The assembly of CD1e is controlled by an N-terminal propeptide which is processed in endosomal compartments


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CD1e displays unique features in comparison with other CD1 proteins. CD1e accumulates in Golgi compartments of immature dendritic cells and is transported directly to lysosomes, where it is cleaved into a soluble form. In these latter compartments, CD1e participates in the processing of glycolipid antigens. In the present study, we show that the N-terminal end of the membrane-associated molecule begins at amino acid 20, whereas the soluble molecule consists of amino acids 32–333. Thus immature CD1e includes an N-terminal propeptide which is cleaved in acidic compartments and so is absent from its mature endosomal form. Mutagenesis experiments demonstrated that the propeptide controls the assembly of the CD1e α-chain with β₂-microglobulin, whereas propeptide-deleted CD1e molecules are immunologically active. Comparison of CD1e cDNAs from different mammalian species indicates that the CD1e propeptide is conserved during evolution, suggesting that it may also optimize the generation of CD1e molecules in other species.

Key words: antigen presentation, CD1, endosome, phosphatidylinositol mannanside (PIM), propeptide.

INTRODUCTION

CD1 molecules are non-classical HLA class I molecules, among which human CD1e displays several distinct features in its trafficking, function and primary structure. Human CD1a, CD1b, CD1c and CD1d cycle between the plasma membrane and different endosomal compartments and are involved in the presentation of glycolipids to T-cells [1]. Conversely, CD1e initially accumulates in the Golgi compartments of immature dendritic cells or transfected cells as a membrane-associated form (mCD1e) and is subsequently transported directly to early and then late endosomes, where it is cleaved into a soluble form (sCD1e) [2,3]. This form facilitates the processing of glycolipids presented by other CD1 molecules transiting through the same compartments. For example, presentation of PIM₆ (phosphatidylinositol hexamannoside) to CD1b-restricted T-cell clones is CD1e-dependent, whereas the presentation of PIM₂ (phosphatidylinositol dimannoside) is not [4]. In vitro experiments have shown that α-mannosidase digests PIM₆ into the immunogenic PIM₇ form, only in the presence of sCD1e [4].

Alignment of the mature α-chains of human CD1 proteins, without the signal peptide, suggests that CD1e has an N-terminal extension of eight to ten amino acids (Table 1a). The assumption that this extension is present on endosomal CD1e is, however, not supported by the N-terminal amino acid sequence of a sheep CD1e [5] which should correspond to endosomal sCD1e on the basis of its sequence homologies and biochemical properties. This latter sequence suggests that the 12 N-terminal amino acids of human CD1e are cleaved. As the gene sequence of sheep CD1e is not known, it is not possible to confirm N-terminus processing of CD1e in this animal.

Comparison of CD1e proteins in different species suggests the existence of an N-terminal extension conserved during evolution (Table 1b), which is cleaved during CD1e maturation. The aim of the present study was to validate this hypothesis, to identify the cellular compartments where this N-terminal extension is cleaved and to elucidate its function.

EXPERIMENTAL

Genetic constructions

CD1e forms used in the present study were derived from the cDNA encoding the isoform with the long cytoplasmic tail (CD1eL) [2]. Propeptide-deleted molecules were derived by PCR mutagenesis of cDNAs encoding CD1e. Various CD1e-derived molecules were expressed in the pEGFP-N3 vector (Clontech-TaKaRa Bio Europe).

Antibodies

The anti-CD1e mAbs (monoclonal antibodies) VIIC7, 2.9 and 20.6 have been described previously [2]. The anti-β₂-microglobulin mAb B1G6 was purchased from Beckman-Coulter. Polyclonal Abs (antibodies) specific for the N-terminus of neosynthesized CD1e were raised by immunizing rabbits with the synthetic

Abbreviations used: Ab, antibody; ADAMTS9, a disintegrin and metalloproteinase with thrombospondin motifs 9; EndoH, endoglycosidase H; ER, endoplasmic reticulum; GM-CSF, granulocyte/macrophage colony-stimulating factor; IFNγ, interferon γ; LC, liquid chromatography; mAb, monoclonal Ab; MALDI, matrix-assisted laser-desorption ionization; mCD1e, membrane-associated CD1e; MS/MS, tandem MS; PIM₆, phosphatidylinositol hexamannoside; PIM₇, phosphatidylinositol hexamannoside; PNGase F, peptide N-glycosidase F; rs, recombinant soluble; sCD1e, soluble CD1e.

1 To whom correspondence should be addressed (email henri.delasalle@efs-alsace.fr).
peptide APQALQSYHLAAEEQLS (NeoMPS) coupled to keyhole-limpet haemocyanin. Anti-peptide Abs were immunopurified from 5 ml of antiserum by adsorption on to 1 mg of peptide coupled to NHS (N-hydroxysuccinimide)-activated Sepharose (GE Healthcare). CD1e molecules were immunopurified with anti-CD1e mAbs (clone 20.6) chemically coupled with dimethyl pimelimidate (Perbio Science) on Protein A–Sepharose (GE Healthcare). CD1e molecules were immunopurified with 20.6 hybridoma cell culture supernatant from 5 ml of antiserum by adsorption on to 1 mg of peptide keyhole-limpet haemocyanin. Anti-peptide Abs were immunopurified from 5 ml of antiserum by adsorption on to 1 mg of peptide coupled to NHS (N-hydroxysuccinimide)-activated Sepharose (GE Healthcare). CD1e molecules were immunopurified with anti-CD1e mAbs (clone 20.6) chemically coupled with dimethyl pimelimidate (Perbio Science) on Protein A–Sepharose (GE Healthcare).

### Transfected cells and biochemical analyses

Transfected M10 cells expressing CD1e and CD1b molecules, alone or together, have been described previously,[2,4]. Mutated forms of CD1e were expressed in M10 cells, expressing or not CD1b, after transfection with FuGENE™ 6 reagent (Roche Applied Science) followed by selection in the presence of 500 μg/ml G418 (Invitrogen). Clones expressing CD1e were identified by immunofluorescence of fixed permeabilized cells (Invitrogen). Clones expressing CD1e were selected and assayed by SDS/PAGE (12.5 % gels) and revealed with Coomassie Blue G250.

### Purification of CD1e molecules from transfected cells

Membrane and cytoplasmic proteins from 7 × 10^6 stably transfected cells were extracted in 30 ml of lysis buffer (20 mM Tris/HisCl, pH 7.5, 150 mM NaCl, 1% Triton X-100 and protease inhibitors), pre-immunoadsorbed on Protein A–Sepharose overnight and then incubated for 2 h with 40 μg of 20.6 mAb covalently coupled to Protein A–Sepharose. After extensive washing, the immunoadsorbed proteins were eluted, separated by SDS/PAGE (12.5 % gels) and revealed with Coomassie Blue G250.

### MS of tryptic digests

Immunopurified recombinant CD1e molecules were separated by SDS/PAGE (12.5 % gels) and stained with Coomassie Blue. As a negative control, a similar procedure was performed using untransfected cells. Major species specific for transfected cell extracts, corresponding to mCD1e and sCD1e, were excised from the gel. Gel slices were washed twice with 50 μl of 25 mM NH_4HCO_3 followed by 50 μl of acetonitrile. Cysteine residues were reduced using 50 μl of 10 mM dithiothreitol at 57 °C and alkylated using 50 μl of 55 mM iodoacetamide. After dehydration with acetonitrile, the proteins were cleared in gel with a 12.5 ng/μl solution of modified trypsin (Promega) in 25 mM NH_4HCO_3. The resulting tryptic peptides were extracted with 60% acetonitrile in 5% methanoic (formic) acid and analysed by LC (liquid chromatography)–MS/MS (tandem MS). Measurements by MALDI (matrix-assisted laser-desorption ionization)-MS were carried out on an Ultraflex TOF/TOF (Bruker) mass spectrometer in reflectron mode with positive-ion spectra. The samples were prepared by standard dried droplet preparation on stainless steel MALDI targets using α-cyano-4-hydroxycinnamic acid as matrix. External calibration of MALDI mass spectra was carried out using singly monoisotopic peaks of a mixture of bradykinin-(1–7) (m/z = 757.400), human angiotensin II (m/z = 1046.542), human angiotensin I (m/z = 1296.685), substance P (m/z = 1347.735), bombesin (m/z = 1619.822), renin (m/z = 1758.933), corticotropin-(1–17) (m/z = 2093.087) and corticotropic-(18–39) (m/z = 2465.199). To achieve mass accuracy, internal calibration was performed with tryptic peptides.

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**Table 1 Predicted N-terminal amino acid sequences of human CD1 proteins and mammalian CD1e molecules after cleavage of their signal peptide**

The N-terminal sequences were predicted using http://www.cbs.dtu.dk/services/SignalP/. The propeptide sequence is in bold.

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<th>Protein</th>
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<td>CD1c</td>
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<td>CD1d</td>
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<td>CD1e</td>
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(a) Human CD1 sequences

(b) Mammalian CD1e sequences

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<td>Human</td>
<td>APQALQSYHLAAEEQLS</td>
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<td>APQALQSYRALPDAEDPLS</td>
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Production of rs (recombinant soluble) CD1e in insect cells

The luminal domains of CD1e without the signal peptide, beginning at Ala21 (rsCD1e) or Glu2 (rsΔ12CD1e) and ending at Ser305, were fused directly to the signal peptide of BiP (immunoglobulin heavy-chain-binding protein) and a C-terminal V5His tag (pMTbipV5HisA, Invitrogen). The two forms were included only the CD1e α-chain and β2-microglobulin, as shown by SDS/PAGE analysis. N-terminal amino acid sequencing confirmed that rsCD1e and rsΔ12CD1e began with the expected amino acid sequence APQALQ or EEQLSF respectively (results not shown).

Antigen-presentation assays

Transfected M10 cells (3 × 10⁶/well) growing in RPMI 1640 medium supplemented with 10% FCS (fetal calf serum), 10 mM Hepes, 2 mM Ultraglutamine II, MEM non-essential amino acids, 1 mM sodium pyruvate (all from Lonza) and 100 μg/ml kanamycin (Invitrogen) were pre-incubated for 2 h at 37°C with different concentrations of purified PIM6 [7,8] or GM1 ganglioside (Matreya), before addition of T-cells (10⁵/well in triplicates). The T-cells used were the CD1b-restricted, PIM6-specific and CD1e-independent clone GG33A [9], which is CD1e-independent (granulocyte/macrophage colony-stimulating factor) or IFNγ (interferon γ) kit (R&D Systems). Data were expressed as means ± S.D. in arbitrary units (for GM-CSF) or pg/ml (for IFNγ) for triplicate samples.

The biological activity of rsCD1e was also tested by pre-incubating CD1b-transfected M10 cells with 20 μg/ml rsCD1e overnight. Cells were then washed and processed for antigen presentation as described above.

Enzymatic digestion of PIM6

Purified PIM6 (100 μg) was sonicated in 50 μl of mannosidase buffer (0.1 M sodium acetate, pH 4.5, and 1 mM ZnSO₄). rsCD1e (4.3 nmol in 150 μl) was equilibrated by dialysis against mannosidase buffer for 1 h and then incubated with PIM6 (100 μg in 50 μl) and with 20 μl of 1.8 mg/ml jackbean α-mannosidase (Sigma) for 1 h at 37°C. Glycolipids were extracted and analysed by MS as described previously [4].

RESULTS

Characterization of the ends of CD1e forms provides evidence for processing of the N-terminus

Analysis of pre-CD1e sequence predicts that cleavage of the signal peptide occurs between Ala21 and Ala25 (Table 1a), whereas data from a CD1e-like sheep protein indicate that the subsequent N-terminal amino acids have been removed from the lysosomal form. To confirm these predictions, CD1e molecules from transfected M10 cells were immunopurified and separated by SDS/PAGE. After Coomassie Blue staining, two protein species were recovered having apparent molecular masses corresponding to those of mCD1e and sCD1e (Figure 1A). Comparison of the MALDI–TOF (time-of-flight) mass spectra of the tryptic digests of the two forms of CD1e revealed four peaks of m/z 1050.54, 1300.69, 1790.89 and 2159.08 Da, specific to the 45 kDa mCD1e form (Figure 1B). These peaks were not predicted by in silico trypsin digestion of mCD1e and hence probably corresponded to semi-tryptic species (with only one trypsin-generated end) resulting from digestion of the N- and C-terminal ends of mCD1e. On the basis of this hypothesis, the m/z value of the predominant 2159.08 Da peak corresponded to the theoretical m/z value of the peptide APOALQSYHLAAEQLSF, generated by trypsin digestion of the predicted N-terminal end of mCD1e (Table 1a). The other three peaks represented shorter N-terminal ends (Table 2). The identities of these peptides were confirmed by de novo sequencing after LC–MS/MS (Figure 1C).

A similar analysis of the tryptic digest of sCD1e revealed three prominent peaks of m/z 908.45, m/z 1585.80 and m/z 1913.00 Da (Figure 1B). LC–MS/MS showed that the 1913.00 Da peak corresponded to the internal trypsic peptide NLQLFQLYHFSR (theoretical molecular mass of 1912.00 Da), which represents amino acids 92–106 of preCD1e (results not shown). Since a glycosylation site is predicted at Asn91, it is possible that the processing of carbohydrates in lysosomes results in a greater accessibility to trypsin of the amino ester bond between amino acids 91 and 92, which would explain why this peak was only observed in the tryptic digest of sCD1e. The two first peaks were not predicted by in silico trypsin digestion of CD1e and thus might result from digestion of the N- or C-terminal end of sCD1e. This conclusion was confirmed by de novo sequencing after LC–MS/MS (Figure 1C and Table 2). The m/z 908.45 Da species corresponded to a peptide derived by proteolysis of the N-terminal end of sCD1e, EEQLSFR, resulting from the cleavage of the 12 N-terminal amino acids of mCD1e. The amino acid sequence of the m/z 1585.80 Da entity was HSSLGHHGLIHWWG (theoretical m/z 1584.78 Da) and hence

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<td>HSSLGHHGLIHWWG</td>
<td>C-terminus</td>
</tr>
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Table 2 N- or C-terminal amino acid sequences of human mCD1e and sCD1e

CD1e molecules were immunopurified from transfected cells and separated by SDS/PAGE (Figure 1A). Tryptic fragments of the membrane-associated and soluble forms were analysed by MALDI-MS (Figure 1B) and de novo sequencing after LC–MS/MS (Figure 1C). 2+ and 3+ indicate doubly and tripoly charged ions respectively. The mass given is the monoisotopic molecular mass of the peptide.
CD1e molecules from solubilized extracts of \(7 \times 10^8\) transfected M10 cells were immunopurified on immobilized mAb 20.6. After separation by SDS/PAGE, proteins were revealed with Coomassie Blue (A). Bands corresponding to mCD1e (m) and sCD1e (s) were recovered, digested with trypsin and analysed by MALDI-MS. Molecular masses are indicated in kDa. (B). Comparison of the two spectra revealed identities specific to mCD1e (\(\Delta\)) and sCD1e (*). (C) De novo sequencing after LC–MS/MS of different precursor ions (i) doubly charged m/z 454.7, (ii) triply charged m/z 529.2 and (iii) triply charged m/z 720.3 confirmed the peptide sequences as (i) EEQLSFR attributed to the N-terminus of sCD1e, (ii) HSSLGGHDLIIHWGG attributed to the C-terminus of the sCD1e and (iii) APQALQSYHLAAEEQLSFR attributed to the N-terminus of mCD1e. The y- and b-ion peaks derived from internal fragmentation of peptide bonds provide amino acid sequence information read from the C-terminus and N-terminus of the peptide respectively.

corresponded to the C-terminal end of sCD1e. This determination confirmed that sCD1e is generated by a proteolytic cleavage of the luminal part of mCD1e, near the transmembrane domain.

Thus newly synthesized CD1e molecules include a 12-amino-acid N-terminal extension, hereafter called the propeptide, which is absent from lysosomal sCD1e. Subsequent experiments

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**Figure 1** Characterization of the N- and C-terminal ends of lysosomal sCD1e

CD1e molecules from solubilized extracts of \(7 \times 10^8\) transfected M10 cells were immunopurified on immobilized mAb 20.6. After separation by SDS/PAGE, proteins were revealed with Coomassie Blue (A). Bands corresponding to mCD1e (m) and sCD1e (s) were recovered, digested with trypsin and analysed by MALDI-MS. Molecular masses are indicated in kDa. (B). Comparison of the two spectra revealed identities specific to mCD1e (\(\Delta\)) and sCD1e (*). (C) De novo sequencing after LC–MS/MS of different precursor ions (i) doubly charged m/z 454.7, (ii) triply charged m/z 529.2 and (iii) triply charged m/z 720.3 confirmed the peptide sequences as (i) EEQLSFR attributed to the N-terminus of sCD1e, (ii) HSSLGGHDLIIHWGG attributed to the C-terminus of the sCD1e and (iii) APQALQSYHLAAEEQLSFR attributed to the N-terminus of mCD1e. The y- and b-ion peaks derived from internal fragmentation of peptide bonds provide amino acid sequence information read from the C-terminus and N-terminus of the peptide respectively.
have a similar cellular distribution, accumulating in TGN46+
molecules in the ER [2]. Immediately after the pulse and during
localized CD1e molecules as well as a small percentage of
mAb 20.6. This mAb immunoprecipitates Golgi- and endosome-
chase labelling experiments, and immunoprecipitation with the
mAb VIIIC7 or the polyclonal Ab A15C recognizing the 12-amino-acid N-terminal peptide. Upper
panels, transfected cells; lower panels, untransfected cells. Molecular masses are indicated in
kDa.

were designed to identify the cellular compartment where the
propeptide is cleaved.

The 12 N-terminal amino acids of CD1e are cleaved in endosomes

To confirm that these 12 amino acids are present on ER (endoplasmic reticulum)-localized CD1e molecules, polyclonal Abs against a synthetic peptide including the 12 N-terminal amino acids of CD1e (A15C) were raised in rabbits. CD1e was immunoprecipitated from extracts of transfected M10 cells expressing CD1e using the mAb VIIIC7. This mAb recognizes the cytoplasmic domain of CD1e and in transfected M10 cells immunoprecipitates molecules which are EndoH-sensitive and thus located in the ER [2]. The immunoadsorbed CD1e was then analysed by Western blotting using either VIIIC7 or the polyclonal anti-A15C Abs (Figure 2). Compared with VIIIC7 mAb, A15C Ab detected CD1e molecules poorly, which may explain why it only revealed EndoH-sensitive CD1e molecules immunoprecipitated with the mAb VIIIC7. Nevertheless, these experiments demonstrated that the anti-propeptide Abs recognized EndoH-
sensitive, ER-localized, CD1e molecules.

To confirm that the N-terminal extension is lacking on lysosomal sCD1e and to identify the cellular compartment in which it is processed, a CD1e form deleted of its 12 N-terminal amino acids (Δ12CD1e) was expressed in M10 cells. Confocal immunofluorescence microscopy showed that CD1e and Δ12CD1e have a similar cellular distribution, accumulating in TGN46+ (trans-Golgi network protein of 46 kDa) compartments of the trans-Golgi network and CD63+ lysosomal compartments (see Supplementary Figure S1 at http://www.BiochemJ.org/bj/419/bj4190661add.htm). Then, the biochemical maturation of Δ12CD1e was compared with that of complete CD1e by pulse–chase labelling experiments, and immunoprecipitation with the mAb 20.6. This mAb immunoprecipitates Golgi- and endosome-localized CD1e molecules as well as a small percentage of molecules in the ER [2]. Immediately after the pulse and during chase, the mCD1e and mΔ12CD1e forms displayed the expected small differences in electrophoretic mobilities (Figure 3A, bands labelled m). In contrast, after 2 h of chase, the cleaved molecules (Figure 3A, bands labelled s) had the same electrophoretic mobility, demonstrating the absence of the 12 N-terminal amino acids on lysosomal CD1e.

Thus, after 4 h of chase, the N-terminal extension is still present on mCD1e molecules, which are EndoH-resistant and thus are transiting, or have transited, through the Golgi apparatus [2] (see also Figure 4). Thus the propeptide is probably cleaved in a post-Golgi compartment. To investigate further this mechanism of CD1e processing, transfected cells expressing CD1e or Δ12CD1e were pulse-labelled and chased for 4 h in the presence of bafilomycin (A) or chased for 2 or 4 h in the presence of bafilomycin (B). The cells were then lysed, and CD1e was immunoprecipitated with mAb 20.6. Samples were left untreated (U) or deglycosylated with PNGase F (F). Bands m and s correspond to mCD1e and endosomal sCD1e respectively. Band p corresponds to a non-N-glycosylated 27 kDa protein which co-immunoprecipitates with CD1e after bafilomycin (Baf) treatment. Molecular masses are indicated in kDa.

The propeptide controls the exit of CD1e from the ER

Propeptides have very diverse functions. They can be involved, for example, in controlling the ER exit of proteins [as in the case of proalbumin, apolipoprotein A-I or ADAMTS9 (a disintegrin and metalloproteinase with thrombospondin motifs 9)], in post-translational modifications (vitamin K-dependent blood clotting factors), or in regulating the activity of pro-proteases. To investigate whether maturation of the CD1e protein is controlled by the propeptide, we performed semi-quantitative biochemical studies using pulse–chase labelling followed by immunoprecipitation with different anti-CD1e mAbs. Immunoprecipitation

Figure 2 Evidence for an N-terminal extension on CD1e

Untransfected and CD1e-transfected M10 cells were lysed, and CD1e was immunoprecipitated with the mAb VIIIC7. The immunoprecipitated proteins were left untreated (U) or deglycosylated with EndoH (H) or PNGase F (F) and then analysed by Western blotting using the mAb VIIIC7 or the polyclonal Ab A15C recognizing the 12-amino-acid N-terminal peptide. Upper panels, transfected cells; lower panels, untransfected cells. Molecular masses are indicated in kDa.

Figure 3 The CD1e propeptide is only present on membrane-associated molecules and is cleaved in late endosomal compartments

Transfected M10 cells expressing the complete CD1e (C) or propeptide-deleted CD1e mutant (Δ) were metabolically labelled with [35S]methionine and [35S]cysteine and recovered immediately (A) or chased for 2 or 4 h in the presence of bafilomycin (B). The cells were then lysed, and CD1e was immunoprecipitated with mAb 20.6. Samples were left untreated (U) or deglycosylated with PNGase F (F). Bands m and s correspond to mCD1e and endosomal sCD1e respectively. Band p corresponds to a non-N-glycosylated 27 kDa protein which co-immunoprecipitates with CD1e after bafilomycin (Baf) treatment. Molecular masses are indicated in kDa.

Double endosomal processing of CD1e

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Figure 4 The biochemical maturation of CD1e is delayed in the absence of the propeptide

(A) Transfected M10 cells expressing CD1e or Δ12CD1e were pulse-labelled and chased for 0, 2, 4 or 8 h. CD1e molecules were recovered from detergent-solubilized cell extracts using the VIIC7 or 20.6 mAb and Protein A–Sepharose. The immunoadsorbed molecules were left untreated (U) or digested with EndoH (H) or PNGase F (F) and separated by SDS/PAGE. Gels were exposed for autoradiography for 11 days (VIIC7) or 23 days (20.6). (B) Untransfected M10 cells (M10) and transfected cells expressing complete (CD1e) or propeptide-deleted CD1e (Δ12CD1e) molecules were pulse-labelled and then chased for 0, 2, 4 or 8 h. After immunodepletion of HLA class I molecules with the W6/32 mAb, CD1e molecules were immunoprecipitated with 20.6 or the anti-β2-microglobulin mAb B1G6 then treated with EndoH (H) or PNGase F (F) or not treated (U), separated by SDS/PAGE and revealed by autoradiography. h, HLA I heavy chain; m, mCD1e; s, sCD1e. (C) The autoradiograms from two pulse–chase-labelling experiments using immunoprecipitation (ipp) with 20.6 (A, left-hand panel) and B1G6 (right-hand panel) were analysed by densitometry scanning of PNGase F-treated samples. At each time point and for wild-type CD1e (C) or Δ12CD1e (Δ), the percentage of soluble among total immunoprecipitated CD1e α-chains was determined. Molecular masses are indicated in kDa. β2m, β2-microglobulin.
with the mAb VIIC7 (Figure 4A), which recognizes all mCD1e molecules, even those which are not assembled with β2-microglobulin, revealed no major differences between CD1e and Δ12CD1e forms. Densimetry scanning showed that, during chase, the amounts of immunoprecipitated CD1e and Δ12CD1e decreased with similar kinetics (results not shown).

Interestingly, immunoprecipitation with the conformation-dependent mAb 20.6 gave more informative results since the kinetics of the generation of soluble Δ12CD1e were significantly slower than for soluble wild-type CD1e (Figure 4). Moreover, the generation of EndoH-resistant Δ12CD1e molecules, i.e. molecules reaching the Golgi compartments, appeared to be delayed throughout the 8 h of chase. In particular, EndoH-sensitive mΔ12CD1e molecules were still detected at the end of the chase (Figure 4A EndoH-treated samples, bands labelled m indicated in lanes F). Hence Δ12CD1e molecules exit the ER more slowly from the ER than wild-type CD1e molecules.

This last finding suggests that the propeptide might control the assembly of β2-microglobulin–CD1e α-chain heterodimers, and/or their exit from the ER. To discriminate between these two possibilities, transfected cells expressing complete or propeptide-deleted CD1e molecules were pulse-labelled and chased for different periods of time (Figure 4B). After immunodepletion with the mAb W6/32, CD1e molecules were immunoprecipitated using the mAb 20.6 or B1G6, which recognize the CD1e α-chain or β2-microglobulin respectively. The immunodepletion of HLA class I molecules was efficient since relatively small amounts of these molecules were immunoprecipitated with the mAb B1G6. Nevertheless, the presence of HLA α-chains in mAbs 20.6 immunoprecipitates suggests that these species resulted from incomplete immunoadsorption of W6/32-HLA class I immune complexes, despite two additional runs of Protein A–Sepharose adsorption. Glycosylated CD1e and HLA class I α-chains migrated at similar positions, but the two species were well resolved after deglycosylation. At all time points, after immunoprecipitation with the mAb 20.6, the ratio of sΔ12CD1e to mΔ12CD1e molecules was less than for complete CD1e (Figure 4C). In contrast, after anti-β2-microglobulin immunoprecipitation, the ratios were equivalent for CD1e and Δ12CD1e after 2 or 4 h of chase (Figure 4C). At 8 h of chase, an unrelated protein was detected migrating as CD1e α-chain and prevented clear interpretation.

Taken together, these data suggest that the absence of the propeptide leads to delayed assembly of CD1e α-chain with β2-microglobulin, and consequently retards the generation of soluble CD1e.

Propeptide-deleted molecules are active

CD1e is necessary for the presentation of the mycobacterial PIM antigen by CD1b to specific T-cells and for the in vitro conversion of PIM into PIMα by α-mannosidase [4]. We used this antigen-processing and presentation model to investigate whether the propeptide is required for proper CD1e function. M10 cells expressing CD1b alone, or co-expressing CD1b with CD1e or Δ12CD1e, were incubated with PIMα and then used to stimulate CD1b-restricted PIM-specific T-cells. The double-transfected M10 cell expressing CD1b and either CD1e or Δ12CD1e presented PIMα with similar efficiency, thus showing that Δ12CD1e is able to assist processing and presentation of this lipid antigen (Figure 5A). In control experiments, transfected M10 cells only expressing CD1b did not present PIMα, confirming the requirement of CD1e for presentation of this antigen. In addition, the CD1b-restricted presentation of GM3 ganglioside, a CD1e-independent antigen, was not influenced by either form of CD1e (Figure 5B).

Figure 5 The Δ12CD1e form is immunologically active

Transfected M10 cells expressing CD1b alone (●) or co-expressing CD1b together with CD1e (○) or with Δ12CD1e (□) were incubated with PIMα (A) or GM3 (B) for 4 h before addition of specific T-cell clones D1.15A30 or GG33a respectively. GM-CSF release was used as a read-out of T-cell activation. (C) In another type of experiment, M10 cells expressing CD1b alone were incubated with 20 μg/ml rsCD1e (○) or rsΔ12CD1e (□) or in the absence of rsCD1e (●). After incubation overnight, cells were washed and incubated with PIMα and specific T-cells. IFNγ release was used as a read-out of T-cell response. (D) PIMα was digested with α-mannosidase (αM) in the presence of rsCD1e or rsΔ12CD1e or in the absence of either. The relative intensities of the peaks, corresponding to the different PIM species generated by digestion were measured by MALDI-MS.

The influence of the propeptide on the CD1e chaperone function was also investigated using two rsCD1e molecules. M10 cells expressing CD1b only were pulsed overnight with rsCD1e or rsΔ12CD1e in order to promote their internalization, then washed and incubated with PIMα together with specific T-cells. The two forms of rsCD1e molecule were equally active in assisting PIMα presentation, confirming that both forms have a similar behaviour in assisting PIMα processing and presentation (Figure 5C). Finally, we looked at the in vitro digestion of PIMα by α-mannosidase. PIMα was incubated with α-mannosidase together with rsCD1e or rsΔ12CD1e, and digestion products were analysed by MS. Both CD1e molecules facilitated PIMα digestion by α-mannosidase as shown by the appearance of PIMα (Figure 5D).

Taken together, these experiments indicate that the absence of the propeptide delays the assembly and maturation of CD1e, but does not alter its immunological function.

DISCUSSION

CD1e is a unique molecule among the CD1 family members. It is the only CD1 protein not expressed on the cell surface, and it is the only protein to be converted into a soluble lysosomal form. Its immunological relevance has been identified [4]; however, it remains unclear how it matures and becomes immunologically functional. In order to better characterize the biochemical maturation of CD1e, we determined the C-terminal sequence of lysosomal sCD1e molecules and compared the N-terminal sequences of the soluble and membrane-associated forms by analysing their tryptic digests by MS. These analyses confirmed that sCD1e is generated by cleavage of the luminal part of the molecule, proximal to the transmembrane domain. Several peaks of the tryptic digest of mCD1e corresponding to the N-terminal sequences were identified. The major one was deduced using a signal peptide prediction algorithm, whereas the shorter ones may result from processing by ER and/or Golgi-localized proteases or from proteolytic events during the purification of CD1e. The major finding of the present study was that CD1e is synthesized as a precursor bearing a N-terminal propeptide of 12 amino acids.
acids. This propeptide is processed within endosomes and occurs nearly concomitantly with the generation of sCD1e. In addition, preliminary data suggest that the two processes are independent.

Pulse–chase labelling experiments demonstrated that, in contrast with wild-type CD1e, more EndoH-sensitive Δ12CD1e molecules were immunoprecipitated by the conformation-dependent mAb 20.6 at all time points. Similar results were obtained when the mAb 2.9 was used in immunoprecipitation experiments (results not shown). In contrast, the kinetics of maturation of CD1e and Δ12CD1e molecules immunoprecipitated with an anti-β2-microglobulin mAb were comparable. This suggests strongly that the exit of CD1e–β2-microglobulin complexes from the ER does not depend on the propeptide, and that the latter mainly facilitates their assembly. A requirement for propeptides in the secretion of other proproteins has been described, and one important issue is how they influence ER retention. The glycosylation of three asparagine residues in its 287-amino-acid-long propeptide controls secretion of ADAMTS9 [12]. Apolipoprotein A-I is synthesized with a six-amino-acid propeptide and accumulates in Golgi compartments before being secreted. Deletion of the propeptide leads to partial retention of apolipoprotein A-I in the ER and to its almost complete disappearance in other cellular compartments [13]. Secretion of rat proalbumin is also controlled by a propeptide, since its deletion induces ER retention and very poor protein release [14]. How the short propeptides influence the secretion of these proteins has not been elucidated.

Although the CD1e propeptide facilitates CD1e assembly, propeptide-deleted CD1e molecules are immunologically functional. Indeed, cells expressing Δ12CD1e, which lacks the propeptide, presented the CD1e-dependent PIM6 antigen with similar efficiency as cells expressing intact CD1e. Furthermore, rsΔ12CD1e produced in insect cells was able to complement M10 cells only expressing CD1b for the presentation of PIM6, similar to rsCD1e molecules [4]. Finally, in vitro, the two forms of rsCD1e similarly facilitated complete digestion of PIM6 by α-mannosidase.

In conclusion, the present study demonstrates that the CD1e propeptide facilitates the assembly of CD1e in the ER, and consequently its egress and transport to Golgi compartments. This is conserved in all species in which the CD1e cDNA has been characterized, thus suggesting that it may optimize the generation of CD1e also in other species. It remains to be elucidated why only CD1e displays such a property among CD1 molecules and other members of the MHC class I family.

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SUPPLEMENTARY ONLINE DATA

The assembly of CD1e is controlled by an N-terminal propeptide which is processed in endosomal compartments

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CONFOCAL MICROSCOPY

Immunofluorescence microscopy and confocal laser-scanning microscopy were performed as described previously [1] using a Leica SP5 AOPS confocal microscope. Briefly, adherent cells were fixed, permeabilized and labelled with the 20.6 mAb, counterstained with Cy3 (indocarbocyanine)-conjugated donkey anti-(mouse IgG), blocked with non-immune mouse serum and then incubated with Alexa Fluor® 488-conjugated H5C6. Alternatively, the cells were first labelled with polyclonal anti-TGN46 (trans-Golgi network protein of 46 kDa) Abs, revealed with Cy5 (indodicarbocyanine)-conjugated donkey anti-(sheep IgG), and then with the 20.6 mAb, revealed with Cy3-conjugated donkey anti-(mouse IgG). Confocal micrographs can be seen in Supplementary Figure S1.
Figure S1  Complete (CD1e) and propeptide-deleted (Δ12CD1e) molecules were stably expressed in M10 cells

Fixed and permeabilized cells were stained with anti-CD1e and anti-TGN46 or anti-CD63 Abs and analysed by confocal microscopy. Merged images are shown in the right-hand panels. Scale bar, 10 μm.

REFERENCE


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