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*J Immunol* 2002; 168:723-733; 
doi: 10.4049/jimmunol.168.2.723
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Lipid Protein Interactions: The Assembly of CD1d with Cellular Phospholipids Occurs in the Endoplasmic Reticulum

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CD1d is a member of a family of lipid Ag-presenting molecules. The cellular ligands associated with CD1d were isolated and characterized by biochemical means as an approach to elucidate the mechanism by which CD1 molecules assemble in vivo. Natural ligands of mouse CD1d included cellular phosphatidylinositol and phosphatidylinositol-glycans that are synthesized in the endoplasmic reticulum. Further biochemical data revealed that the two CD1d mutants, one defective in recycling from-and-to the plasma membrane and the other in efficiently negotiating the secretory pathway, associated with phosphatidylinositol. Thus phosphatidylinositol associated with CD1d in the early secretory pathway. Phosphatidylinositol also associated with CD1d in Pig-A-deficient cells that are defective in the first glycosylation step of glycosylphosphatidylinositol biosynthesis. Moreover, cellular phosphatidylinositol-glycans are not Vα14Jα15 T cell Ags. Therefore, we predict that cellular lipids occlude the hydrophobic Ag-binding groove of CD1 during assembly until they are exchanged for a glycolipid Ag(s) within the recycling compartment for display on the plasma membrane. In this manner, cellular lipids might play a chaperone-like role in the assembly of CD1d in vivo, akin to the function of invariant chain in MHC class II assembly. The Journal of Immunology, 2002, 168: 723–733.

Received for publication August 24, 2001. Accepted for publication November 1, 2001.

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1 This work was supported by grants from the National Institutes of Health (AI42284 to S.J., AI46555 to R.R.B., and DK56309 to M.E.M.), Juvenile Diabetes Research Foundation International, and the Human Frontiers in Science Program.

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5 Abbreviations used in this paper: β₂m, β₂-microglobulin; PI, phosphatidylinositol; DPM, dolichylphosphorylmannose; ER, endoplasmic reticulum; hAs, heteroan-tiserum; endo H, endoglycosidase H; C:M, chloroform:methanol; PI-PLC, PI-specific phospholipase C; PL-Aγ, phospholipase Aγ; oGalCer, α-galactosylceramide; Rα, relative migration.

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by specific T lymphocytes. To elucidate the basis for CD1d1 function, our approach has been to isolate and characterize the associated ligand by biochemical methods. The data revealed that CD1d1 expressed in mammalian cells assembled with a phospholipid, which was identified as GPI (19). Consistent with this data, a recent study demonstrated that a Vα14Jα15 NKT cell hybridoma recognized PI presented by CD1d1 as its Ag (18). Notwithstanding, PI does not represent a major NKT cell Ag (20) and, hence, the role of phospholipid(s) in CD1d1 function remains elusive. Thus, to clarify the role of CD1d1-associated PI and PI-glycans in CD1 function, we have studied the assembly of this molecule in vivo. The data are consistent with the idea that the natural CD1d1-associated ligands play a chaperone-like role during its assembly in vivo akin to the function of invariant chain in MHC class II assembly.

**Materials and Methods**

*Expression constructs*

The full-length βm-cDNA from pEE6-βm (21) was digested with HindIII and BamHI, and the resulting fragment was subcloned into HindIII-BamHI-digested pEE12 (CellTech, Slough, England). The resulting pEE12-βm was checked for integrity by restriction mapping. Full-length CD1d1 cDNA (pBluescript-mCD1d1; kindly provided by Dr. S. Balk, Harvard Medical School, Boston, MA), was subcloned into the pVLI1393 vector (kindly provided by Dr. M. D. Summers, Texas A&M University, College Station, TX) using the XhoI-Nool restriction site. The EcoRI-EcorV fragment from pVLI1393-CD1d1 containing the first four exons of CD1d1 was subcloned into pCR3 (Invitrogen, Carlsbad, CA). Finally, the EcoRI-Nool fragment containing exons 5 and 6 of CD1d1 was subcloned into pCR3 containing exons 1–4 of CD1d1 resulting in pCR3-mCD1d1; thus, the full-length cDNA encoding wild-type CD1d1 was generated. The cDNA encoding soluble CD1d1 (pBluescript-sCD1d1; also provided by Dr. S. Balk) was digested with XhoI and blunted with Klenow polymerase. The XhoI blunt-Nool fragment containing the sCD1d1 cDNA was subcloned into pCR3, resulting in pCR3-sCD1d1. For cloning, the pCR3 vector was prepared by digesting with HindIII and then was blunted with Klenow polymerase and digested with Nool. The cDNA for an endoplasmic reticulum (ER)-retained CD1d1 molecule was constructed by appending the ER retention signal KDEL to the carboxyl terminus of sCD1d1. This construct also contained a c-Myc tag between sCD1d1 and the ER retention signal to facilitate detection of the expressed CD1d1. The sCD1d1-er cDNA was constructed using a 70-bp-long DNA encoding the c-Myc tag and KDEL. It entailed the annealing of the following four oligonucleotides: a, 5'-AAGACTGAATGGAGCAGAATGCCTTTTGGAA-3'; b, 5'-GAGGACCTGATTTGGAGAGTGGATCTGAAAGATC-3'; c, 5'-CGGCGGAGCTGATTTGGAGAGTGGATCTGAAAGATC-3'; and d, 5'-CTTTCTTCAAGTAAAGCCTTTTGGATCTGAAAGATC-3'. This resulted in a 70-bp-long DNA encoding the c-Myc tag and KDEL. The resulting fragment was cloned into the EcoRV-Nool site of pCR3-mCD1d1, resulting in pCR3-sCD1d1-er. A CD1d1 mutant lacking its internalization signal was constructed by substituting the transmembrane and cytosolic region of CD1d1 with that of H2Kβ, resulting in CD1d1-Kβail. The CD1d1-Kβail cDNA was constructed by subcloning an EcoRV-Nool fragment containing exons 5–8 of H2Kβ into the EcoRV-Nool site of pCR3-sCD1d1. In sCD1d1 cDNA, the EcoRV site lies downstream of exon 4, which encodes the α3 domain of the mature protein. cDNA encoding the soluble H2Dβ was constructed by PCR amplification of exons 1–4 using 5'-CAACAAGTCTGGATGAT CCGGGGCGATGCTCCCG-3' forward and 5'-CGGGATCCGGCTCGGAGAGG-3' reverse primers. The resulting product was cleaved with HindIII and BamHI and cloned into the HindIII and BamHI site of pCR3. An authentic pCR3-Dh-sol determined by Sanger dideoxy nucleotide sequence analysis was used for gene transfer.

**Cell lines**

See Table I for a description of CD1- and H2 class I-expressing cell lines. Qβ that expresses mouse βm was generated by gene transfer into NSO plasmaclmya as described (21). Transfected cells were selected 24 h later in 250 μg/ml G418. Qβ cell lines expressing soluble H2Kβ as wild-type and mutant CD1d1 were generated by electroporation of the respective cDNA constructs. Transfected cells were selected in the absence of l-glutamine but in the presence of 0.8 mg/ml of genetin (G418; Life Technologies, Rockville, MD). Qβ and derived lines were maintained in 1-glutamine-deficient DMEM (Media-Tech, Herndon, VA) containing 10% dialysed FBS (HyClone Laboratories, Deer Park, PA, or Life Technologies) and 0.5 mg/ml G418. Kb-high and Db-high cells were maintained as described (21). Pig A-S49a and Pig E-BSW147e mutants (22) were from American Type Culture Collection (Manassas, VA) and maintained in RPMI 1640 containing 0.5 mg/ml G418; stable lines were maintained in 0.25 mg/ml G418.

All cell lines used in this study express wild-type and mutant CD1d1 as well as H2Kα and H2Dβ molecules in the appropriate cell lines, which was confirmed by flow cytometry and/or immune precipitation methods using specific Abs (data not shown).

NKT cell hybridomas DN32.D3 and 431.A11 (gifts from A. Bendelac of Princeton University, Princeton, NJ) as well as N38-2C12 and N37-1A12 (generously provided by K. Hayakawa of Fox Chase Cancer Institute, Philadelphia, PA) were maintained in RPMI 1640 containing 10% FBS.

**Flow cytometric analysis**

To determine CD1d1 expression, ~5 × 10⁶ cells were reacted with ~0.5 μg of biotinylated 1B1, a CD1d1-reactive mAb, and biotinylated Af6, a H2K-reactive mAb, and were detected with streptavidin-CyChrome using a FACSscan flow cytometer (BD Biosciences, San Jose, CA). All reagents were from BD Pharmingen (San Diego, CA).

**Labeling CD1d1-associated ligand with [*H]*mannonse, [*32P]*orthophosphate, [*3H]*inositol, [*3H]*ethanolamine, and [*3H]*mevalonic acid**

At least ~2 × 10⁷ CD1d1-expressing cells or control cells were tritium labeled with 250 μCi of [*3H]*mannose or [*3H]*ethanolamine (American Radiolabeled Chemicals, St. Louis, MO) as described (19). Cells were labeled with 250 μCi of [*3H]*inositol (NEN, Boston, MA) in isoinositol-free DMEM (Life Technologies) or with 250 μCi of [*3H]*mevalonic acid (American Radiolabeled Chemicals) in DMEM. [*32P]*Orhophosphate labeling was accomplished using phosphate-free DMEM (Life Technologies) as described (25). Culture supernatants from tritium-labeled cells were collected for the purification of soluble molecules. The harvested cells were solubilized in 2.5–3 ml of PBS containing 1% (w/v) 3-[3-cholamidopropyl]dimethylammonio]-1-hydroxy-2-propanesulfonate (Sigma-Aldrich, St. Louis, MO). Cell lysates were clarified by centrifugation. Membrane-bound CD1d1 and class I molecules were isolated from the postnuclear fraction, whereas total cellular lipids were extracted from the nuclear and membrane pellet.

**Mouse CD1d1-specific Abs**

Polyclonal heteroantiserum (hAs) against CD1d1 was generated in a rabbit immunized i.m. with ~0.1 mg of Ni-affinity purified sCD1d1 (this Ag was >95% pure) emulsified in Ribi adjuvant. The sCD1d1 immune rabbit was boosted three times with 50 μg of sCD1d1 at 3- to 4-wk intervals. The

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**Table I. Description of cell lines generated and used in this study**

<table>
<thead>
<tr>
<th>Cell Lines</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oβ</td>
<td>NSO plasmaclmya expressing mouse βm</td>
</tr>
<tr>
<td>mCD1d1</td>
<td>Qβ expressing wild-type mouse CD1d1</td>
</tr>
<tr>
<td>sCD1d1</td>
<td>Qβ expressing soluble mouse CD1d1; hexahistidine-tagged</td>
</tr>
<tr>
<td>sCD1d1-er</td>
<td>Qβ expressing sCD1d1 fused to c-Myc tag and KDEL ER retention sequence at the carboxyl terminus</td>
</tr>
<tr>
<td>sCD1d1-Kb tail</td>
<td>Qβ expressing sCD1d1 fused to the transmembrane and cytosolic tail sequence of H2Kα at the carboxyl terminus</td>
</tr>
<tr>
<td>Ks62</td>
<td>Human erythroleukemia cell line</td>
</tr>
<tr>
<td>Ks62-mCD1d1</td>
<td>Ks62 expressing wild-type mouse CD1d1</td>
</tr>
<tr>
<td>IA</td>
<td>Pig-A-deficient derivative of Ks62</td>
</tr>
<tr>
<td>IA-mCD1d1</td>
<td>IA expressing wild-type mouse CD1d1</td>
</tr>
</tbody>
</table>
immune rabbit was terminally bled 2 wk after the last boost. The specificity of the resulting hAs was determined using CD1d1-positive cell lines by flow cytometry as well as by immune precipitation of $^{[35]}$S-cysteine/ $^{[35]}$S-methionine-labeled proteins. It only precipitates CD1d1 from cell lines and no other molecule. In fact, it does not even cross-react with the human homolog CD1d or paralog CD1b (data not shown). Therefore, it is less likely to cross-react directly with a lipid, lipoprotein, or another lipid binding protein.

**Biosynthetic labeling of proteins**

Steady-state as well as pulse labeling of cells with $^{[35]}$S-cysteine/$^{[35]}$S-methionine-labeled cell lysates were performed as described (21). After preclearing with normal mouse serum, samples were immune precipitated with the anti-CD1d1 hAs or an appropriate control Ab as described. An H2K$^b$-specific (Y3) (26) or H2D$^d$-specific (B22-249) (26) mAb was used when experiments were performed with mouse cell lines. W6/32 (generously provided by J. Yewdell of National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD), an anti-HL-A class I-specific mAb (27), was used when experiments were performed in human cell lines. Immune precipitates were separated by 15% SDS-PAGE and visualized by autoradiography. In pulse-chase experiments, the immune precipitates were subjected to 1–2 μU or varying concentrations of endoglycosidase H (endo H) for 14–16 h and were processed as above.

**Affinity chromatography**

CD1d1 molecules and the control class I molecules from each sample were sequentially purified by affinity immune chromatography as previously described (28). CD1d1-specific and class I-specific affinity columns were prepared by prebinding ~0.1–0.15 mg of purified Ab to 1 ml of 50% protein A-Sepharose slurry (Repligen, Needham, MA). Unbound Ab and nonspecifically bound proteins were washed away with PBS and resuspended in an equal volume of the same buffer. Each Ab-bound protein A-Sepharose was packed into a 5-ml disposable column (Pierce, Rockford, IL) and used to purify CD1d1 and class I molecules. Immune affinity chromatography was performed as described earlier (28). Secreted CD1d1 from the tritium labeling supernatants was purified using Hi-Trap metal chelating columns (Amersham Pharmacia Biotech, Piscataway, NJ) according to the manufacturer’s instructions. Ten 0.5-ml fractions were collected; one-tenth of each fraction was dissolved in Econo-safe scintillation cocktail according to the manufacturer's directions, and exposed to autoradiography. In pulse-chase experiments, the immune precipitates were subjected to 1–2 μU or varying concentrations of endoglycosidase H (endo H) for 14–16 h and were processed as above.

**Lipid extraction from affinity-purified proteins**

Radioactive fractions from each affinity-purified sample were pooled. An equal number of fractions not containing radioactivity were pooled separately. Lipids were extracted by Bligh-Dyer method (29) with two volumes of chloroform:methanol (2:1 C:M) with a 30 min ultrasonic bath. The aqueous phase was extracted with chloroform:methanol (2:1 C:M). After thorough mixing, the top aqueous organic phases were allowed to separate. The organic phase was carefully transferred into another tube. The aqueous phase was extracted two or three more times. The resulting organic phase was pooled with the first and dried under vacuum or a gentle stream of N$_2$ gas. The dried extract was redissolved in 0.5 ml of C.M. Radioactivity was monitored by scintillation counting using $^{10}$–$^{20}$ μl of the extracted lipids.

**Enzymatic digestions of lipids**

C:M in two equal aliquots of the lipid extracts were evaporated under vacuum or N$_2$ gas. One aliquot was dissolved in 50 μl of PBS (pH 7–7.4) and digested with $^{55}$μl of PI-specific phospholipase C (PI-PLC; Glyko, Novato, CA) at 37°C for 1 h. Likewise, the second aliquot was dissolved in 50 μl of buffer containing 4.9 mM CaCl$_2$ and 147 mM NaCl (pH 8.9) and digested with $^{1}$ U of phospholipase A$_2$ (PLA$_2$) (Sigma-Aldrich) at 25°C for 1 h. The enzymatic reactions were stopped by C:M extraction. The organic phase was evaporated, resuspended in 20–40 μl of C:M, and spotted onto a 20-cm $\times$ 20-cm K6 silica gel TLC plate (Whatman, Clifton, NJ). Approximately 25 μg of PI was spotted and served as a standard. Additionally, $^{0.025}$ μCi of $[^{3}H]$PI or $[^{3}H]$DPM (American Radiolabeled Chemicals) was used as radioactive standard.

**TLC**

TLC was performed in a chamber saturated with 100–150 ml of neutral mobile phase containing chloroform:methanol:water in 10:10:3 ratio (30). The plate was dried for at least 1 h. Sprayed with EN$[^{3}H]$ANCE (NEN Life Sciences) according to the manufacturer’s directions, and exposed to autoradiographic film or a phosphorimager TR plate specifically sensitive to tritium (Fuji Medical Systems, Stamford, CT). Phosphorimaging was facilitated by an FLA 2000 Fluorescent Image Analyzer (Fuji Medical Systems).

**NKT cell stimulation and ELISA**

Equal numbers ($\sim$5 X 10$^3$ cells per well) of stimulator cells and responder NKT cell hybridomas were cocultured for 18–20 h at 37°C. Stimulation of hybridomas was measured by monitoring IL-2 secretion by ELISA. ELISA was performed using JES6-1A12 and JES5-5H4 (BD Pharmingen) as IL-2 capture and detection mAbs, respectively, according to the manufacturer’s instructions.

**Results**

A mannosylated phospholipid associates with mouse CD1d1

Initial characterization of the CD1-associated ligand(s) relied on determining the mass of the compounds eluted from CD1d1. Interpretation of the mass spectral data suggested the natural ligand of CD1d1 to be GPI (19). To study CD1-lipid interactions in vivo as well as to characterize the natural CD1-associated ligand(s), a biochemical method was established. For this purpose, cells that express the soluble form of CD1d1 and H2D$^d$ class I molecules, sCD1d1 and Db-sol, respectively, were generated. Soluble molecules were used to establish the biochemical method because it provides an abundant source of CD1d1 and, hence, the associated ligand(s). Additionally, in a previous study we had demonstrated that $[^{3}H]2$-mannose-labeled ligands specifically associated with sCD1d1 (19).

Thus, to characterize the natural CD1d1-associated ligand(s), both sCD1d1 and Db-sol were radiolabeled with $[^{3}H]$2-mannose. $[^{3}H]$2-mannose was used in this experiment because 1) its label is seldom lost to another sugar but fucose (31) and 2) it labels GPI as well as DPM-glycans. sCD1d1 and control Db-sol were affinity purified from the supernatant of $[^{3}H]$2-mannose-labeled cells. The associated ligand(s) were extracted by the Bligh-Dyer method, separated by TLC in a neutral mobile phase along with nonradioactive mammalian PI as a standard, and visualized by fluorography. The data revealed that $[^{3}H]$2-mannose was incorporated into a dominant lipid species along with two minor ones that were associated with sCD1d1 (Fig. 1, lane 1) but not with Db-sol (Fig. 1, lane 2). Interestingly, the three CD1d1-associated lipid species have a migratory pattern on the TLC that was distinct from the nonradioactive PI standard (Fig. 1, arrow) with a relative migration (R.J) suggestive of a polar compound.

One possible explanation of the above analysis is that $[^{3}H]$-labeled lipids spilled into the tissue culture medium from cells

![FIGURE 1. Mannose-containing lipids associate with CD1d1. Lipids were extracted from affinity-purified soluble CD1d1 and soluble H2D$^d$ expressed by $[^{3}H]$2-mannose-labeled sCD1d1 (lane 1) and Db-sol (lane 2) cells. They were separated by TLC using a neutral mobile phase (C:M: HOH, 10:10:3 ratio). Tritium signal was amplified with EN$[^{3}H]$ANCE spray and detected by phosphorimaging. Nonradioactive PI was used as the standard in this experiment; its position after TLC is indicated by an arrow.](https://www.jimmunol.org/content/jimmunol/725/1/275/F1.large.jpg)
used as standards (lanes 4). Lipids were extracted from the postnuclear fraction of [3H]2-mannose-labeled proteins in the culture supernatant are in low concentrations (S. Joyce, unpublished data). Despite nonspecific purification, [3H]-labeled lipids were not found in Db-sol supernatants passed over Ni-Sepharose columns (Ref. 19 and data not shown). Second, CD1d should bind all the [3H]2-mannose-labeled lipids synthesized by the cell (Fig. 2B) were it nonspecifically “mopping-up” the numerous [3H]-labeled lipids spilled into the tissue culture medium. This it does not, and hence the [3H]-labeled lipids shown in Fig. 2A are specifically bound to CD1d.

PI-PLC-sensitive ligand associates with CD1d. Mannose can randomize to glucose, galactose, and fucose. However, because the label in [3H]2-mannose is on the hydrogen of C-atom 2, the label would be predominantly found in mannose and fucose. In rare instances, the label from [3H]2-mannose can be found in glucose and lactic acid, suggesting that during oxidation and reduction reaction resulting in the epimerization of mannose to glucose, the label may be transferred from C-atom 2 to C-atom 1 (see Ref. 31). Thus, the major labeled spot in Fig. 1 could be due to a glycan modifying a lipid and/or a protein. For three reasons we believe that the [3H]2-mannose-labeled compound is a glycolipid and not a glycopeptide or glycoprotein. First, the control Db-sol, also a glycoprotein with two N-linked glycans, processed in the same manner as sCD1d, does not contain the tritated spots seen with the CD1d sample (Fig. 1). Second, the CD1d-associated [3H]2-mannose-labeled ligands are PI-PLC sensitive as described below. Third, sialic acid-containing glycans have poor mobility on TLC and hence remain at or a bit above the origin in the mobile phase used. For example, ceramide migrates to the front of the TLC, whereas sialic acid containing glycoceramides remains close to the origin (S. Joyce, unpublished data). Because each glycan moiety added to proteins contains three sialic acid units, it is less likely to migrate to the center of the TLC as did the [3H]2-mannose-labeled ligand extracted from sCD1d. Therefore, we conclude that the [3H]2-mannose-labeled compound associated with CD1d is not its own glycan.

To determine the chemical nature of the [3H]2-mannose-labeled lipid(s) associated with CD1d, in the second experiment the Bligh-Dyer extracted ligand(s) was subjected to PI-PLC or PLA2 digestion. PI-PLC specifically cleaves inositol-containing phospholipids; its enzymatic activity is sensitive to acyl-modification of the inositol head group (32). In contrast, PLA2 specifically cleaves fatty acyl modification at C-atom 2 of glycerolipids; it is sensitive to the stereochemistry of the asymmetric C-atom 2 (33). The enzymatic reaction was stopped by lipid extraction; the products were separated by TLC in a neutral gradient along with PI as well as [3H]PI and [3H]DPM standards and were detected by autoradiography. The data revealed that cleavage with PI-PLC resulted in the loss of the [3H]2-mannose label from the sCD1d-associated ligand (Fig. 2A, lane 1). This result is consistent with release of the water-soluble [3H]2-mannose-labeled phosphoinositol-glycan from mannosylated PI because the glycan does not partition into the organic phase. In contrast, cleavage with PLA2 did not result in the loss of the [3H]2-mannose label (Fig. 2A, lane 2). Both the PLA2-cleaved and uncleaved [3H]2-mannose-labeled PI partition into the organic phase during Bligh-Dyer extraction. Therefore, it is unclear whether the sCD1d-associated mannosylated PI is sensitive to PLA2 or not. Thus, together with the previously published mass spectral data, we conclude that GPI is a major [3H]2-mannose-labeled lipid associated with CD1d.

To determine the diversity of cellular lipids that incorporate [3H]2-mannose, one-twentieth of the postnuclear detergent lysate of [3H]2-mannose-labeled sCD1d and Db-sol cell lines was subjected to Bligh-Dyer extraction. The organic phase of this extract was separated by TLC in a neutral mobile phase and visualized by autoradiography. The data revealed that both sCD1d and Db-sol cell lines incorporated [3H]2-mannose into several glycolipids (Fig. 2B) whose identities, because they are not essential to this study, were not determined. Thus CD1d selects a single dominant ligand from a cellular pool of several [3H]2-mannose-labeled glycolipids.

Stoichiometry of CD1d-GPI association. Previously, we reported that 90% of CD1d molecules are occupied by GPI. Being a glycoprotein, [3H]2-mannose labels the glycan moiety of CD1d α-chain as well. Therefore, the ratio of the [3H]2-mannose label associated with CD1d to that of the extracted ligands provides a measure of what percentage of CD1d molecules is associated with GPI. To determine the number of glycan groups on CD1d, it was immune precipitated from cells pulse labeled with [35S]cysteine/[35S]methionine and either not chased or chased for 2 h. The immune precipitates were then subjected to digestion with varying concentrations of endo H. H2K9, which has one N-glycan modification (34), was used as the control. The data revealed five endo H-sensitive radioactive bands in the case of CD1d (Fig. 3A) and one, as expected, in the case of H2K9 (Fig. 3B). Thus, all predicted glycosylation sites of CD1d are modified by N-linked glycans.

To determine the stoichiometry of CD1d and GPI, the amount of radioactivity in the aqueous and the organic phases of the Bligh-Dyer extract were monitored. Because CD1d is modified by five glycans, it would contain ~15 mannose residues. GPI, in contrast, contains a minimum of three mannose residues. Therefore, a 1:1 stoichiometry of CD1d1 to GPI should yield five times as much radioactivity in the aqueous phase compared with the organic.

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**FIGURE 2.** A natural ligand of CD1d1 is mannosylated-PI glycan. A. Lipids were extracted from affinity-purified soluble CD1d1 and soluble H2D+ secreted by [3H]2-mannose-labeled sCD1d1 and Db-sol cells. Lipids extracted from sCD1d1 were divided in two aliquots. One aliquot was subjected to PI-PLC digestion (lane 1) and the other to PLA2 digestion (lane 2), and then they were re-extracted and separated by TLC using a neutral mobile phase (C:M:HOH, 10:10:3 ratio) along with untreated lipid extract of Db-sol (lane 3). About 0.025 μCi of [3H]PI and [3H]DPM were used as standards (lanes 4 and 5, respectively). Tritium signal was amplified with ENHRANCE spray and detected by autoradiography. B. Total lipids were extracted from the postnuclear fraction of [3H]2-mannose-labeled Db-sol (lane 1) and sCD1d1 (lane 2) cells solubilized in detergent. The two lipid samples along with [3H]PI and [3H]DPM were separated by TLC and visualized as in A.
phase. In over three separate experiments, approximately seven to 10 times the radioactivity was found in the aqueous phase compared with that in the organic phase (data not shown). Thus, greater than half the total CD1d1 molecules were associated with [3H]2-mannose-labeled GPI. Considering 70–80% efficiency of Bligh-Dyer extraction, consistent with the previous report (19), >90% of CD1d1 molecules are associated with GPI.

Calculations of stoichiometry require steady-state labeling of constituents being compared. Note that DPM and dolichylphosphophorylglycans that contain mannose are precursors that feed into the biosynthesis of glycans added onto PI as well as glycoproteins. Their rates of synthesis are about the same within cells and, hence, the precursor pools should be the same after pulse with [3H]2-mannose, which in our experiments was for 24 h. Therefore, the results presented herein from our calculations will not differ from the actual stoichiometry of CD1d1 and the associated compound.

Select few cellular lipids associate with CD1d1

As noted above, the majority of CD1d1 assemble with GPI. However, because [3H]2-mannose does not label all cellular lipids, it does not reveal the repertoire of ligands that associate with CD1d1. Additionally, our previous study revealed an ion pertaining to free PI and several unidentified ions. These observations raised the question regarding the diversity of the repertoire of CD1d1-associated cellular lipids. To address this question, cell lines expressing wild-type CD1d1 and H2Kb were pulse-labeled for 30 min with [35S]cysteine/[35S]methionine, washed with PBS, and divided in two equal aliquots. One aliquot was maintained on ice, whereas the other was chased for 2 h. Both were solubilized in detergent, and the postnuclear fraction was divided into six equal aliquots. CD1d1 (A) and H2Kb (B) were immune precipitated from the respective postnuclear fractions. The immune precipitates were subjected to cleavage of N-linked glycans with varying concentrations of endo H, separated by 10% SDS-PAGE under reducing conditions and detected by autoradiography. The arrows show the various N-glycosylated forms of CD1d1 H chain. Data are representative of two similar experiments.

FIGURE 3. CD1d1 is modified at all predicted N-linked glycosylation sites. Cells expressing wild-type CD1d1 and H2Kb were pulse-labeled for 30 min with [35S]cysteine/[35S]methionine, washed with PBS, and divided in two equal aliquots. One aliquot was maintained on ice, whereas the other was chased for 2 h. Both were solubilized in detergent, and the postnuclear fraction was divided into six equal aliquots. CD1d1 (A) and H2Kb (B) were immune precipitated from the respective postnuclear fractions. The immune precipitates were subjected to cleavage of N-linked glycans with varying concentrations of endo H, separated by 10% SDS-PAGE under reducing conditions and detected by autoradiography. The arrows show the various N-glycosylated forms of CD1d1 H chain. Data are representative of two similar experiments.

piolate phospholipids. The data revealed that three [3H]inositol-labeled lipids were associated with CD1d1 (Fig. 4A, lane 2). Two of the [3H]inositol-labeled lipids were specifically extracted from CD1d1, but the third faster migrating lipid was also found in extracts of H2Kb (Fig. 4A, lanes 1 and 2). The two [3H]inositol-labeled lipids were selected by CD1d1 among several 8–10 biosynthetically labeled lipids synthesized by the cell (Fig. 4C, lane 1). Of the two [3H]inositol-labeled lipids associated with CD1d1, one has the same Rf as the [3H]PI standard, suggesting that this lipid may be PI. The [3H]PI standard consists of stearic and arachidonic acid at C-atoms 1 and 2 (according to the supplier), the two fatty acids predicted from the mass spectral data of CD1d1-associated ligand(s) reported previously (19).

To determine whether the [3H]inositol-labeled lipids were PI, they were subjected to PI-PLC digestion. The lipid spot that comigrates with the [3H]PI standard was specifically lost upon digestion with PI-PLC (Fig. 4A, lanes 2 and 4). However, the slow migrating lipid spot was partially resistant to PI-PLC (Fig. 4A, lane 4). PI-PLC activity on CD1d1-associated [3H]inositol-labeled lipid is specific because it cleaves [3H]PI standard (Fig. 4B, lane 2) but not [3H]DPM (data not shown). Also note that PI-PLC digestion of [3H]PI results in a slow migrating spot (Fig. 4B, arrow) similar to that observed upon cleavage of CD1d1-associated ligand with PI-PLC (Fig. 4A, arrow). This new species could be [3H]inositolphosphate. Together, the data suggest that the faster migrating CD1d1-associated lipid that comigrates with the [3H]PI standard is mammalian PI, whereas the identity of the second lipid species is unknown. Thus, PI is another CD1d1-associated natural ligand.

The extent to which it occupies CD1d1 was not measurable in the current experiment.

Of the two CD1d1-associated lipid spots, only the slowest migrating species was partially susceptible to PLA2 (Fig. 4A, lanes 2 and 3). Resistance to PLA2 was not due to inactivity of the enzyme, because a larger quantity of [3H]PI standard compared with

FIGURE 4. CD1d1 assembles with PI. A, [3H]inositol-labeled lipids were extracted from affinity-purified CD1d1 (lanes 2–4) as well as control class I H2Kb (lane 1), and the CD1d1-derived lipids were divided into three aliquots. One aliquot was treated with buffer alone (lane 2), the second with PLA2 (lane 3), and the third with PI-PLC (lane 4). After enzymatic digestion, the lipids were extracted and separated by TLC as in Fig. 2B. [3H]-Labeled lipids were detected as in Fig. 1 B. [3H]PI standard was undigested (lane 1) or digested with either PI-PLC (lane 2) or PLA2 (lane 3), extracted, separated, and visualized as described in Fig. 2B. C, Total lipids were extracted from the postnuclear fraction of [3H]inositol-labeled CD1d1 (lane 1) and Kb-high (lane 2) cells solubilized in detergent. The two lipid samples were separated by TLC and visualized as in Fig. 2B. Standards included –0.025 μCi of [3H]PI and [3H]DPM (lanes 5 and 6 in A, lanes 4 and 5 in B, and lanes 3 and 4 in C). Note that a slow migrating polar compound, presumably [3H]inositol-1-phosphate, results from the digestion of CD1d1-associated PI as well as the [3H]PI standard (see Fig. 4, A and C). F, Front; O, origin; a, [3H]DPM standard; b, [3H]PI standard.
the CD1d1-associated ligand was sensitive to PLA₂ (Fig. 4B, lane 3). Because PLA₂ is sensitive to the stereochemistry of C-atom 2 of glycerophosphatides, the configuration of this atom in CD1d1-associated PI may be distinct from the standard mammalian PI.

It was puzzling that the [3H]inositol label was not incorporated into GPI. If GPI was labeled, it being more polar, would migrate more slowly than PI upon TLC. Such a slow migrating lipid with an Rₜ similar to the mannosylated-PI-glycan observed in Fig. 1 was not present among CD1d1-associated [3H]inositol-labeled lipids (Fig. 4A). The reason for this is currently unknown.

Dolichylphosphorylglycans and phosphatidylethanolamine do not assemble with CD1d1. Because dolichylphosphorylglycans and phosphatidylethanolamine are indigenous to the ER, whether they also assemble with CD1d1 was determined. The data revealed that neither CD1d1 nor H2Kb contained any [3H]mevalonic acid-labeled lipid (Fig. 5A), despite the fact that the cell lines that express them contained numerous biosynthetically labeled lipids (Fig. 5B). The fast migrating predominant spot extracted from CD1d1 has an Rₜ predicted for cholesterol; it was not found consistently. Additionally, this lipid was also found in extracts of the control class I molecules in the fraction of [3H]mevalonic acid-labeled mCD1d1 (expresses a mutant CD1d1 that had its transmembrane and cytosolic regions replaced by a KDEL signal (37, 38). At its carboxyl terminus (sCD1d1-er). The second line pressed CD1d1 in the ER by virtue of containing the KDEL signal chaperones, then GPI and/or PI would assemble with CD1d1 in the ER. Thus, dolichylphosphorylglycans and phosphatidylethanolamine may be minor, if at all, ligands of CD1d1.

CD1d1 assembles with PI in the ER

It is clear that neither GPI nor PI is the natural Ag of NK cells (20). However, it is possible that GPI and PI have a chaperone-like function in the assembly of CD1d1. If they do indeed function as chaperones, then GPI and/or PI would assemble with CD1d1 in the ER. Thus, two cell lines were generated. One line retains the expressed CD1d1 in the ER by virtue of containing the KDEL signal (37, 38), at its cytosolic carboxyl terminus (sCD1d1-er). The second line expresses a mutant CD1d1 that has its transmembrane and cytosolic tail replaced with that from the nonrecycling H2Kb (sCD1d1-Kb tail) and, hence, has lost its endosome/lysosome-targeting signal. The expression and intracellular trafficking patterns of the wild-type CD1d1 as well as sCD1d1-er and sCD1d1-Kb tail mutants were determined. For this purpose, cell lines expressing the wild-type and the mutant CD1d1 molecules were pulse labeled with [35S]cysteine/[35S]methionine and chased for the indicated time periods. Cells were solubilized with detergent, and the postnuclear fraction was subjected to immune precipitation with CD1d1-specific hAs. The sCD1d1-er mutant was also immune precipitated from the labeling supernatant. Immune precipitates were subjected to endo H digestion. Both CD1d1 and sCD1d1-Kb tail traffic through the secretory pathway with similar kinetics; their t₁/₂ in the ER is between 1 and 2 h (Fig. 6A, upper two panels). Thus, replacement of the transmembrane and the cytosolic regions of CD1d1 with those of H2Kb did not significantly alter the intracellular traffic of the mutant CD1d1 molecule.

In contrast, the traffic pattern assessed by pulse-chase experiments described above is significantly altered in the case of sCD1d1-er. Its t₁/₂ in the ER is ~4–8 h (Fig. 6A). The delayed egress of the sCD1d1-er mutant from the ER is consistent with the function of the KDEL ER-retention signal (37, 38). A reason that the ER retention signal was appended to the secreted form of CD1d1 is that, if it did egress from the ER, it would not be retrieved but secreted into the growth medium so that it will not confound the experiment described in Fig. 6C. The sCD1d1-er mutant is indeed secreted into the labeling supernatant upon achieving endo H resistance (Fig. 6A).

Whether sCD1d1-Kb tail had lost its ability to traffic through the endosome/lysosome compartment was determined using CD1d1-restricted NKT cell hybridomas as probes. One Vα14J4e15 NKT cell hybridoma, DN32.D3, recognizes an endosomal/lysosomal Ag presented by CD1d1 (39) and, hence, should not be activated by sCD1d1-Kb tail. A second CD1d1-restricted NKT cell hybridoma, 431.A11, which expresses the Vα3 receptor, recognizes an Ag present in the anterograde secretory pathway (39). It should be activated by sCD1d1-Kb tail. Thus, both DN32.D3 and 431.A11 recognize mCD1d1 (Fig. 6B). Moreover, as expected, DN32.D3 did not recognize, whereas 431.A11 did recognize, sCD1d1-Kb tail (Fig. 6B). Thus, sCD1d1-Kb tail inefficiently, if at all, traffics through the endosomal/lysosomal compartments.

Thus, the wild-type and the two mutant CD1d1 molecules provide a good system to determine the site of CD1d1 assembly with cellular lipids. Cell lines expressing the three forms of CD1d1 as well as control H2Kb were metabolically labeled with [3H]inositol and solubilized in detergent, and CD1d1 as well as H2Kb were sequentially affinity purified from their postnuclear fractions. The eluted fractions were collected and the amount of radioactivity in each of 10 fractions was measured. The data revealed that [3H]inositol was incorporated into molecules associated with CD1d1 but not significantly into H2Kb (Fig. 6C). The parent cell line used for ectopic expression of CD1d1 and H2Kb expresses a small amount of endogenous CD1d1 (data not shown). Because [3H]inositol is incorporated into PI, assembly of CD1d1 with PI occurs in the ER. Moreover, GPI is associated with sCD1d1, a form that does not enter the endosome/lysosomal compartment. Thus, we conclude that the assembly of PI and GPI with CD1d1 occurs in the ER.

PI is sufficient for complete assembly and intracellular traffic of CD1d1

Because GPI is the major natural ligand of CD1d1 and PI assembles with CD1d1 in the ER, it is possible that these cellular phospholipids play a chaperone-like role in the assembly of CD1d1. Self-peptides derived from the cytosol play a critical role in the assembly of MHC class I molecules in the ER. Proper assembly of class I molecules is inhibited by the lack of peptides in the ER (40). Thus, we reasoned that the absence of GPI and PI in cells would adversely affect CD1d1 assembly in GPI-deficient cell lines.

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activation, was detected by ELISA. Data are representative of at least three similar experiments. The supernatants were collected after 12–18 h of coculture, and secreted IL-2, a measure of NKT cell activation, was measured.

Kbtail, and sCD1d1-er, as well as Kb-high, were labeled with [3H]inositol. Similar experiments. CD1d1 and H2Kb were affinity purified from the labeled cells, and their endogenous CD1d1 and H2 class I molecules were affinity purified from postnuclear fractions of detergent lysates, and the amount of radioactivity in each eluted fraction was monitored by scintillation counting. The data revealed that the [3H]inositol label was associated with CD1d1 and not with H2Kκ (Fig. 7D, a and d). Interestingly, about the same amount of [3H]inositol-labeled compound was associated with CD1d1 expressed by GPI-positive K562-mCD1d1 and GPI-negative IA-mCD1d1 cells (Fig. 7D, b and c). Thus, CD1d1 assembles with an inositol-containing lipid even in the absence of GPI.

To conclusively prove that the CD1d1-associated [3H]inositol is indeed incorporated into PI, the associated ligand(s) was extracted, separated by TLC, and detected by phosphorimaging. The data revealed that one of the three detectable lipids extracted from CD1d1 is indeed PI, because the Rf of this lipid is similar to the mammalian [3H]PI standard (Fig. 7D) and because this spot extracted from mCD1d1 is sensitive to PI-PLC (Fig. 4A). Thus, CD1d1 assembles with PI in GPI-deficient cells.

The association of CD1d1 with PI in the IA-mCD1d1 cell line could be due to its assembly with human β2m. Therefore, mouse cell lines S49a and Bw5147e deficient in Pig A and Pig E (complementation group E mutant that is unable to mannosylate glucosaminylated PI), respectively (22), were labeled with [3H]inositol, and their endogenous CD1d1 and H2 class I molecules were affinity purified. Scintillation counting of the eluted fractions revealed that [3H]inositol-derived radioactivity was associated with affinity purified CD1d1 only but not with control class I molecules (data not shown). Thus, the association of CD1d1 with PI in IA-mCD1d1 is not due to assembly with human β2m. Instead, the data suggest that CD1d1 uses lipids indigenous to the ER to assemble stable CD1d1 in vivo.

**Discussion**

In summary, the data presented herein affirm that CD1d1, a member of the CD1 family of lipid binding proteins, selectively associates with PI and GPI in vivo. Additional data revealed that the association of CD1d1 with the phospholipids occurs during the assembly of CD1 in the ER. These findings are consistent with...
the discontinuous electron density observed within the ligand-binding site in the mouse CD1d1 crystal structure. Note that this structure was determined without the addition of exogenous lipids during protein purification (7). Together the data suggest that lipids indigenous to the ER may play a chaperone-like role in the assembly of CD1d1 in vivo akin to the function of invariant chain in the assembly of MHC class II molecules in the ER.

Selective binding of lipids to CD1d1 in vivo

Among the numerous [3H]2-mannose-, [3H]inositol-, and [3H]mevalonic acid-labeled lipids, only PI and GPI assemble with CD1d1. The label in [3H]2-mannose does not transfer into another sugar but into fucose (31). Therefore, if a fucosylated lipid(s) is indeed synthesized by the cell, it is not associated with CD1d1 because such lipids are resistant to PI-PLC. Among the mannosylated lipids synthesized in the ER are GPI and DPM-glycan. The latter are precursors of N-linked glycans attached to glycoproteins (36). They were not associated with CD1d1 in the [3H]2-mannose and the [3H]mevalonic acid labeling experiments. Thus CD1d1 selects GPI over DPM-glycans.

Two [3H]inositol-labeled lipids specifically associate with CD1d1, one of which is PI-PLC-sensitive PI. The character of the second, slow-migrating, [3H]inositol-labeled compound is unknown because no inositol-containing lipid other than PI is known to exist in mammalian cells. That notwithstanding, inositol-containing ceramides are synthesized by yeast (41) in lieu of sphingomyelin (42). Although not described in mammalian cells, inositolceramide or a similar compound may be synthesized by certain mammalian cells (e.g., tumor cells such as in NS0 plasmacytoma and K562 erythroleukemia lines) used in the present study. Alternatively, because inositol can be converted to glucose, this novel compound associated with CD1d1 could be a glucose-containing lipid other than a PI-glycan. Additionally, this novel compound is resistant to both PI-PLC and PLA2. Thus, further biochemical studies are required to solve the structure of the novel CD1d1-associated compound.

Mammalian cells synthesize nonacylated and acylated inositol-containing GPI anchors for proteins (43), and protein-free GPI anchors although usually contain acylated inositol (44) can contain nonacylated inositol. The GPI extracted from CD1d1 is sensitive to PI-PLC. Therefore, the GPI associated with CD1d1 does not contain an acylated inositol. Furthermore, the PI associated with CD1d1 is resistant to PLA2 (45). PLA2 is sensitive to the stereochemistry of the asymmetric C-atom 2 of glycerophosphatides.
Therefore, one plausible reason for the resistance of the CD1d1-associated ligand to PLA2 might be the stereochemistry of PI. Whereas the glycerol in cellular PI has its asymmetric C-atom 2 in \(\sigma\)-configuration (45), that in the CD1d1 ligand might be in the \(\tau\)-configuration. Alternatively, C-atom 2 in CD1d1-associated PI may be modified by a PLA2-resistant ether linkage to an alkyl group as opposed to the PL\(\alpha\)2-sensitive ester linkage. The CD1d1-associated PI is less likely to contain an alkyl group because its mass would be distinct from the observed mass of CD1d1-associated PI reported previously (19).

Thus, CD1d1 exhibits selectivity in the type of natural ligands it binds: selectivity is observed in the type of lipids that bind (e.g., CD1d1 selects PI and GPI over DPM-glycans and numerous other cellular lipids). CD1d1-associated PI is resistant to PL\(\alpha\)2, and hence the C-atom 2 has a distinct configuration. Finally, CD1d1 associates with GPI distinct from the common mammalian GPI (i.e., it does not contain an acylated inositol common to mammalian GPI).

Model for CD1 assembly, intracellular traffic, and Ag presentation

The association of PI and GPI with CD1d1 raised interest in the biological significance of this finding. A thorough search for the function of CD1d1-GPI complex in vivo revealed that it is inert to immune system. Thus, NKT cells do not recognize the CD1d1-GPI complex (Ref. 20 and data not shown). However, the possibility remains that the CD1d1-PI can be recognized by some NKT cells. Consistent with this possibility, one study reported the recognition of PI in the context of CD1d1 by an autoreactive V\(\alpha\)14J\(\alpha\)15-positive NKT cell hybridoma (18). Thus, this finding supports the biological relevance of the CD1d1-associated PI described herein. That notwithstanding, the prevalence of PI-reactive NKT cells in vivo and their physiological role remain undefined.

The data presented herein support the idea that the lipids associated with CD1d1 play a chaperone-like role in the assembly of CD1d1. Being a type I integral membrane protein, akin to the classical Ag-presenting molecules for which much is known regarding assembly in vivo, the assembly of CD1d1 begins as it is cotranslationally inserted into the ER. Soon thereafter it binds calnexin, a step inferred from the latter’s association with a group I CD1 synthesized in the absence of \(\beta\)m (46) as well as with MHC class I molecules (47–49). Calnexin probably prevents aggregation of unassembled CD1 H chains. The monomeric H chain then assembles with \(\beta\)m, forming a heterodimer that is receptive to lipids of the ER and/or the downstream vesicular compartment(s).

The ability to extract PI from both wild-type CD1d1 and derived mutants that do not negotiate the recycling compartment (19) suggests that the association of the cellular ligand with CD1d1 occurred in the secretory pathway. The association of lipids with CD1d1 during biosynthesis probably satisfies two structural requirements: 1) protecting the deep, nonpolar hydrophobic Ag binding groove from collapse; and 2) occupying that groove with a ligand that can easily be exchanged for another lipid. Thus, PI and GPI might play a chaperone-like role (Fig. 8) akin to the function of MHC class II-associated invariant chain in class II assembly in vivo (50).

CD1d1 expression in PIG-A mutants is normal. Additionally, V\(\alpha\)14J\(\alpha\)15-positive and -negative NKT cells recognize CD1d1 expressed by the mutant as effectively as they recognize those expressed by wild-type cells. Thus, GPI, albeit a major natural ligand of CD1d1, is neither essential for the assembly of CD1d1 in vivo nor the natural Ag of NKT cells (20). Therefore, in the absence of GPI, PI or other lipids of the ER may assist CD1d1 assembly in vivo. After assembly of CD1d1 in the ER, it negotiates the secretory pathway and arrives at the MHC class II-enriched vesicles either directly from the trans-Golgi or via the plasma membrane. Targeting to the class II-enriched vesicles depends on the Yxx\(\phi\) internalization sequence found at the cytosolic tail of CD1d (39, 51). Here, CD1d meets the naturally processed Ag(s) whence they arrive at the plasma membrane for presentation to NKT cells (Fig. 8) (39, 51). Because human CD1 show structural (11, 52–56) and functional (57–59) similarities to CD1d1, we predict that the above assembly and intracellular traffic mechanism may be a common feature of this family of lipid Ag-presenting molecules.

To support our model that PI and GPI-lysosomes act as chaperones, an in vitro competition assay was performed. sCD1d1, when plate bound, does not activate NKT cell hybridomas. The addition of GalCer but not PI to plate-bound sCD1d1 specifically activates V\(\alpha\)14J\(\alpha\)15 NKT cell hybridomas. This stimulatory activity of GalCer can be inhibited by titrating amounts of PI (A. K. Stanic, L. Van Kaer, and S. Joyce, unpublished data). Considering that PI and GalCer have similar affinities for CD1d1 (19, 60), the reverse can also occur. Thus, PI and PI-glycans assembled with CD1d1 in the ER can be exchanged for another lipid.

Two recent reports indicate that CD1d molecules can be assembled in vitro by oxidative refolding chromatography (61, 62). Albeit an inefficient process, these reports suggest that CD1d can assemble in the ER of mammalian cells without cellular lipids; i.e., CD1d assemble as empty molecules in the ER. However, it is possible that the lipids contained within the H and L chain inclusion bodies could have been included in the in vitro assembly process, thus achieving a low level of folding of CD1a. A similar argument can be levied against our data as well, in that lipids resulting from cell death could have contributed to the elution of radio-labeled lipids from CD1d1. However, this co-elution is less likely because, as discussed above, CD1d1 did show selectivity in associating with
cellular lipids and did not bind all lipids that incorporated the test label. Additionally, the previously discussed selectivity also rules out the possibility that lipids nonspecifically associate with CD1d1 because they cosegregate within detergent micelles during CD1 extraction.

How lipid loading onto CD1d1 in the ER is accomplished and how these lipids are exchanged for Ag in endocytic vesicles remain important unsolved questions. Because lipids are membrane embedded, they need to be “plucked out” of the membrane and loaded into the Ag-binding groove of CD1d1. This event might require an elaborate molecular machinery similar to the peptide loading complex essential for peptide Ag assembly with MHC class I and II molecules (40, 50). Solution of the mechanism should provide insights into the basis for lipid-protein interactions in vivo as well as into the evolution of the lipid Ag-presenting system in vertebrates.

In conclusion, we have developed a biochemical method to study protein lipid interactions in vivo. Using this method, the role of cellular lipids in CD1d1 assembly was studied. The results revealed that cellular lipids play a chaperone-like role during the assembly of CD1d1 family of proteins. Finally, and most importantly, the development of this method sets the stage for the isolation and characterization of the elusive natural Ag(s) of CD1d-restricted NKT cells.

Acknowledgments

We thank M. H. Hunsinger, A. J. Joyce, and J. Weaver for technical help; M. E. Embers for generating sCD1d1-Kbtail and sCD1d1-er cell lines; and M. E. Embers, W. Khan, and G. Oltz laboratories at Vanderbilt University for helpful discussions. Excellent secretarial help from A. L. Karr is gratefully acknowledged.

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