Characterization of CD1e, a Third Type of CD1 Molecule Expressed in Dendritic Cells*

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Dendritic cells express several alternatively spliced CD1e mRNAs. These molecules encode proteins characterized by the presence of either one, two, or three α domains and either a 51- or 63-amino acid cytoplasmic domain. Moreover, mRNAs encoding isoforms lacking the transmembrane domain are observed. Several of these CD1e isoforms were expressed in transfected cells, and two of them, with three α domains, displayed a particular processing pathway. These latter isoforms slowly leave the endoplasmic reticulum due to the presence of atypical dilysine motifs in the cytoplasmic tail. These molecules are associated with the β2-microglobulin and accumulate in late Golgi and late endosomal compartments. In the latter compartments, they are cleaved into soluble forms that appear to be stable. In dendritic cells, these isoforms are mainly located in the Golgi apparatus, and upon maturation they are redistributed to late endosomal compartments. This work demonstrates the existence of CD1e molecules. As compared with other CD1 molecules, CD1e displays fundamentally different properties and therefore may represent a third type of CD1 molecules.

CD1 molecules (reviewed in Ref. 1) are nonclassical major histocompatibility complex class I molecules, composed of a membrane-associated heavy chain comprising three immunoglobulin-like extracellular α domains, which associate with β2-microglobulin (β2m). The number of CD1 genes varies among species, only two closely related CD1 genes having been described in mice but five different genes in humans. The human CD1A, B, -C, -D, and -E genes are constitutively expressed in a limited number of cell types, including cortical thymocytes and dendritic cells (DCs), and they can be induced by granulocyte-macrophage colony-stimulating factor in monocytes.

The CD1A, -B, and -C genes encode structurally and functionally related proteins and are classified as type I CD1 genes. CD1a, -b, and -c molecules are found in the plasma membrane and in the endosomal compartments of DCs. However, depending on the DC type and stage of maturation, these molecules display differences in terms of their intracellular localization and traffic (2). These proteins can present glycolipids of microbial origin and, in the case of CD1b, also self-glycolipids (3). The presentation of glycolipids by CD1b is dependent on its internalization into acidic late endosomal compartments and recycling to the cell surface (2). On the basis of homology studies, CD1d has been proposed to represent a second type of CD1 molecule, and structural and functional studies have confirmed this classification. Notably, whereas association with β2m is required for the cell surface expression of type I CD1 molecules (4), human β2m-free CD1d can be expressed at the plasma membrane as a nonglycosylated protein (5). In polarized human epithelial cells, nonglycosylated β2m-free CD1d molecules are observed on the apical cell surface, while glycosylated CD1d molecules are present on both sides of the cells (6). In transfected melanoma cells, β2m-free CD1d molecules expressed at the cell surface are endoglycosidase H (Endo H)-sensitive, while β2m-associated CD1d molecules are Endo H-resistant (7). Thus, three biochemically different forms of CD1d molecules have been described in humans. In human intestinal epithelial cells, CD1d is expressed on the cell surface and internalized into endosomal compartments. This traffic is controlled by a YXXZ motif in the cytoplasmic tail, which is likewise found in CD1b and CD1c (1). The localization of CD1d in basolateral membranes is also regulated by the cytoplasmic domain (8). The murine molecules orthologous to CD1d are the CD1.1 and CD1.2 proteins. In mice, β2m-associated CD1 molecules are expressed at the surface of cells of different types including DCs, while in transfected cells murine CD1 is found on the plasma membrane and in endosomal compartments (9). There is indirect evidence that β2m-free CD1d molecules exist in mice in that CD1-restricted T cells can develop in aged β2m-deficient mice (10). Murine CD1d molecules bind different kinds of hydrophobic antigens, including peptides with hydrophobic anchor residues, glycosyl phosphatidylinositol, and ceramide-containing glycolipids, which are also presented by human CD1d molecules (11–13). The presentation of ceramide glycolipids by mouse CD1 or human CD1d antigens stimulates NKT cells, a T cell subpopulation expressing an invariant T cell receptor α chain and producing interleukin-4 and interferon-γ upon stimulation. In mice, the activation of NKT cells by CD1 proteins appears to play key role in the induction of systemic immune tolerance following immunization through an immune-privileged site (14), although the ligands of CD1 involved in this process are not known. At least in mice, CD1 molecules also stimulate other T cell subsets (15). CD1e molecules have not been studied to date.

The human CD1E gene was described more than 14 years ago and shown to be transcribed in Jurkat and MolT4 tumor T cell lines (16, 17), and several partially characterized tran-
scripts were registered in GenBank™. However, the existence of protein(s) encoded by the CD1E gene has not yet been demonstrated. The aims of the present work were thus to analyze the pattern of CD1e transcripts in DCs, to determine whether CD1e gene product(s) could direct the synthesis of proteins, and to define the different cellular and biochemical properties of CD1e molecules.

**EXPERIMENTAL PROCEDURES**

**CD1e cDNAs Clones and Expression Vectors**—Total RNA from DCs were prepared using RNeasy extraction kit (Qiagen, Les Ulis, France). Reverse transcription of total RNA was reverse transcribed with avian myeloblastosis virus reverse transcriptase (Eurogentec, Seraing, Belgium) using random hexanucleotides as primers. CD1e cDNAs were amplified using 100 ng of reverse transcribed RNA with Taq DNA polymerase (Goldstar, Eurogentec) and GGGGGGATCCCTCTTTAAGAGCTTCA and ATTTTGGGATCATCAGAAG oligonucleotides (94 °C for 30 s, 56 °C for 30 s, and 72 °C for 2 min, 35 cycles). Amplified products were digested with EcoRI and cloned into the homologous restriction sites of pEGFP-N3 (CLONTECH, Palo Alto, CA).

To express CD1e/CD1c or CD1c/CD1e fusion molecules, first a plasmid encoding CD1c with Eco47III and ScaI restriction sites located in the N-terminal and the C-terminal parts of the transmembrane domain, respectively, was constructed. CD1c extracellular domain was obtained by polymerase chain reaction mutagenesis, enodes for the exact sequence of CD1c protein. Plasmids encoding CD1c (extracellular and transmembrane domains)/CD1e (part of the CD1e cytoplasmic domain) fusion molecules were then obtained by inserting fragments encoding part of the CD1c cytoplasmic domain and amplified by polymerase chain reaction in the ScaI site. Before cloning into the ScaI site, the fragments encoding the N-terminal half of the long or short cytoplasmic domain (DSRLKKQSSNKILPSHTPSVFMLGANTQDTKN and DSR-LKKQQFVPLMGANTQDTKN, respectively) were cut with EcoRI and treated with T4 DNA polymerase, producing a stop codon at the end of the fragments. The amino acid sequence of the Eco-end fragment (C-terminal half of the CD1e cytoplasmic domain) fused to CD1c was NSRHHQFLARYVKNVLLKKWKTRNLQWL. The plasmid pL213 encodes the first 305 amino acids of CD1e, four extra unrelated amino acids (DLEAK), and then the amino acids of the transmembrane and cytoplasmic domains of CD1e fused to eGFP.

Natural CD1e isoforms were expressed by inserting the different reconstituted full-length CD1e cDNA clones downstream from the cytomegalovirus promoter of pEGFP-N3 expression vector.

Plasmid cDNAs were transfected in cell lines using Fugene (Roche Diagnostics, Meylan, France) or Exgen (Euromedex, Schlittingheim, France) reagents. Stable M10 transfectants were isolated using 50 μg/ml G418 (Life Technologies, Paisley, UK). The transfected clones expressing the membrane-associated isoforms were selected by immunofluorescence staining on fixed and permeabilized cells using VIIC7 monoclonal antibody (mAb). Clones expressing pL213 were first selected using the presence of eGFP. Fluorescence microscopy showed the hybrid molecule to be expressed on the cell surface.

**Cell Lines and Culture Medium**—HeLa cells were obtained from ATCC (number CCL-2). The melanoma M10 cell line was kindly provided by Dr. T. Hercend (Villejuif, France). HeLa cells were grown in Dulbecco's culture medium, and M10 cells were grown in RPMI 1640, all supplemented with 10% fetal calf serum (Life Technologies). Monocyte-derived DCs and epidermal Langerhans cells (LCs) were prepared as previously (18, 19). Maturation of DCs was induced with 1 μg/ml Escherichia coli LPS (Sigma) or 30 ng/ml TNFα (R&D Systems, Abingdon, UK). Mature LCs were obtained by 48-h culture in RPMI 1640 supplemented with 10% fetal calf serum, 50 ng/ml granulocyte-macrophage colony-stimulating factor (generously provided by Novartis, Rueil Malmaison, France), and 30 ng/ml TNFα.

**Antibodies**—The following mAbs were used: B1G6 (anti-β2m, IgG2a) (Immunotech, Marseille, France); mouse IgG1 (anti-GFP, clones 7.1 and 13.1) (Roche Molecular Biochemicals); L161 (anti-CD1c, IgG1) (Immunotech); W6/32 (pan-anti-HLA class I, IgG2a) (Dako, Trappes, France); biotinylated goat anti-mouse IgG (Pharmingen, San Diego, CA); fluorescein isothiocyanate-conjugated Fab' antibody anti-mouse IgG (Silenus Ltd., Sydney, Australia); phycoerythrin-conjugated Fab' antibody anti-mouse IgG (Jackson Immunoresearch, West Baltimore, PA); Alexa-594-conjugated goat anti-mouse IgG and Alexa-488-conjugated goat anti-mouse IgG (Molecular Probes, Inc., Eugene, OR); and control IgG1 and IgG2a (Immunotech). H5C6 (IgG1, anti-CD63), was kindly provided by Dr. F. Lanza, EFS-Alsace, Strasbourg) and the rabbit anti-EEA1 antiserum (20) by Dr. H. Stenmark (EMBL, Heidelberg, Germany). H5C6 was directly coupled to cyanin 3 using a Cy3 labeling kit (Amersham Pharmacia Biotech) according to the manufacturer's instructions. Polyclonal rabbit IgG against Rab-6 was produced and purified as described (21). Polyclonal rabbit anti-TGN-46 (22) was kindly provided by Dr. J. Lucocq (University of Dundee, United Kingdom), and Dr. S. Carlson, (University of Umea, Sweden). The mAb VIIC7 (IgG1, anti-CD1e cytoplasmic domain) was obtained by immunizing mice with a synthetic peptide (YIKKNRLKKWKLRT, corresponding to amino acids 171–179 of the cytoplasmic domain) coupled to keyhole limpet hemocyanin. Hybridomas were screened with a dot blot assay using a glutathione S-transferase-CD1e cytoplasmic domain fusion protein expressed in E. coli.

The mAbs 1.2 (IgG1), 2.9 (IgG2a), and 20.6 (IgG1 (anti-CD1α domains) were obtained by immunizing mice with an M10 cell line transfected with pL213. Hybridomas were screened by incubating untransfected or pL213-transfected M10 cells first with hybridoma supernatants and then with biotinylated anti-mouse Abs and finally with Cy-Chrome-conjugated streptavidin (Pharmingen). The specificities of these mAbs were determined using HeLa cells transfected with plasmids obtained in the cloning step. All membrane-associated CD1e isoforms were expressed as protein lacking the C-terminal end of the cytoplasmic domain (downstream from the Ero1 restriction site) fused to enhanced green fluorescent protein (eGFP) (CD1e domains). The cells were fixed, permeabilized, and incubated with antibody 1.22, 2.9, or 20.6 followed by Cy3-conjugated anti-mouse Abs. Simultaneous eGFP and Cy3 labeling revealed the specificities of the mAbs for the different membrane-associated isoforms of CD1e.

**Flow Cytometry and Immunostaining**—Cells were washed in cold phosphate-buffered saline (PBS) and incubated with the relevant mAb in PBS for 30 min at 4 °C. In indirect staining experiments, the cells were incubated with fluorescein isothiocyanate- or phycoerythrin-conjugated goat anti-mouse IgG for 30 min at 4 °C. Controls included staining with an isotype-matched irrelevant Ab.

In intracellular staining, the cells were first fixed with 1% paraformaldehyde in PBS for 15 min and washed in cold PBS, and the primary Ab was incubated in staining buffer (2% FCS, 0.2% sodium azide, 0.1% saponin) for 30 min at 4 °C. The cells were then washed twice in saponin buffer (0.1% saponin in PBS), and the secondary Ab was added in the same buffer. The cells were washed twice in saponin buffer and twice in PBS before analysis on a FACScan cytometer (Becton Dickinson).

**Immunofluorescence (IF) microscopy of fixed permeabilized DCs** was carried out as described previously (18). Confocal laser scanning microscopy and IF analyses (24) were performed as described on a Leica TCS4D confocal microscope (Leica Laser Tecknik, Heidelberg, Germany).

**Metabolic Labeling, Immunoprecipitation, and Endo H and F Treatment**—Confluent 75-cm² flasks of transfected or untransfected cells were twice washed in PBS and incubated for 1 h in 20 ml of methionine and cysteine-free medium supplemented with 10% dialyzed fetal calf serum and 1 mM glutamine. The cells were washed twice and labeled with 250 μCi of [35S]methionine and cysteine (Promix; Amersham Pharmacia Biotech) in 3 ml of medium for 30 min to 1 h. The reaction was stopped by the addition of ice-cold PBS followed by two washing steps. After chase in 20 ml of RPMI containing 10% fetal calf serum, the cells were lysed in 1 ml of lysis buffer (20 mM Tris, pH 8.0, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, 1 μg/ml pepstatin, 2 μg/ml leupeptin) for 20 min on ice.

Lysates were centrifuged at 20,000 × g for 15 min and were incubated twice with 50 μl of protein A-Sepharose (Amersham Pharmacia Biotech) for 2 h. Supernatants were incubated with protein A-Sepharose and 5 μg of mAb or irrelevant isotype-matched mAb for 2 h. After extensive washing, the immunoprecipitates were treated or not with Endo H or Endo F (BioLabs, Beverly, CA). Samples were separated on 12.5% SDS-polyacrylamide gel electrophoresis (PAGE) gels under reducing conditions. Gels were treated with Amplify (Amersham Pharmacia Biotech) and exposed for autoradiography.

**RESULTS**

**Alternative Splicing of the CD1E Pre-mRNA**—Since human CD1A, B, and C genes are expressed in DCs, we first tested whether this was the case for the CD1E gene. RNA from monocyte-derived DCs was reverse transcribed, and CD1e cDNA was amplified by polymerase chain reaction using oligonucleotides hybridizing with the 5′- and 3′-untranslated regions of the gene. Several fragments were co-amplified, digested with

**Intracellular Distribution of CD1e**

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Schematic representation of some recombinant CD1e molecules. In order to study the localization of the different isoforms, several mAbs were selected and then tested on transfected cells expressing individual CD1e isoforms, fused or not with eGFP. The eGFP-fused molecules were examined first for practical reasons, as they could be expressed using the recombinant plasmids obtained in the cloning step. Membrane-associated CD1e isoforms expressed using these vectors were fused to eGFP at the EcoRI restriction site in the middle of the sequence encoding the cytoplasmic domain. Consequently, these constructions lacked the 28 C-terminal amino acids of the cytoplasmic domain encoded by the sequences downstream from the EcoRI restriction site and were designated using the “ΔCyt” suffix. All isoforms were further designated by listing their different constitutive domains (Fig. 1B; only a few representative isoforms are shown).

A mouse mAb (VIIC7) was raised against a peptide of the CD1e cytoplasmic domain (at positions 5−17). Additional experiments confirmed that this mAb could be used to follow the expression of membrane-associated CD1e isoforms in transfected cells by IF, immunoprecipitation, and Western blotting. Abs specific for the α domains were obtained by constructing a plasmid encoding a hybrid protein comprising the three α domains of CD1e, fused to the transmembrane and intracellular domains of CD1c and to eGFP. M10 cells transfected with this expression vector were shown by IF microscopy and biochemical experiments to express the hybrid molecule on the cell surface. Mice were immunized with the transfected cells and hybridomas were prepared from one mouse. Three mAbs, named (1.22, 2.9, and 20.6) were selected, which stained the transfected cells but not untransfected M10 cells. The specificity of these mAbs was tested on HeLa cells transiently transfected with plasmids encoding the different isoforms. In these experiments, the membrane-associated molecules were expressed as CD1e ΔCyt-eGFP fusion proteins. The cells were fixed, permeabilized, stained with the different mAbs, and analyzed by IF microscopy (Table I). None of the mAbs stained transfected HeLa cells expressing the α2α3 isoform, while in IF studies the mAb 1.22 recognized all other isoforms, thus appearing to be specific for the α domain. The mAb 20.6 stained cells expressing the α2α2α3, α2α2α3, and α2α2α3 isoforms hence was more selective than 1.22. Although the mAb 2.9 specifically recognized the three α domain isoforms, the intensity of the signals were poor relative to those of the other mAbs, and 2.9 was therefore inappropriate for IF staining. On the basis of eGFP fluorescence or mAb staining, the CD1e α2α2, α2α3, and α2α2α3-ΔCyt-eGFP molecules appeared to be retained in the ER. In contrast, CD1e α2α2α3-ΔCyt-eGFP were found on the cell surface and intracellularly.

| mAb | α2α3 | α2α3’ | α2α3’’ | α2α3’’’ | α3 | α3’ |
|-----|------|-------|--------|---------|----|-----|
| 1.22 | +++  | +++   | +++    | +++     | +  | +   |
| 2.9  | +    | -     | +      | -       | -  | -   |
| 20.6 | +++  | +++   | +++    | -       | -  | -   |

* C. Angénieux, unpublished observations.
Additional experiments using transfected M10 cells showed that all three mAbs could be used to detect CD1e isoforms by immunoprecipitation and displayed identical specificity as in IF, whereas none of them could be used for Western blotting. Moreover, cytofluorimetry on transfected cells expressing either CD1α, -β, or -γ molecules demonstrated that these mAbs do not react with the other CD1 molecules expressed by DCs.3

**Detection of Retention Sequences in the 3'-End of the Cytoplasmic Domain of CD1e—**Preliminary IF experiments using the VIIC7 mAb showed that whereas CD1e α3ΔCytl and CD1e-ΔCytS molecules transiently expressed in HeLa cells could reach the plasma membrane, complete CD1e molecules expressed in transfected cells appeared to be retained in the ER (data not shown). This suggested that the 28 C-terminal amino acids were involved in the retention of the full-length CD1e molecules. Since initially we had no mAb against the α domains of CD1e, it was not possible to directly determine which part of the protein mediated its retention in the ER. Therefore, we constructed plasmids expressing hybrid molecules consisting of the extracellular and transmembrane domains of CD1c fused to different parts of the CD1e cytoplasmic domain were stably transfected into M10 melanoma cells. A, extracellular and transmembrane domains of CD1c were fused to the part of the cytoplasmic domain of CytL or CytS upstream from the EcoRI site (CD1c-ΔCytL and CD1c-ΔCytS, respectively) or to the end of the cytoplasmic domain, downstream from the EcoRI site (CD1c-Eco-end). An asterisk indicates a stop codon introduced at the level of the EcoRI site; other symbols are described in Fig. 1. B, expression of the fusion proteins was quantified by flow cytometry of transfected cells permeabilized (total) or not (surface) and stained with L161 (anti-CD1c) and phycoerythrin-conjugated F(ab’2) goat anti-mouse IgG. C, CD1c was fused to the CD1e cytoplasmic domain deleted in the five C-terminal amino acids (1) or of the six next amino acids including the dilyisine motifs (KKWKTR) (2). Cell surface expression and intracellular distribution of the fusion molecules were determined by flow cytometry and confocal microscopy.

In M10 cells, CD1c molecules as well as CD1c-ΔCytL and CD1c-ΔCytS hybrid molecules were found on the cell surface (Fig. 2B). In contrast, CD1c-Eco-end was only weakly present on the cell surface, although the intracellular staining showed it to be expressed equally as strongly as the other molecules. Confocal microscopy revealed intracellular CD1c molecules to

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**Fig. 2. Detection of a retention motif in the 3'-end of the CD1e cytoplasmic domain.** Fusion molecules encoding the extracellular and transmembrane domains of CD1c fused to different parts of the CD1e cytoplasmic domain were stably transfected into M10 melanoma cells. A, extracellular and transmembrane domains of CD1c were fused to the part of the cytoplasmic domain of CytL or CytS upstream from the EcoRI site (CD1c-ΔCytL and CD1c-ΔCytS, respectively) or to the end of the cytoplasmic domain, downstream from the EcoRI site (CD1c-Eco-end). An asterisk indicates a stop codon introduced at the level of the EcoRI site; other symbols are described in Fig. 1. B, expression of the fusion proteins was quantified by flow cytometry of transfected cells permeabilized (total) or not (surface) and stained with L161 (anti-CD1c) and phycoerythrin-conjugated F(ab’2) goat anti-mouse IgG. C, CD1c was fused to the CD1e cytoplasmic domain deleted in the five C-terminal amino acids (1) or of the six next amino acids including the dilyisine motifs (KKWKTR) (2). Cell surface expression and intracellular distribution of the fusion molecules were determined by flow cytometry and confocal microscopy.
be localized in HLA-DR compartments, as expected, while CD1c-Eco-end molecules were principally in the ER. The C-terminal region of this part of the cytoplasmic domain of CD1e contains two overlapping dilysine motifs (KKXX), both of which are known to mediate the retention of molecules in the ER (25). Dilysine motifs are generally located at amino acids \(-3/-4\) (KK motif) or \(-3/-5\) (KXX motif) of the cytoplasmic domain, whereas the KXXK sequence of CD1e lies at positions \(-8/-11\). The function of this sequence was explored by expressing in M10 cells other hybrid molecules comprising the extracellular and transmembrane domains of CD1e fused to the cytoplasmic domain CyTL, complete or truncated at different positions in the C-terminal end. Hybrid fusion proteins containing the complete cytoplasmic domain of CD1e (short or long) could not be detected on the surface of transfected M10 cells. Similarly, a CD1e long cytoplasmic tail deleted of the first five C-terminal amino acids induced a complete intracellular retention of CD1c (Fig. 2C). Confocal microscopy confirmed the ER localization of these fusion molecules. Deletion of the next six amino acids (KKWKTR) partly allowed cell surface expression of the fusion molecules. Although these molecules also displayed strong ER localization, they appeared to be more heterogeneously distributed among cell membranes including the Golgi apparatus and plasma membrane. This distribution indicated that CD1e is present throughout the biosynthetic secretory pathway. These experiments suggested that a sequence containing dilysine motifs mediates the retention in the ER, while other C-proximal sequences of the cytoplasmic domain might further slow down or hinder its transport to the cell surface.

**CD1e Molecules Accumulate in the Golgi and Late Endosomal Compartments of Transfected Melanoma Cells**—With the development of mAbs specific of the \(\alpha\) domains, we got additional tools to follow the expression of CD1e molecules in transfected cells. The mAb 20.6 against the CD1e \(\alpha\) domains and VIIC7 against the CD1e cytoplasmic domain were used to detect CD1e in a transfected M10 cell line expressing CD1e \(\alpha_{1\alpha_{2}\alpha_{3}}\)-CyTL or CD1e \(\alpha_{1\alpha_{2}\alpha_{3}}\)-CytS. The cells were fixed, permeabilized, and incubated with the mAbs. Interestingly, while VIIC7 strongly stained the ER and dense structures, which were probably Golgi compartments, 20.6 only weakly labeled the ER but brightly stained the Golgi compartment and other structures not revealed by VIIC7 (Fig. 3A). Untransfected M10 cells were not labeled by the mAbs (not shown). The unknown structures were identified by double immunostaining with 20.6 and Abs specific for Rab-6 (Golgi compartments), TGN-46 (trans-Golgi network), EEA1 (sorting endosomes), CD63 (late endosomes), Lamp-1 (lysosomes), or HLA-DR (HLA class II enriched compartments). Confocal microscopy showed that CD1e partially co-localized with Rab-6 and TGN-46 as well as CD63, and to a much lesser extent with Lamp-1 and HLA-DR molecules, but not with EEA1 (data shown only for CD1e \(\alpha_{1\alpha_{2}\alpha_{3}}\)-CyTL, Fig. 3B). Thus, in transfected M10 cells, CD1e was found not only in the ER, but also in the late Golgi and late endosomal compartments.

**CD1e Is Cleaved in Acidic Compartments**—In an attempt to explain the discrepancies between the immunostaining results obtained with the anti-cytoplasmic domain mAb VIIC7 and the other mAbs, CD1e molecules were analyzed biochemically. Transfected M10 cells were metabolically labeled for 1 h with \(^{35}\)S and chased for 0, 2, 4, or 20 h. Membrane proteins were solubilized in 1% Triton X-100 and immunoprecipitated with either VIIC7 or 2.9 mAb, and the glycosylation of the immunoprecipitated proteins was examined by treatment with Endo H or F (Fig. 4A). Most of the molecules immunoprecipitated with VIIC7 mAb remain Endo H-sensitive, and only a faint Endo H-resistant signal was detected after 2 h of chase. These mol-
Acrylamide gels under reducing conditions. Fluorographs were developed with VIIC7 or 2.9 and the samples were resolved on 10% SDS-polyacrylamide gels in the presence of an irrelevant isotype-matched control mAb. Moreover, CD1e molecules were immunoprecipitated with either VIIC7 or 2.9. Autoradiography revealed that removal of β2m eliminated all 2.9 reactive molecules but did not seem to affect VIIC7 reactive molecules (Fig. 4B). These experiments demonstrated that (i) most CD1e molecules present in the ER and reacting with VIIC7 are not associated with β2m, (ii) the mAb 2.9 recognizes complexes between the CD1e heavy chain and β2m, and (iii) soluble CD1e molecules are associated with β2m.

According to these findings, cleavage of CD1e occurred late in its biosynthesis, which suggested that lysosomal proteolytic enzymes acting in an acidic environment could be responsible for this post-translational modification. This was confirmed using three different inhibitors of cellular acidification. M10-CD1e cells were pulse-labeled for 1 h and chased for 2 or 4 h in the absence or in the presence of chloroquine, NH4Cl, or bafilomycin, and CD1e molecules were immunoprecipitated with 2.9 (Fig. 4C). In absence of inhibitors of acidification, most of the CD1e molecules were cleaved during the first 2 h of chase, while in their presence no cleavage products were observed even after 4 h. Hence, CD1e must be processed in acidic compartments, most probably in late CD63⁺ endosomal compartments. CD1e α1α2α3-CytL behaves like CD1e α1α2α3-CytL in terms of processing.

Expression of CD1e in Dendritic Cells—In view of the multiplicity of hypothetical CD1e molecules able to be produced in DCs, an analysis of the CD1e polypeptide chains translated in DCs was performed. The synthesis of CD1e molecules was demonstrated by metabolic labeling of DCs and immunoprecipitation with the mAb VIIC7, and more than seven forms were detected (Fig. 5A). The two proteins of highest molecular mass corresponded to isoforms containing three α domains, while the other polypeptides displayed the molecular masses of isoforms with two α domains or a shortened α3 domain. Since VIIC7 is specific for the CD1e cytoplasmic domain (at positions -5/-17), it was not possible to fully identify the different isoforms separated by SDS-PAGE. However, it was possible to follow the two longer CD1e isoforms using the mAb 20.6 in pulse-chase labeling experiments. Immunoprecipitation of CD1e with 20.6 revealed that the two longer isoforms (Fig. 5B, band U), which were present after the pulse, became Endo H-resistant and were cleaved in the absence of the bafilomycin within 2 h of chase (band P). After 2 h of chase in the presence of bafilomycin, only complete Endo H-resistant molecules were detected. Interestingly, a higher number of CD1e molecules was immunoprecipitated from bafilomycin-treated immature DCs, suggesting that in these cells CD1e was unstable. A 27-kDa Endo F-resistant protein unexpectedly coprecipitated with CD1e after 2 h of chase in the presence of bafilomycin (band B). Since the same result was observed using VIIC7 or 20.6 mAb, but not an irrelevant control mAb (Fig. 5A), this molecule was not a degradation product of CD1e molecules but could be a protein that associates with CD1e in the late steps of its transport.

Finally, we investigated the localization of CD1e in DCs. Monocytes, DCs derived from monocytes, and freshly isolated epidermal LCs were fixed, permeabilized, and stained with VIIC7 and 20.6. No staining was observed in monocytes, while if the mAb VIIC7 stained DCs, the cellular localization obtained by IF was ambiguous. Confocal microscopy and double staining with an anti-calnexin Ab showed that the molecules recognized by VIIC7 accumulated partly in the calnexin-positive ER but also in other unidentified calnexin-negative compartments (Fig. 5C). The mAb 20.6 stained compartments in DCs derived from monocytes and in LCs (Fig. 5C).

The intracellular distribution of CD1e in these cells was characterized by confocal microscopy (Fig. 5D). In immature DCs, CD1e colocalized mainly with Rab-6 and TGN-46 and

\[ \text{Intracellular Distribution of CD1e} \]

![Image](http://www.jbc.org/)

**Fig. 4. Biosynthesis of CD1e α1α2α3-CytL in transfected M10 cells.** A. M10 or M10-CD1e α1α2α3-CytL cells were pulse-labeled for 1 h with [35S]methionine and cysteine and chased for various period of times. After immunoprecipitation of CD1e with VIIC7 or 2.9, the precipitates were digested or not with Endo H or Endo F and resolved on a 12% SDS-PAGE gel under reducing conditions. B. M10-CD1e α1α2α3-CytL cells were metabolically labeled and chased for different times. Lysates were depleted of β2m by two anti-β2m immunoprecipitations (β2m). Controls were obtained by performing two adsorptions on protein A-Sepharose in the presence of an irrelevant isotype-matched mAb (lg). The remaining CD1e molecules were immunoprecipitated with VIIC7 or 2.9 and the samples were resolved on 10% SDS-polyacrylamide gels under reducing conditions. Fluorographs were developed after 4 days (VIIC7) or 10 days (2.9). The 33-kDa band found in 2.9 immunoprecipitates is nonspecific and was sometimes observed in untransfected M10 cells. C. M10 or M10-CD1e cells were metabolically labeled for 1 h and chased for 2 or 4 h in the presence of 1 μM bafilomycin (BAF), 100 μM chloroquine (CHL), or 10 mM NH4Cl (NH4). CD1e molecules were immunoprecipitated with 2.9 and treated (+) or not (−) with Endo F. NT, not treated by drugs.
most strongly with TGN-46. Conversely, in LPS-treated mature DCs, CD1e was found in CD63+ and Lamp-1+ compartments and was almost absent from the Golgi. When DCs were treated with TNFα for 24 h, CD1e also colocalized with CD63, suggesting a redistribution of CD1e molecules from the late Golgi compartments to late endosomal structures during maturation. The absence of cell surface CD1e was confirmed by flow cytometry of immature and mature (TNFα- or LPS-treated) DCs.

**DISCUSSION**

The biosynthesis of CD1e molecules has not yet been demonstrated, although the gene was characterized in 1986. Multiple alternative splicing of the pre-mRNA was observed in T cell lines, but the resulting mRNA molecules were not characterized (17). In this work, we therefore chose to first characterize the CD1e transcripts produced in DCs derived from monocytes. More than 15 alternatively spliced mRNA molecules were identified, and the translation products may be classified either as membrane-associated proteins or as putative soluble proteins. Transcripts homologous to human CD1e were recently described in guinea pigs (26). Although only one CD1e cDNA was characterized, an extensive analysis of CD1e transcripts from this animal might reveal other alternatively spliced sequences. It is worthy of note that human and guinea pig CD1e both have a long cytoplasmic tail, which differ by the length of the cytoplasmic tail, was investigated in transfected melanoma cells. Interestingly, in these cells, the mAb VIIC7 against the cytoplasmic domain and the mAb 20.6 against α domains showed different patterns of distribution of CD1e molecules. Whereas VIIC7 revealed CD1e in the ER and Golgi compartments, 20.6 revealed CD1e mainly in the Golgi and CD63− late endosomal compartments. Fluorescence-activated cell sorting analyses of transfected M10 cells labeled with the mAb 20.6 failed to detect CD1e α1α2α3-Cytl on the cell surface. To understand this particular distribution, a biochemical analysis of CD1e synthesis and maturation was performed. Experiments using transfected cells showed that most of the CD1e molecules immunoprecipitated by VIIC7 were Endo H-sensitive. A minor part of these molecules were immunoprecipitated with the 2.9 mAb, these molecules being converted into an Endo H-resistant form. The latter molecules were then cleaved into a soluble form in acidic compartments. While most VIIC7-immunoprecipitated molecules appeared to be free of β2m, those immunoprecipitated with 2.9 mAb were associated with β2m. These observations suggest that most of the CD1e molecules in the ER slowly acquire the right conformation; this rate-limiting step could be the association of CD1e with the CD1c heavy chain with β2m. Once they are properly folded, these molecules are exported to an acidic compartment, where they are cleaved into a soluble form, probably the late CD63− endosomal compartments, where they colocalize. In these compartments, the CD1e molecules are rather stable, since they can be still detected in cell extracts after a 20-h chase while they could not be found in culture supernatants.

Experiments using CD1c-CD1e hybrid proteins demonstrated that a short sequence including two overlapping dilysine motifs (KXXK) motifs located in the C-terminal end of the cytoplasmic domain was involved in the intracellular traffic
of CD1e molecules. This sequence was involved in the ER retention of the hybrid molecules. However, whereas all such dilynine motifs (KK or KKK) characterized to date lie 1–2 amino acids from the C-terminal end of the cytoplasmic domain (25), those of CD1e lie 8–10 amino acids upstream of the C-terminal end. This unusual position raises the question as to whether these signals act like classical di-lysine motifs. Notably, preliminary experiments in transfected M10 cells have shown that the CD1e α1α2α3-CytLeGFP fusion protein is converted into a 2.9 reactive form more rapidly than CD1e α1α2α3-CytL, suggesting that the C-terminal amino acids play a role in the folding of CD1e α domains or in the association of the CD1e heavy chain with β2m. Recently, CD1e-ΔCyt molecules have been expressed in M10 cells; these molecules were observed on the cell surface as well as in endosomal compartments. This last observation suggests that the dilynine motifs could also be involved in the control of the traffic of CD1e molecules in post-Golgi compartments, allowing a retrograde transport of molecules en route toward the cell surface. We can hypothesize that the cleavage of CD1e in the acidic compartment prevents these molecules from a retrograde transport from the endosomes to the ER. One of these dilynine motifs is also present in guinea pig CD1e (NRLKLKKWKRNLQNLW/human/KNRLLKKWKRNLQFW (guinea pig) ), and it would be interesting to know if the guinea pig CD1e molecules present a similar intracellular distribution. Experiments are now in progress to define more exactly the role of the dilynine motifs and those of other sequences of the cytoplasmic domain in the cellular distribution of human CD1e molecules.

Molecules with only two α domains or with a truncated α3 domain were retained in the ER, even when the dilynine motif was removed (CD1e-ΔCyt-eGFP hybrid proteins). Moreover, when the hypothetical soluble isof orm CD1e α1α2α3 was expressed in M10-transfected cells, this molecule remained fully Endo-H-sensitive after 4 hr of chase and was not secreted into the culture medium. Immunoprecipitation with the 20.6 mAb did not co-prefecture β2m, and conversely immunoprecipitation of β2m did not co-prefecture CD1e α1α2α3. Hence, this isoform does not associate or only very loosely associates with β2m. Since the membrane-associated form CD1e α1α2α3-CytS contains a shortened α3 domain, it is also unlikely to associate with β2m. Hence, the 1.22 and 20.6 mAbs, detecting these truncated isoforms, also recognize β2m-free CD1e molecules.

In immature DCs, metabolic labeling and immunoprecipitation with VIIC7 revealed numerous CD1e polypeptides corresponding, in terms of molecular mass, to the different isoforms predicted from cDNA cloning. The mAb 20.6 detected only the biosynthesis of long CD1e isoforms, and these molecules were expressed in M10 cells; these molecules were also recognized by the mAb VIIC7, specific for the CD1e cytoplasmic domain. After treatment of the cells with LPS, CD1e was found mainly in CD63 positive compartments. An identical distribution of CD1e molecules was also observed in freshly isolated Langerhans cells as in immature monocyte-derived DCs, and the same redistribution when maturation of the cells was induced by 2 days of culture in the presence of granulocyte-macrophage colony-stimulating factor and TNFα. Thus, CD1e appears to be implicated in the maturation program of dendritic cells, which involves changes in the expression and traffic of antigen-presenting molecules such as CD1a, β1, or -c molecules as well as HLA-DR molecules and other proteins of unknown function such as DC-Lamp, all of which traffic through the endosomal pathway. Finally, metabolic and IF studies suggest that the apparent differences in the distribution of CD1e in mature and immature DCs reflect different stabilities of CD1e molecules in the two types of cells.

This work first demonstrates the biosynthesis of the CD1e proteins not only in transfected cells but also in differentiated DCs. The distribution of this protein belonging to a family of antigen-presenting molecules was unexpected. Other CD1 molecules are first expressed on the surface of DCs and may then internalize into early or late endosomal compartments. In contrast, CD1e proteins expressed in DCs or in transfected cells do not reach the cell surface but seem to move directly from the Golgi compartments to the late endosomes. This unusual distribution makes it difficult to predict the function of CD1e proteins. However, the differences in localization between immature and mature cells strongly suggest that CD1e plays a physiological role in DCs. The absence of expression on the cell surface argues against a direct role in antigen presentation, unlike other CD1 molecules, whereas the presence of CD1e in late endosomal compartments points to its involvement in antigen processing. Characterization of molecules that may interact with CD1e will be necessary to determine its exact biological function.

Acknowledgments—We thank Dr. A. Bohbot for providing elutriated monocytes and C. Schwartz for technical assistance.

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*J. Biol. Chem. 2000, 275:37757-37764.*
doi: 10.1074/jbc.M007082200 originally published online August 17, 2000

Access the most updated version of this article at doi: 10.1074/jbc.M007082200

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