Lipid Protein Interactions: The Assembly of CD1d1 with Cellular Phospholipids Occurs in the Endoplasmic Reticulum

A. Dharshan De Silva, J.-June Park, Naoto Matsuki, Aleksandar K. Stanic, Randy R. Brutkiewicz, M. Edward Medof and Sebastian Joyce

*J Immunol* 2002; 168:723-733; doi: 10.4049/jimmunol.168.2.723

http://www.jimmunol.org/content/168/2/723

<table>
<thead>
<tr>
<th>References</th>
<th>This article cites 58 articles, 34 of which you can access for free at: <a href="http://www.jimmunol.org/content/168/2/723.full#ref-list-1">http://www.jimmunol.org/content/168/2/723.full#ref-list-1</a></th>
</tr>
</thead>
<tbody>
<tr>
<td>Subscription</td>
<td>Information about subscribing to <em>The Journal of Immunology</em> is online at: <a href="http://jimmunol.org/subscription">http://jimmunol.org/subscription</a></td>
</tr>
<tr>
<td>Permissions</td>
<td>Submit copyright permission requests at: <a href="http://www.aai.org/About/Publications/II/copyright.html">http://www.aai.org/About/Publications/II/copyright.html</a></td>
</tr>
<tr>
<td>Email Alerts</td>
<td>Receive free email-alerts when new articles cite this article. Sign up at: <a href="http://jimmunol.org/alerts">http://jimmunol.org/alerts</a></td>
</tr>
</tbody>
</table>
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 Pig-A-deficient cells that are defective in the first glycosylation step of glycosylphosphatidylinositol biosynthesis. Moreover, cellular phosphatidylinositol-glycans are not Vα14Jα15 natural 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 CD1d1 in vivo, akin to the function of invariant chain in MHC class II assembly. The Journal of Immunology, 2002, 168: 723–733.

Humans express group I CD1a, CD1b, and CD1c and group II CD1d molecules (2–4). Mice and rats express only CD1d (5, 6). Topologically, CD1 resembles the classical MHC-encoded Ag-presenting molecules. Its domain organization and its association with β2-microglobulin (β2m) (7) for complete assembly are similar to MHC class I molecules. A distinguishing feature of CD1d1 is its exclusively nonpolar hydrophobic Ag-binding groove. The groove consists of a large pocket A that is almost completely covered from all sides except for a narrow lateral opening connecting it to a short apically exposed pocket F (7). Thus, CD1d molecules have evolved to present hydrophobic Ags to the mammalian immune system. Indeed, structure function studies pioneered by Brenner and colleagues (reviewed in Refs. 8 and 9–11) have revealed that human CD1b and CD1c present lipid and glycolipid Ags to specific T cells reactive to mycobacterial Ags. Ags presented by CD1b include mycolic acid, glucosylmannonolactate, phosphatidylinositol (PI)-mannans, and lipoarabinomannans (9–11). CD1b also presents self glycolipids such as GM1 to autoreactive T cells (12). CD1c, in contrast, presents mycobacterial dolichylphosphorylmannose (DPM) to specific T cells (13). Current evidence suggests that mouse and human CD1d present α-galactosylceramide (αGalCer) and potently activate mouse Vα14Jα15 and human Vα24JαQ NKT cells, respectively (14–17). Additionally, CD1d1 also presents PI and activates a small proportion of Vα14Jα15 NKT cells (18). Thus, self and nonself lipids presented by CD1 are T cell Ags.

The ability to study lipid CD1 interactions in vitro has illuminated several physico-chemical aspects of lipid presentation and recognition (reviewed in Ref. 1). Notwithstanding, all of the above studies have relied on purification of mycobacterial or cellular lipids and their ability to reconstitute functional CD1 molecules in vitro, either on live cells or using a cell-free system, recognizable.
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 Vε14Jα15 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 pEFβ-β.m (21) was digested with HindIII and BamHI, and the resulting fragment was subcloned into HindIII-BamHI-digested pE12 (CellTech, Slough, England). The resulting pEF12-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 pVL1393 vector (kindly provided by Dr. M. D. Summers, Texas A&M University, College Station, TX) using the XhoI-NotI restriction sites. The EcoRI-EcoRV fragment from pVL1393-CD1d1 containing the first four exons of CD1d1 was subcloned into pCR3 (Invitrogen, Carlsbad, CA). Finally, the EcoRV-NcoI 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-NotI 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 NotI. 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 the ER 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′-AAAGACTGAATGGAACAAAGCTTATATTTGAA-3′; b, 5′-GAGGACCTGAATTCGGAAGGATGACCTGAGAATGTCG-3′; c, 5′-GGCGGCAGCTGATTGACCTGCTTCTCTCCAG-3′; and d, 5′-TTCCTTCCAGAATGAAGCTTTCTGCTCTATTGAGCTCT-3′. This 70-bp annealing was digested with a 5′ blunt end and a 3′ NotI overhang. The oligonucleotides were phosphorylated and ligated together to form the 70-bp fragment. The resulting fragment was cloned into the EcoRV-NotI 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 H2Kb, resulting in CD1d1-Kbtail. The CD1d1-Kb-tail cDNA was constructed by subcloning an EcoRV-NotI fragment containing exons 5–8 of H2Kb into the EcoRV-NotI 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 H2Dd was constructed by PCR amplification of exons 1–4 using 5′-CAACGGTGTTCTGGTTGGTTCGGGGGCGATGGATCCGCTCCGG-3′ forward and 5′-CGGGATCCCGTCA-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 dideoxynucleotide 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 NS0 plasmacytoma as described (21). Transfected cells were selected 24 h later in nonselective medium. Qβ cell lines expressing soluble H2Kb 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 geneticin (G418; Life Technologies, Rockville, MD). Qβ and derived lines were maintained in t-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- (S9a) and Pig E- (BWS147e) mutants (22) were from American Type Culture Collection (Manassas, VA) and maintained in RPMI 1640 (Mediatech, Herndon, VA) containing 10% FBS.

All cell lines used in this study express wild-type and mutant CD1d1 as well as H2Kb and H2Dd 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 at 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 × 10^7 cells were reacted with ~0.5 µg of biotinylated 1B1, a CD1d1-reactive mAb, and biotinylated Af, a H2K-reactive mAb, and were detected with streptavidin-CyChrome using a FACScan flow cytometer (BD Biosciences, San Jose, CA). All reagents were from BD PharMingen (San Diego, CA).


At least ~2 × 10^7 CD1d1-expressing cells or control cells were tritium labeled with 250 µCi of [3H]mannose or [3H]mevalonic acid (American Radiolabeled Chemicals, St. Louis, MO) as described (19). Cells were labeled with 250 µCi of [3H]inositol (NEN, Boston, MA) in isoinositol-deficient DMEM (Life Technologies) or with 250 µCi of [3H]mevalonic acid (American Radiolabeled Chemicals) in DMEM. [32P]Orthophosphate 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]dimidylammonio]-2-hydroxy-1-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 heteroantisera (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

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>Qβ</td>
<td>NS0 plasmacytoma expressing mouse βm</td>
</tr>
<tr>
<td>nCD1d1</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 H2Kb at the carboxyl terminus</td>
</tr>
<tr>
<td>K562</td>
<td>Human erythroleukemia cell line</td>
</tr>
<tr>
<td>K562-mCD1d1</td>
<td>K562 expressing wild-type mouse CD1d1</td>
</tr>
<tr>
<td>IA</td>
<td>Pig-A-deficient derivative of K562</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 [35S]cysteine/[35S]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 [35S]cysteine/[35S]methionine yielded high amounts of labeled proteins were performed as described (21). After precipitation with normal mouse serum, samples were incubated with the anti-CD1d1 hAs or an appropriate control Ab as described. An H2Kb-specific (Y3) (26) or H2Dc-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-HLA 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 mU 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 immune affinity 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 chromatography columns (Amersham Pharmacia Biotech, Piscataway, NJ) according to the manufacturer’s instructions. Ten 0.5-ml fractions were collected; tenth of each fraction was dissolved in Econo-safe scintillation fluid (RPI, Mount Prospect, IL) to measure radioactivity using a scintillation counter (LS6500; Beckman Coulter, Fullerton, CA).

**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). After thorough mixing, the top aqueous and bottom 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 N2 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 N2 gas. One aliquot was dissolved in 50 μl of PBS (pH 7–7.4) and digested with ~55 mU 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 CaCl2 and 147 mM NaCl (pH 8.9) and digested with ~1 U of phospholipase A2 (PLA2) (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 × 20-cm Kilo silica gel TLC plate (Whatman, Clifton, NJ). Approximately 25 μg of PI was spotted and served as a standard. Additionally, ~0.025 μCi of [3H]PI or [3H]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[3H]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 (~5 × 10^5 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 H2Dd 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 [3H]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 [3H]2-mannose. [3H]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 [3H]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 [3H]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 (Rf) suggestive of a polar compound.

One possible explanation of the above analysis is that [3H]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 H2Dd expressed by [3H]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[3H]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.
dying during the labeling period could nonspecifically bind CD1d1. However, this is less likely because of the following observations. First, such lipids would be captured by BSA used in the tissue culture medium. BSA binds nonspecifically to numerous lipids and glycolipids that were tested in an in vitro binding assay (S. Joyce, unpublished data). It copurifies during affinity chromatography with Ni-Sepharose, especially when hexahistidine-tagged 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, CD1d1 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 CD1d1.

PI-PLC-sensitive ligand associates with CD1d1. 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 sCD1d1, does not contain the triitated spots seen with the CD1 sample (Fig. 1). Second, the CD1d1-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 glycosphingolipids 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 sCD1d1. Therefore, we conclude that the [3H]2-mannose-labeled compound associated with CD1d1 is not its own glycan.

To determine the chemical nature of the [3H]2-mannose-labeled lipid(s) associated with CD1d1, 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 glicerolipids; 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 sCD1d1-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 sCD1d1-associated mannosylated PI is sensitive to PLA2 or not. Thus, together with the previously published mass spectral data, we conclude that GPL is a major [3H]2-mannose-labeled lipid associated with CD1d1.

To determine the diversity of cellular lipids that incorporate [3H]2-mannose, one-tenth of the postnuclear detergent lysate of [3H]2-mannose-labeled sCD1d1 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 sCD1d1 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 CD1d1 selects a single dominant ligand from a cellular pool of several [3H]2-mannose-labeled glycolipids.

**Stoichiometry of CD1d1-GPI association.** Previously, we reported that >90% of CD1d1 molecules are occupied by GPI. Being a glycoprotein, [3H]2-mannose labels the glycan moiety of CD1d1 α-chain as well. Therefore, the ratio of the [3H]2-mannose label associated with CD1d1 to that of the extracted ligands provides a measure of what percentage of CD1d1 molecules is associated with GPI. To determine the number of glycan groups on CD1d1, 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. H2Kβ, 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 CD1d1 (Fig. 3A) and one, as expected, in the case of H2Kβ (Fig. 3B). Thus, all predicted glycosylation sites of CD1d1 are modified by N-linked glycans.

To determine the stoichiometry of CD1d1 and GPI, the amount of radioactivity in the aqueous and the organic phases of the Bligh-Dyer extract were monitored. Because CD1d1 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 phase.
were repeated using labels that are precursors of specific classes of phospholipids. The data revealed that three \[^{3}H\]inositol-labeled lipids were associated with CD1d1 (Fig. 4.A, lane 2). Two of the \[^{3}H\]inositol-labeled lipids were specifically extracted from CD1d1, but the third faster migrating lipid was also found in extracts of H2K\(^{b}\) (Fig. 4A, lanes 1 and 2). The two \[^{3}H\]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 \[^{3}H\]inositol-labeled lipids associated with CD1d1, one has the same R\(_f\) as the \[^{3}H\]PI standard, suggesting that this lipid may be PI. The \[^{3}H\]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 \[^{3}H\]inositol-labeled lipids were PI, they were subjected to PI-PLC digestion. The lipid spot that comigrates with the \[^{3}H\]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 \[^{3}H\]inositol-labeled ligand is specific because it cleaves \[^{3}H\]PI standard (Fig. 4B, lane 2) but not \[^{3}H\]DPM (data not shown). Also note that PI-PLC digestion of \[^{3}H\]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 \[^{3}H\]inositolphosphate. Together, the data suggest that the faster migrating CD1d1-associated lipid that comigrates with the \[^{3}H\]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 PLA\(_{2}\) (Fig. 4A, lanes 2 and 3). Resistance to PLA\(_{2}\) was not due to inactivity of the enzyme, because a larger quantity of \[^{3}H\]PI standard compared with

Select few cellular lipids associate with CD1d1

As noted above, the majority of CD1d1 assemble with GPI. However, because \[^{3}H\]mannose does not label all cellular lipids, it does not reveal the repertoire of ligands that are associated 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, cells lines expressing wild-type CD1d1 and control H2K\(^{b}\) were generated. These were radiolabeled with \[^{32}P\]orthophosphate (a precursor of phospholipids), \[^{3}H\]inositol (a precursor of PI and GPL), \[^{3}H\]ethanolamine (a precursor of phosphatidylethanolamine and, to a lesser extent, of phosphatidylycerine and phosphatidylcholine), or \[^{3}H\]malonic acid (a precursor of dolichol and cholesterol) (35, 36). The associated ligand(s) was isolated and characterized as described above. Because the same lipid spots of very similar intensity were extracted from CD1d1 and the control H2K\(^{b}\) (data not shown), it was difficult to ascertain the phospholipid repertoire specifically associated with CD1d1 by \[^{32}P\]orthophosphate labeling method.

**CD1d1 associates with PI.** Therefore, the above experiments were repeated using labels that are precursors of specific classes of cellular lipids. To address this question, cell lines expressing wild-type CD1d1 and control H2K\(^{b}\) were generated. These were radiolabeled with \[^{3}H\]mannose (a precursor of glycolipids), \[^{3}H\]inositol (a precursor of PI and GPI), \[^{3}H\]ethanolamine (a precursor of phosphatidylethanolamine and, to a lesser extent, of phosphatidylycerine and phosphatidylcholine), or \[^{3}H\]malonic acid (a precursor of dolichol and cholesterol) (35, 36). The associated ligand(s) was isolated and characterized as described above. Because the same lipid spots of very similar intensity were extracted from CD1d1 and the control H2K\(^{b}\) (data not shown), it was difficult to ascertain the phospholipid repertoire specifically associated with CD1d1 by \[^{3}P\]orthophosphate labeling method.

**CD1d1 associates with PI.** Therefore, the above experiments were repeated using labels that are precursors of specific classes of cellular lipids. To address this question, cell lines expressing wild-type CD1d1 and control H2K\(^{b}\) were generated. These were radiolabeled with \[^{3}H\]mannose (a precursor of glycolipids), \[^{3}H\]inositol (a precursor of PI and GPI), \[^{3}H\]ethanolamine (a precursor of phosphatidylethanolamine and, to a lesser extent, of phosphatidylycerine and phosphatidylcholine), or \[^{3}H\]malonic acid (a precursor of dolichol and cholesterol) (35, 36). The associated ligand(s) was isolated and characterized as described above. Because the same lipid spots of very similar intensity were extracted from CD1d1 and the control H2K\(^{b}\) (data not shown), it was difficult to ascertain the phospholipid repertoire specifically associated with CD1d1 by \[^{3}P\]orthophosphate labeling method.

**CD1d1 associates with PI.** Therefore, the above experiments were repeated using labels that are precursors of specific classes of cellular lipids. To address this question, cell lines expressing wild-type CD1d1 and control H2K\(^{b}\) were generated. These were radiolabeled with \[^{3}H\]mannose (a precursor of glycolipids), \[^{3}H\]inositol (a precursor of PI and GPI), \[^{3}H\]ethanolamine (a precursor of phosphatidylethanolamine and, to a lesser extent, of phosphatidylycerine and phosphatidylcholine), or \[^{3}H\]malonic acid (a precursor of dolichol and cholesterol) (35, 36). The associated ligand(s) was isolated and characterized as described above. Because the same lipid spots of very similar intensity were extracted from CD1d1 and the control H2K\(^{b}\) (data not shown), it was difficult to ascertain the phospholipid repertoire specifically associated with CD1d1 by \[^{3}P\]orthophosphate labeling method.

**CD1d1 associates with PI.** Therefore, the above experiments were repeated using labels that are precursors of specific classes of cellular lipids. To address this question, cell lines expressing wild-type CD1d1 and control H2K\(^{b}\) were generated. These were radiolabeled with \[^{3}H\]mannose (a precursor of glycolipids), \[^{3}H\]inositol (a precursor of PI and GPI), \[^{3}H\]ethanolamine (a precursor of phosphatidylethanolamine and, to a lesser extent, of phosphatidylycerine and phosphatidylcholine), or \[^{3}H\]malonic acid (a precursor of dolichol and cholesterol) (35, 36). The associated ligand(s) was isolated and characterized as described above. Because the same lipid spots of very similar intensity were extracted from CD1d1 and the control H2K\(^{b}\) (data not shown), it was difficult to ascertain the phospholipid repertoire specifically associated with CD1d1 by \[^{3}P\]orthophosphate labeling method.

**CD1d1 associates with PI.** Therefore, the above experiments were repeated using labels that are precursors of specific classes of cellular lipids. To address this question, cell lines expressing wild-type CD1d1 and control H2K\(^{b}\) were generated. These were radiolabeled with \[^{3}H\]mannose (a precursor of glycolipids), \[^{3}H\]inositol (a precursor of PI and GPI), \[^{3}H\]ethanolamine (a precursor of phosphatidylethanolamine and, to a lesser extent, of phosphatidylycerine and phosphatidylcholine), or \[^{3}H\]malonic acid (a precursor of dolichol and cholesterol) (35, 36). The associated ligand(s) was isolated and characterized as described above. Because the same lipid spots of very similar intensity were extracted from CD1d1 and the control H2K\(^{b}\) (data not shown), it was difficult to ascertain the phospholipid repertoire specifically associated with CD1d1 by \[^{3}P\]orthophosphate labeling method.

**CD1d1 associates with PI.** Therefore, the above experiments were repeated using labels that are precursors of specific classes of cellular lipids. To address this question, cell lines expressing wild-type CD1d1 and control H2K\(^{b}\) were generated. These were radiolabeled with \[^{3}H\]mannose (a precursor of glycolipids), \[^{3}H\]inositol (a precursor of PI and GPI), \[^{3}H\]ethanolamine (a precursor of phosphatidylethanolamine and, to a lesser extent, of phosphatidylycerine and phosphatidylcholine), or \[^{3}H\]malonic acid (a precursor of dolichol and cholesterol) (35, 36). The associated ligand(s) was isolated and characterized as described above. Because the same lipid spots of very similar intensity were extracted from CD1d1 and the control H2K\(^{b}\) (data not shown), it was difficult to ascertain the phospholipid repertoire specifically associated with CD1d1 by \[^{3}P\]orthophosphate labeling method.

**CD1d1 associates with PI.** Therefore, the above experiments were repeated using labels that are precursors of specific classes of cellular lipids. To address this question, cell lines expressing wild-type CD1d1 and control H2K\(^{b}\) were generated. These were radiolabeled with \[^{3}H\]mannose (a precursor of glycolipids), \[^{3}H\]inositol (a precursor of PI and GPI), \[^{3}H\]ethanolamine (a precursor of phosphatidylethanolamine and, to a lesser extent, of phosphatidylycerine and phosphatidylcholine), or \[^{3}H\]malonic acid (a precursor of dolichol and cholesterol) (35, 36). The associated ligand(s) was isolated and characterized as described above. Because the same lipid spots of very similar intensity were extracted from CD1d1 and the control H2K\(^{b}\) (data not shown), it was difficult to ascertain the phospholipid repertoire specifically associated with CD1d1 by \[^{3}P\]orthophosphate labeling method.
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 ^[3]HIinositol 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 ^[3]HIinositol-labeled lipids (Fig. 4A). The reason for this is currently unknown.

**Dolichylphosphorylglycans and phosphatidylethanolamine do not assemble with CD1d1.** Because dolicholipophosphorylglycan 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 ^[3]HImevalonic 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 repeat of this experiment (data not shown). Additional data revealed that, similar to the ^[31]Porthophosphate labeling experiments, the ^[3]Hethanolamine label was nonspecifically associated with lipids extracted from CD1d1 as well as H2Kb. Thus, dolicholphosphorylglycans 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 NKT 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 carboxyl terminus (sCD1d1-er). The second line expresses a mutant CD1d1 that had its transmembrane and cytosolic tail replaced with that from the nonrecycling H2Kb (sCD1d1-Kbtail) and, hence, has lost its endosomal/lysosome-targeting signal. The expression and intracellular trafficking patterns of the wild-type CD1d1 as well as sCD1d1-er and sCD1d1-Kbtail mutants were determined. For this purpose, cell lines expressing the wild-type and the mutant CD1d1 molecules were pulse labeled with ^[35]S|cysteine|^[35]S|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-Kbtail traffic through the secretory pathway with similar kinetics; their tᵢ/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 tᵢ/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-Kbtail had lost its ability to traffic through the endosome/lysosome compartment was determined using CD1d1-restricted NKT cell hybridomas as probes. One Vα14Jβ15 NKT cell hybridoma, DN32.D3, recognizes an endosomal/lysosomal Ag presented by CD1d1 (39) and, hence, should not be activated by sCD1d1-Kbtail. 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-Kbtail. 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-Kbtail (Fig. 6B). Thus, sCD1d1-Kbtail 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 ^[3]HIinositol 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 ^[3]HIinositol 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 ^[3]HIinositol 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/lysosome 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.
Therefore, the assembly and traffic of wild-type CD1d1 was studied in human GPI-positive K562 and the derived GPI-deficient cell line IA. IA lacks functional Pig A and, hence, they are deficient in the first glycosylated product of PI (23). Upon CD1d1 cDNA transfer and selection, both wild-type (K562-mCD1d1) and the Pig A mutant (IA-mCD1d1) cells express similar levels of CD1d1 (Fig. 7A). This expression pattern is consistent with the ability of Va14Jα15 NKT cells and derived hybridomas to recognize CD1d1 expressed by Pig A-deficient mouse cell lines (Fig. 7B and Ref. 20).

An explanation for normal expression of CD1d1 in GPI-positive and GPI-deficient cells could be that a few CD1 molecules assemble in the absence of GPI and, hence, traffic to the plasma membrane. Because CD1d1 has a long half-life (>1 day; data not shown), they can accumulate at the cell surface over time. Thus the rate of intracellular traffic of newly synthesized CD1d1 was determined by pulse-chase analysis. The data revealed that CD1d1 in both GPI-positive and GPI-negative cells egress from the ER with similar kinetics (Fig. 7C). The t1/2 of ER residence is ~2 h (Fig. 7C). Thus, CD1d1 assembles normally in GPI-deficient cell lines.

**CD1d1 assembles with PI in GPI-deficient cells**

To determine whether CD1d1 expressed in GPI-negative cells assembles with a cellular lipid, K562-mCD1d1 and IA-mCD1d1 cell lines were labeled with [3H]inositol. CD1d1 from both cell lines 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 H2Kb (Fig. 7Da and d). Interestingly, about the same amount of [3H]inositol-labeled compound was associated with CD1d1 expressed by GPI-positive K562-mCD1d1 and GPI-deficient IA-mCD1d1 cells (Fig. 7Da and d). 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 βm. 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-derivived 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 βm. Instead, the data suggest that CD1d1 use 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.
(33). 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 \( \tau \)-configuration (45), that in the CD1d1 ligand might be in the \( \pi \)-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 PLA2-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 PLA2, 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 the 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 cell lines in vivo. After assembly of CD1d 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 shows 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 PI-glycans 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\( \alpha \)14\( \alpha \)15-positive NKT cell hybridomas. This stimulatory activity of GalCer can be inhibited by titrating amounts of PI (A. K. Stanci, 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 CD1 molecules can be assembled in vitro by oxidative refolding chromatography (61, 62). Although an inefficient process, these reports suggest that CD1 can assemble in the ER of mammalian cells without cellular lipids; i.e., CD1 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 CD1. 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 CD1d because they cosegregate within detergent micelles during CD1 extraction.

How lipid loading onto CD1d in the ER is accomplished and how these lipids are exchanged for Ag in endocytic vesicles remains an important unsolved question. Because lipids are membrane embedded, they need to be “plucked out” of the membrane and loaded into the Ag-binding groove of CD1d. This event might require an elaborate molecular machinery similar to the peptide loading complex essential for peptide Ag assembly with MHC class I and class 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 CD1d assembly was studied. The results revealed that cellular lipids play a chaperone-like role during the assembly of CD1 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 scCD1d-KbtaII and scCD1d-er cell lines; and A. Bendelac and K. Hayakawa for NKT cell hybridomas. We are grateful to Drs. J. R. Bennink, J. Van Kaer, and J. W. Yewdell for continued discussions and encouragement during the course of this project as well as for the critical evaluation of the data and comments on this manuscript. We also thank Dr. C. Aiken for critical reading of this manuscript, as well as members of the M. Boothby, W. Khan, and G. Oltz laboratories at Vanderbilt University for helpful discussions. Excellent secretarial help from A. L. Kams is gratefully acknowledged.

References

1. Joyce, S. 2001. CD1d and natural T cells: how their properties jump start the study protein lipid interactions in vivo. Using this method, the role of cellular lipids in CD1d assembly was studied. The results revealed that cellular lipids play a chaperone-like role during the assembly of CD1 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 scCD1d-KbtaII and scCD1d-er cell lines; and A. Bendelac and K. Hayakawa for NKT cell hybridomas. We are grateful to Drs. J. R. Bennink, J. Van Kaer, and J. W. Yewdell for continued discussions and encouragement during the course of this project as well as for the critical evaluation of the data and comments on this manuscript. We also thank Dr. C. Aiken for critical reading of this manuscript, as well as members of the M. Boothby, W. Khan, and G. Oltz laboratories at Vanderbilt University for helpful discussions. Excellent secretarial help from A. L. Kams is gratefully acknowledged.

References


