL-10 and IL-12-p40 from R&D Systems, and for TNF-α from BD PharMingen (San Diego, CA). As standards, we used human recombinant cytokines purchased from R&D Systems. Assays were performed in duplicates according to the recommendations of the manufacturers. The lower limit of detection was 10 pg/ml for IL-1β and 20 pg/ml for IL-10, IL-12-p40, and TNF-α.

Measurement of choline incorporation

Cells (1 × 10⁶) were incubated in 1 ml of RPMI 1640 medium/10% FCS containing 0.5 µCi of [³⁵S]choline chloride (Amersham) for 10, 30, or 60 min at 37°C. Afterward, the cells were washed twice with PBS. The samples were divided and one-tenth of the cells was used to determine the protein content in a Bradford assay (Pierce) for normalization. The other parts of the cell preparation (nine-tenths) were pelleted and resuspended in protein content in a Bradford assay (Pierce) for normalization. The other parts of the cell preparation (nine-tenths) were pelleted and resuspended in protein.

Results and Discussion

Cellular reactivity of the CDw92 mAbs

The primary cellular reactivities of the CDw92 mAbs VIM15 and VIM15b were described before (1). However, binding analyses to individual subsets of lymphocytes were not performed in detail. Thus, we examined the distribution of CDw92 on human peripheral blood leukocyte subsets by double-staining immunofluorescence analysis using markers to individual leukocyte subsets.

FIGURE 1. Expression pattern of the CDw92 molecule on human peripheral blood leukocytes. PE-labeled CDw92 mAb VIM15 was used in combination with FITC-labeled mAbs to markers of leukocyte subpopulations (CD15, granulocytes; CD14, monocytes; CD19, B cells; CD16, NK cells; and CD4/CD8, T cell subsets) to double stain whole blood samples. Lymphocytes were gated according to their side and forward scatter characteristics before analysis. Immunofluorescence was assessed on a FACScan flow cytometer (BD Immunocytometry Systems, San Jose, CA). Data are representative of three separate experiments.

FIGURE 2. RT-PCR amplification of the CDw92 cDNA. The CDw92 mouse transductant as well as untransduced mouse cells (negative control) were subjected to RT-PCR using primers flanking the multiple cloning site of the vector pBabeMN-lacZ. The 3-kb fragment specifically amplified from the transductant, as well as the size markers, are indicated.

Isolation of the CDw92 cDNA

To study the structure and function of the CDw92 molecule, we isolated the CDw92 cDNA by using a retroviral cloning system that had been recently established in our laboratory. This system is based on the retroviral vector pBabeMN-lacZ and the packaging cell line Phoenix, both of which were developed by G. Nolan and colleagues (10). We constructed a pBabeMN-lacZ retroviral cDNA library from myeloid KG1a cells that strongly expressed the CDw92 molecule. We transiently transfected the library into virus-producing Phoenix cells and used the resulting supernatants (which contain virions representing the library) to infect different mouse target cell lines. Those transduced cells, which bound the CDw92 mAb VIM15, were sorted by using the MACS magnetic beads technology. Upon four MACS-sorting cycles, 60–90% (depending on the mouse cell line used) of cells scored positively with VIM15 mAb.

The MACS-sorted population was subjected to limiting dilution to separate one single-cell clone that was strongly stained by the CDw92 mAbs. This cell clone was expanded and the RNA was isolated to amplify the CDw92 cDNA insert by RT-PCR using primers flanking the multiple cloning site of the retroviral vector. As can be seen in Fig. 2, a 3-kb DNA fragment was obtained. To analyze whether this fragment represents the CDw92 cDNA, we subcloned it back into pBabeMN-lacZ. The resulting plasmid was transfected into Phoenix cells to produce retroviral supernatants for transduction of BW5147 mouse cells. The reactivities of the...
CDw92 mAbs VIM15 and VIM15b with the established transductant were analyzed by immunofluorescence staining and flow cytometric analysis. A strong staining of both mAbs was observed with the transduced cells but not with untransduced BW5147 mouse cells used as a control (Fig. 3). This specific binding of both CDw92 mAbs to the transduced cells indicated that the established plasmid, termed CDw92A6, contained the CDw92 cDNA as insert.

Analysis of the native and recombinant CDw92 molecule by immunoprecipitation

To further prove that plasmid CDw92A6 encodes the CDw92 protein, we compared by SDS-PAGE the recombinant protein encoded by CDw92A6 with the native CDw92 protein on KG1a cells. Lysates of CDw92A6-transduced and -untransduced BW5147 mouse cells as well as of human myeloid KG1a cells were subjected to immunoprecipitation using CDw92 mAb VIM15b. Precipitates were resolved under nonreducing SDS-PAGE conditions. VIM15b immunoprecipitates derived from lysates of CDw92A6-transduced BW5147 cells displayed a polypeptide band of 70 kDa that migrated as the native CDw92 protein precipitated from myeloid KG1a cells. This 70-kDa band was not seen in lysates of untransduced BW5147 cells and in immunoprecipitates obtained by using a control mAb (Fig. 4). Thus, this experiment confirmed that we had cloned the cDNA encoding the CDw92 protein.

Analysis of the nucleotide and deduced amino acid sequence of CDw92

Sequencing of the cDNA insert of plasmid CDw92A6 revealed a length of 2679 bp with an open reading frame of 1959 bp coding for 652 amino acid residues. This sequence has been deposited at the EMBL/GenBank nucleotide sequence database (accession no. AJ272365). The predicted molecular mass of the CDw92 polypeptide chain is 72.98 kDa. This computational calculation in comparison with the molecular mass of the native CDw92 molecule of 70 kDa determined by SDS-PAGE shows that CDw92 is not or is only weakly glycosylated on one of the three potential N-glycosylation sites found in the sequence (Fig. 5).

Hydropathy and topography analyses of the CDw92 protein sequence were performed by using several predictive algorithms. Using the Kyte-Doolittle scale, the TopPred II program (5) predicted eight certain TMDs with hydrophobicity values above 1.5 (TMDs 1–6, 11, and 12) and four putative TMDs with hydrophobicity values between 1.1 and 1.5 (TMDs 7–10; Fig. 5 and Table I). Based on this prediction, 16 different membrane topologies are possible containing either all four (Fig. 6, structure 1), some (Fig. 6, structures 2–15), or none (Fig. 6, structure 16) of the putative TMDs. By applying the “positive-inside” rule, i.e., the observation that positively charged amino acids are more abundant in cytoplasmic segments (11–13), the best topology is predicted for structure 11, which has the largest difference (Fig. 6; KR Diff = 20) of lysines (Fig. 6, K) and arginines (Fig. 6, R) in cytoplasmic over exoplasmic segments. Structure 11 contains 10 TMDs (TMDs 1–7, 9, 11, and 12) and cytoplasmic positions for both the N and C termini (Fig. 6). When we used PHDTopography (8) as second algorithm, a similar 10-transmembrane topology was predicted. According to this model, TMDs 1–4, 6–8, and 11 and 12 and an additional region between amino acids 178 and 195 are membrane integrated. However, the KR difference of this structure is 12 (data not shown). In any case, each algorithm used assigned a cytoplasmic position for both termini. This is of particular interest because the C-terminal segment contains, at position 587–593, the amino acid stretch SIYEMV, which closely resembles the consensus sequence V/I/YSxxL/V of the immunoreceptor tyrosine-based inhibitory motif (ITIM).

ITIMs have been shown to play key roles in down-regulation of kinase-mediated signals of immunoreceptors such as the Ag receptors of T and B cells by recruitment of SH2-containing phosphotyrosines (for review, see 14–16). ITIM-related sequences containing a serine instead of V/I two amino acid positions upstream of the tyrosine as in CDw92 are found in several receptors and signaling molecules including the IL-9R α-chain or the rat mast-cell function-associated Ag (MAFA). Occasionally, serines are also found at the critical position three amino acids downstream of the tyrosine (e.g., in SLAM or IL6-R α-chain). Thus, Sinclair (15) proposed to redefine the consensus sequence of ITIM by V/I/L/SxxL/V/I/S. This consideration has been supported recently by...
the finding that tyrosine-phosphorylated peptides of the putative MAFA-ITIM sequence SIYSTL bound the phosphatases SHP-1, SHP-2, and SHIP (16). This suggests that SIYSTL in MAFA functions as classical ITIM motif, which might be involved in the capacity of MAFA to inhibit the IgE-mediated mast cell degranulation (17).

Therefore, we speculated that the amino acid stretch SIYEMV in CDw92 could link CDw92 to ITIM-mediated signal transduction cascades. To test this, we ectopically expressed CDw92 in the murine mast cell CPII that was stably transfected with a TNF-α/H9251 reporter gene (18), and we analyzed whether incubation by mAbs VIM15 or VIM15b inhibits IgE-mediated activation of this gene in these cells. Application of the mAbs neither cross-linked (using rabbit anti mouse Ig F(ab')2) nor in monomeric form led to a significant inhibition of the FcγRI-dependent transcriptional induction of TNF-α/H9251 compared with an isotype control mAb. In addition, no differences were observed between the CDw92-transfected CPII mast cells vs the parental cell line. To exclude a lack of an ITIM-based negative regulating signaling cascade in this particular cell line, the effect of cross-linking of the FcγRI to the endogenous ITIM-containing FcγRII was studied (19). In this experimental setting, TNF-α/H9251 transcription was reduced to 50% (data not shown).

Interestingly, it was found that CDw92 coprecipitated with protein kinases (20), which are required for phosphorylation of ITIM motifs (21). Thus, it is at the moment not clear whether this ITIM-related sequence plays a role in the function of CDw92.

Comparison of the CDw92 protein sequence

Comparison of the CDw92 protein sequence using the basic local alignment search tool program at the National Center for Biotechnology Information (Bethesda, MD) revealed 99.1% identity to the human choline transporter-like protein 1 (hCTL1). The differences to the protein sequence of hCTL1 are in Cys69 vs Arg, Val 79 vs Ile, Ala 644 vs Ser, and Ile 651 vs Leu, and there are two additional amino acids (Lys 653 and Arg 654) in the C terminus of hCTL1 (Fig. 7).

hCTL1 is the human ortholog of CTL1, a gene that was cloned by O'Regan et al. (22) from an expression library of the electrical lobes of Torpedo marmorata by functional complementation of a choline transport-deficient yeast. In human cells, there were found two further homologs of CTL1, hCTL2, and hCTL4, and additional ortholog and homolog genes were also identified in several other species, including Caenorhabditis elegans, Drosophila, rat, mice, and yeast (22). O'Regan et al. (22) also proposed a 10-transmembrane model for hCTL1. According to the prediction of these authors, hCTL1 uses TMDs 1–4, 6–8, and 10–12 for anchorage in the membrane, though this model has, in contrast to that one proposed by us (Fig. 6), a KR difference of only 16. In any case, each prediction favors a 10-transmembrane segment arrangement for hCTL1/CDw92. Ten-transmembrane helical models are discussed for several transporters such as the bicarbonate transporter (PFAM database, http://www.sanger.ac.uk/cgi-bin/Pfam/) in Table I.

Table I. Characteristics of the potential TMDs of CDw92

<table>
<thead>
<tr>
<th>TMD No.</th>
<th>Probability</th>
<th>Amino Acid Stretch</th>
<th>Hydrophobicity</th>
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<tr>
<td>1</td>
<td>Certain</td>
<td>29–49</td>
<td>2.5052</td>
</tr>
<tr>
<td>2</td>
<td>Certain</td>
<td>213–230</td>
<td>2.8083</td>
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<tr>
<td>3</td>
<td>Certain</td>
<td>239–259</td>
<td>2.2010</td>
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<td>4</td>
<td>Certain</td>
<td>290–310</td>
<td>2.5479</td>
</tr>
<tr>
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<td>Certain</td>
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</tr>
<tr>
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<td>7</td>
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<td>385–405</td>
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<td>567–587</td>
<td>2.7865</td>
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getacc?PF00955), which is the principal regulator of pH in animal cells; the sodium:solute symporters (PFAM database http://www.sanger.ac.uk/cgi-bin/Pfam/getacc?PF00474), which mediate the intake of a wide variety of molecules with the concomitant uptake of sodium ions; or the glucose 6-phosphate transporter (23). Thus, the structural feature of hCTL1/CDw92 together with the finding that expression of Torpedo marmorata CTL1 increased the high-affinity uptake of choline in a yeast mutant suggests a transporter function of hCTL1/CDw92, in particular for choline, in human cells.

To analyze a potential function of CDw92 as choline transporter, we assessed in a first experiment uptake of 14C-choline chloride by CDw92 transductants in comparison with the untransduced parental cells. We found a reproducible 10–15% increased incorporation of 14C-choline by the CDw92-transduced mast cell line CPII, but not by CDw92 BW5147 mouse thymoma transductants. The enhanced 14C-choline uptake was only seen 60 min after onset of the culture and not at other time points. Therefore, this experiment does give a further hint but does not clearly attest to CDw92 as a choline transporter. Inasmuch as several choline transporters do exist (22) that might be able to substitute each other, the revelation of CDw92 as choline transporter requires a separate set of carefully designed experiments.

Comparison of the CDw92 nucleotide sequence

A comparison of the nucleotide sequences of CDw92 and hCTL1 revealed that the discrepancies in the amino acid sequences resulted from the following base changes: codon TGT (Cys69) vs CGT (Arg69), codon GTA (Val79) vs ATA (Ile79), and codon GCC (Ala644) vs TCC (Ser644). Furthermore, a silent point mutation in codon GCC vs GCT (Ala7) was found. Whether these differences reflect a polymorphism or are the result of a cloning artifact is not clear. However, replacement of the C-terminal codons ATT (Ile651) and AAG (Lys652) of CDw92 vs CTG (Leu651), AAG (Lys652), AAA (Lys653), and AGG (Arg654) of hCTL1, and the completely unrelated 3'-untranslated regions show that despite the strong homology, CDw92 and hCTL1 are not identical (Fig. 8).

Nonetheless, both sequence-stretches downstream of Met650 match 100% to segments contained in the genomic clone RP11-287A8 (accession no. AL161627, version GI:10443402). This clone was constructed from chromosome 9 and was sequenced by the Sanger Center Chromosome 9 Mapping Group (Hinxton Hall, UK; http://www.sanger.ac.uk/HGP/Chr9). Although the CDw92 3' segment downstream of Met650 is located between bp 8010 and 8528 of clone RP11-287A8, the hCTL1 3' segment is separated by 48,000 bp and is located between bp 56,387 and 56,610 (Fig. 9). Both 3' segments are flanked by splice junction consensus sequences. This shows that the extreme C termini and the 3'-untranslated regions are determined by individual exons and that the CDw92 and the hCTL1 clone are splice variants. To check whether other 3' exons do exist, we compared the sequence section between the last common exon of hCTL1/CDw92 and the 3' exon of CDw92 (bp 3609–8009 of genomic clone RP11-287A8; Fig. 9) as well as the 48,000 bp spacing the 3' exons of CDw92 and hCTL1 (bp 8,529–56,386 of genomic clone RP11-287A8; Fig. 9) with expressed sequence tag (EST) entries contained in the sequence databases. We found a number of different ESTs.
As can be seen in Fig. 8, inside within the 5′-untranslated region and function.

Sequence is a critical level to control hCTL1/CDw92 protein expression and function. The 3′-untranslated region suggests that alternate usage of these sequences is a critical level to control hCTL1/CDw92 protein expression and function. The 3′-untranslated region is implicated in the stability of mRNA and the regulation of translation (27). The 3′-untranslated region is a multimembrane protein have been identified recently (25). One of these exons also contains in the C-terminal region a consensus PDZ-interaction domain, a motif that appears to be crucial for linking proteins within signaling cascades (26). The 3′-untranslated region is a consensus PDZ-interaction domain, a motif that appears to be crucial for linking proteins within signaling cascades (26).

FIGURE 9. A diagram of the alignment of the CDw92 and hCTL1 cDNA clones. Identities are represented by dots. Positions of nucleotides are indicated on the right.

FIGURE 8. Alignment of the 5′ and 3′ ends of the CDw92 and hCTL1 cDNA clones. Identities are represented by dots. Positions of nucleotides are indicated on the right.

Alignment of the 5′ untranslated regions of CDw92 and hCTL1

Alignment of the 3′ untranslated regions of CDw92 and hCTL1

Not only do complex and extremely stable secondary structures within 5′-untranslated regions stall enzymes used for cloning, but so does the 43S ribosome subunit when scanning the transcript for the AUG start codon in vivo. Thus, proteins whose transcripts form secondary structures within the 5′ end must initiate translation through mechanisms different to the ribosome scanning or cap-dependent mechanism, which is initiated by the eIF-4F protein complex that facilitates binding of the 43S to mRNA (32). One of these alternative mechanisms capable of bypassing the impediment of RNA secondary structures in the 5′-untranslated region is internal ribosome entry. It appeared recently that this interplay between RNA secondary structures and internal ribosome entry guarantees translation of key cellular proteins even under conditions in which overall cellular protein synthesis, including formation of the eIF-4F complex, is compromised. Growth arrest, heat shock, stress, apoptosis, hypoxia, and virus infection are examples of adverse conditions diminishing eIF-4F complex activity. By internal ribosome entry, e-myot was efficiently translated under apoptosis (34) and the angiogenic growth factor VEGF under hypoxic conditions (35). Inasmuch as internal ribosome entry was shown to be important for translation of c-jun that has a similar GC-rich sequence as hCTL1/CDw92 (33), it is tempting to speculate that this mechanism also contributes to the regulation of expression of hCTL1/CDw92. In respect to a putative role of hCTL1/CDw92 in transport of cholines for phospholipid and/or acetylcholine synthesis, one can envisage that efficient translation of hCTL1/CDw92 must also be secured under unfavorable cellular conditions.

Analysis of CDw92 expression upon cell activation/differentiation

Because both the 5′ end and the 3′ end of the hCTL1/CDw92 message suggested a complexity of levels for controlling hCTL1/CDw92 expression, we were keen on studying regulation of CDw92 upon cell activation/differentiation. First, we analyzed the binding of the CDw92 mAbs to differentially activated PBLs in comparison with nontreated cells. No evidence of altered CDw92 binding of the CDw92 mAbs to differentially activated PBLs in comparison with nontreated cells. No evidence of altered CDw92 binding of the CDw92 mAbs to differentially activated PBLs in comparison with nontreated cells. No evidence of altered CDw92 binding of the CDw92 mAbs to differentially activated PBLs in comparison with nontreated cells. No evidence of altered CDw92 binding of the CDw92 mAbs to differentially activated PBLs in comparison with nontreated cells.
GM-CSF plus IL-4 (38). Finally, we examined CDw92 expression on differentially activated peripheral blood monocytes and Mo-DCs. We cultured both types of these cells for 1 day in the presence of LPS, LPS/TNF-α, IFN-α, IFN-γ, calcium ionophore, or ionomycin. On monocytes, CDw92 expression was not modified by these stimuli (data not shown). Also, Mo-DCs did not respond in terms of CDw92 expression to LPS, LPS/TNF-α, IL-10, IFN-γ, or IFN-α (Fig. 11 and data not shown). However, when we treated Mo-DCs for 1 day with ionomycin or calcium ionophore, CDw92 expression was down-regulated. Interestingly, however, CDw92 was reinduced when ionomycin- or calcium ionophore-treated Mo-DCs were cultured in the presence of IL-10 (Fig. 11).

CD83 and CD86 are generally accepted as markers of a mature phenotype of potent immunostimulatory DCs. Both CD83 and CD86 were strongly up-regulated on Mo-DCs upon ionomycin, as well as with LPS treatment (Fig. 11). However, only the Ca^{2+}-releasing agent caused down-regulation of CDw92 demonstrating that different maturation states are induced by ionomycin and LPS in DCs.

**Modulation of DC function by CDw92 mAbs**

Based on this selective regulation of CDw92 on Mo-DCs, we assumed a particular function of CDw92 on these cells. To study this, we used our CDw92 mAbs as surrogate ligands and we analyzed their influence on the function of Mo-DCs. The most interesting finding obtained by these experiments was the 2.8-fold augmentation of LPS-induced production of IL-10 (1167 ± 388 vs 411 ± 41 pg/ml; n = 3) by one of our CDw92 mAbs.
mAbs, VIM15b, with Mo-DCs. VIM15, the second CDw92 mAbs, VIM15b, with Mo-DCs. VIM15, the second CDw92 mAb, had no significant effect on production of these cytokines on monocytes pretreated with or without LPS, IFN-γ, or LPS plus IFN-γ (Fig. 12 and data not shown).

Moreover, we tested both CDw92 mAbs for their capacity to modulate T cell proliferation induced by allogeneic Mo-DCs or IL-10-treated Mo-DCs, their capacity to inhibit CTL induction by immature Mo-DCs, their capacity to influence the GM-CSF plus IL-4-induced differentiation of monocytes to DCs or the LPS-induced maturation of Mo-DCs, and their capacity to affect presentation of tetanus toxoid or mannosylated tetanus toxoid to autologous T cells by Mo-DCs. Finally, we analyzed whether the CDw92 mAbs can modulate Mo-DCs in terms of macroinocytosis using lucifer yellow as read-out reagent and Ag uptake via the mannose receptor using mannosylated BSA-FITC. In none of these assays did the CDw92 mAbs display any modulatory function, indicating that the enhancement of IL-10 production on LPS-treated Mo-DCs by CDw92 mAb VIM15b is quite unique. This finding, together with the reinduction of the CDw92 molecule by IL-10 after ionomycin down-regulation, suggests that CDw92 is part of an autoregulatory signaling loop that controls expression and maintenance of IL-10 production in DCs.

In conclusion, these results indicate a constitutive and relatively stable expression of hCTLL/Cdw92 on various cells of the hematopoietic system, but a subtle regulation by immunomodulating substances, ionomycin, and IL-10 on DCs. Ionomycin is known as a strong activator of DC immune responses, up-regulating Ag-presenting and costimulatory molecules (39 and Fig. 11), whereas IL-10 negatively controls DC responses (40). The stable expression of hCTLL/Cdw92 on various cells of the hematopoietic system, but a subtle regulation by immunomodulating substances, ionomycin, and IL-10 on DCs. Ionomycin is known as a strong activator of DC immune responses, up-regulating Ag-presenting and costimulatory molecules (39 and Fig. 11), whereas IL-10 negatively controls DC responses (40 – 42). The specific regulation by these agents, together with the structural information including the potential ITIM motif and the observed involvement in IL-10 expression by DCs, suggests that hCTLL/Cdw92 might not only act as a mere transporter of choline for membrane phospholipid synthesis of immune cells, but may also be implicated in specific regulation of immune functions, in particular negative signaling pathways.

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References


