

Biochemical Characterization of CD1d Expression in the Absence of β_2 -Microglobulin*

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CD1d is a major histocompatibility complex class I-like molecule that exhibits a distinct antigen processing pathway that functions in the presentation of hydrophobic antigens to T cells. CD1d has been previously shown to be expressed on the cell surface of human intestinal epithelial cell lines *in vivo* and a transfected cell line *in vitro* independently of β_2 -microglobulin (β_2m). To define the relationship between CD1d and β_2m and characterize the biochemical structure of CD1d in the absence of β_2m , we have used a newly generated series of CD1d transfectants and CD1d-specific antibodies. These studies show that in the absence of β_2m , CD1d is expressed on the cell surface as a 45-kDa glycoprotein that is sensitive to endoglycosidase-H and is reduced to 37-kDa after N-glycanase digestion. In contrast, in the presence of β_2m , CD1d is expressed on the cell surface as a 48-kDa endoglycosidase-H-resistant glycoprotein. Pulse-chase metabolic labeling studies demonstrate that acquisition of endoglycosidase-H resistance of CD1d is observed in the presence of β_2m but not in the absence of β_2m even after a 24-h chase period. Thus, CD1d is able to be transported to the cell surface independently of β_2m ; however, in the absence of β_2m , the glycosylation pattern of CD1d is altered and consistent with an immature glycoprotein.

First identified on cortical thymocytes, the CD1 gene family encodes a group of nonpolymorphic proteins, which have several features in common with both the major histocompatibility complex (MHC)¹ class I and class II molecules (1). CD1 is a system of proteins encoded by five genes, CD1A–E, which, although homologous to classic MHC molecules, are likely distinct in their structure and function. The human CD1 gene family falls into two groups based on sequence homology:

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¹ The abbreviations used are: MHC, major histocompatibility complex; β_2m , β_2 -microglobulin; NK, natural killer; GST, glutathione S-transferase; Ab, antibody; mAb, monoclonal antibody; CHO, Chinese hamster ovary; PAGE, polyacrylamide gel electrophoresis; Endo-H, endoglycosidase-H.

CD1A–C and CD1D. In mice, two CD1D homologues, MCD1.1 and MCD1.2, have been identified (2, 3). There is increasing recognition that the CD1 molecules may serve a unique role in antigen presentation distinct from both MHC class I and class II by presenting nonpeptide antigens to a discrete subset of T cells. CD1b and CD1c, for example, have been shown to mediate CD4[−]CD8[−] double negative T cell recognition of bacterial lipid and glycolipid antigens (4–6). Similar functions are predicted for the CD1d subtype based on the recent crystallographic structural characterization of mouse CD1d (7) and the recent identification of glycoposphatidylinositol anchors (8) and glycosylceramides (9) as antigens involved in CD1d presentation. In addition, unlike MHC class I, which presents peptide antigens in transporter in antigen processing protein-dependent manner, CD1d has been shown by several investigators to be transporter in antigen processing-independent with respect to CD1d surface expression and antigen presentation (10–12).

In rodents, a subset of CD4⁺ T cells exist that express the natural killer cell (NK) marker 1.1, called NK1.1⁺ CD4⁺ T cells, and carry an invariant T cell receptor- α chain in co-association with a limited range of T cell receptor- β chain variable regions (13). These T cells directly recognize β_2 -microglobulin (β_2m)-associated mouse CD1 in the absence of an exogenous antigen (14). CD1d mediated activation of these V α 14⁺ NK1.1⁺ T cells by glycosylceramides has also been demonstrated (9). Highly enriched in the liver, these mouse CD1d reactive NK1.1⁺ CD4⁺ T cells have a high likelihood of playing a central role in immunoregulation of cytokine responses through production of interleukin-4 and/or interferon γ and in anti-tumor immunity (14–17). Recently, double negative T cells with similar use of a highly homologous T cell receptor- α chain and recognition of CD1d has been identified in humans (18–20). These studies suggest, therefore, that CD1d in association with β_2m represents the cognate ligand for a distinct subset of T cells.

Previous studies have shown that CD1d is also expressed on intestinal epithelial cells of human (21, 22), mouse (23), and rat (24, 25). However, in this location, the major form of CD1d identifiable on the cell surface of human intestinal epithelial cells has been defined as a 37-kDa, nonglycosylated, β_2m -independent molecule (26). Although, it has been recently shown that intestinal epithelial cells also express a β_2m -associated, fully glycosylated form of CD1d (27), the fact that a β_2m -unassociated form of CD1d could be identified on the cell surface of a native cell type raises the possibility that post-translational modifications of CD1d, and the interactions between CD1d and β_2m are likely to be key factors in determining and/or modifying the function of CD1d as a ligand for T cells. For example, in addition to NK1.1⁺ T cells, which appear to

recognize β_2 m-associated CD1d, T cell clones have been identified that may be capable of recognizing CD1d on the cell surface of β_2 m-deficient antigen presenting cells (28, 29).

Given these previous observations on the biochemical characterization of CD1d in intestinal epithelium and the recognition that T cell clones can be identified that appear to be capable of recognizing β_2 m-independent forms of CD1d, we have investigated the relationship between cell surface expression of CD1d and β_2 m. To examine this interaction, we have established a transfected model system using stably transfected cell lines that express CD1d and/or β_2 m. FO-1 is a CD1d and β_2 m-negative cell line derived from human melanoma cells, which we have stably transfected with either CD1d alone or CD1d plus β_2 m. These studies clearly show that CD1d is capable of being expressed on the cell surface independently of β_2 m. In addition, we have shown that, although the absence of β_2 m does not prevent CD1d glycosylation, the glycosylation pattern is altered such that CD1d is expressed on the cell surface with an immature glycosylation pattern.

MATERIALS AND METHODS

Transfected Cells—The β_2 m-deficient human melanoma cell line, FO-1 (30) (kindly provided by Dr. Soldano Ferrone, New York Medical College, Valhalla, NY), and CD1d transfectant of this cell line have been described previously (26). FO-1 clones expressing CD1d and β_2 m were generated similarly by co-transfection of the CD1d cDNA in the pSR α -neo vector (31) with the human β_2 m gene in pEMBL9 (kindly provided by Dr. Hidde Ploegh, Harvard Medical School, Boston, MA) (32). C1R, a human HLA A- and B-deficient lymphoblastoid cell line (33) was transfected with CD1d cDNA in the pSR α -neo vector by electroporation as described previously (18). Chinese hamster ovary (CHO) cells expressing CD1d were generated by calcium phosphate transfection method as described previously (18).

Antibodies—A series of monoclonal and polyclonal antibodies were generated against CD1d-glutathione *S*-transferase (GST) fusion protein. CD1d-GST fusion proteins containing the $\alpha 1$ domain alone, the $\alpha 1$ and $\alpha 2$ domains, or the $\alpha 1$ - $\alpha 3$ domains were constructed using convenient restriction sites in the CD1d cDNA (34) or sites were introduced by polymerase chain reaction. These proteins were expressed as GST fusion proteins and purified on glutathione agarose beads (Sigma) (35). The purified proteins were eluted sequentially with glutathione and SDS, and the pooled proteins were used to immunize rabbits and mice. Rabbit antiserum was affinity purified by multiple passes through a GST column followed by adsorption to a column of agarose beads (Affi-Gel, Bio-Rad) covalently conjugated with a mixture of the three CD1d-GST fusion proteins. Bound Abs were acid eluted by a pH step gradient from pH 4.2 to 2.6, followed by alkaline elution from pH 9.5 to 11.5, and pooled as described previously (36).

Monoclonal Ab (mAb) against the pooled CD1d-GST fusion proteins was generated by fusing hyperimmune BALB/c spleen cells to murine myeloma (NS-1) cells. Wells were screened by enzyme-linked immunosorbent assay against the pooled fusion proteins, and positives were subsequently screened against GST to eliminate GST-reactive clones. One Ab from this screen, D5 (IgG2b isotype) reacts in immunoblots to an epitope in the CD1d $\alpha 1$ domain,² and it immunoprecipitates native CD1d solubilized in ionic or nonionic detergents.³

CD1d-Ig fusion protein was produced by generating a *Bam*HI site in the 3' end of the CD1d $\alpha 3$ domain by polymerase chain reaction. This *Bam*HI site was then used to fuse the CD1d leader sequence through the $\alpha 3$ domain to the Fc portion of murine IgG2b, using an Ig Fc expression vector (kindly provided by Dr. Terry Strom, Beth Israel Deaconess Medical Center, Boston) (37). The fusion protein was secreted as a β_2 m-associated disulfide-linked dimer when expressed in hamster (CHO), murine (NSO), or human (C1R) cells.² Mice were immunized with the fusion protein produced in murine NSO cells, and hybridomas were subsequently screened by enzyme-linked immunosorbent assay using the protein. The anti-CD1d mAbs raised against the CD1d-Ig fusion protein, 55.1, 42.1, and 51.1, were of the IgG1 isotype as described previously (18). BBM.1 is a mouse anti-human β_2 m mAb of the IgG1 isotype (38).

Flow Cytometry—Analyses were carried out using approximately $1 \times$

10^6 cells in 50–100 μ l of PBS containing 0.05% sodium azide and 1% fetal calf serum. The primary Abs were each used at 10–20 μ g/ml for 20–30 min at 4 °C. The secondary Abs were fluorescein isothiocyanate-conjugated anti-mouse or anti-rabbit F(ab')₂ fragments (DAKO, Carpinteria, CA). Flow cytometry analyses were performed using a Becton Dickinson immunocytometry system (Franklin Lakes, NJ).

Radiolabeling, Immunoprecipitation, Western Blotting, and SDS-Polyacrylamide Gel Electrophoresis—Using $1-2 \times 10^7$ cells, surface proteins were radiolabeled with 1–2 mCi of ¹²⁵I using the lactoperoxidase-catalyzed method, as described previously (26). After washing, labeled cells were lysed in immunoprecipitation buffer (0.15 M NaCl, 1 mM EDTA, 50 mM Tris, pH 7.8, 10 mM iodoacetamide) containing protease inhibitors (phenylmethylsulfonyl fluoride (0.17 mg/ml), leupeptin (2 μ g/ml), aprotinin (2 μ g/ml), chymostatin (2 μ g/ml), pepstatin (2 μ g/ml), antipain (2 μ g/ml), and diisopropyl fluorophosphate (1 μ l/ml)) and 1% Nonidet-P40 as detergent, as described previously (26). Lysis was performed at 4 °C for 30 min and sequentially centrifuged at 14,000 \times g and 100,000 \times g for 15 and 30 min, respectively.

Immunoprecipitations were performed using Abs coupled to protein G (IgG1 mAb) or protein A-Sepharose beads (Pierce). To minimize background from eluted IgG, the Ab in some cases was covalently coupled to the beads using dimethylpiperimidate. Lysates were cleared by 2–3 incubations with nonimmune serum bound to protein A and/or G-Sepharose beads. Lysates were then incubated with specific antibodies coupled to Sepharose beads, washed 10 times with immunoprecipitation buffer, resuspended in Laemmli buffer in the absence or presence of reducing agents and analyzed by autoradiography after resolution of proteins by SDS-PAGE under reducing or nonreducing conditions.

Nonlabeled CD1d was detected by Western blotting as described previously using the affinity purified rabbit anti-CD1d antibody, followed by an anti-rabbit horseradish peroxidase conjugate and ECL. β_2 m was detected by Western blotting of proteins resolved under non-reducing conditions with the BBM.1 mAb. *N*-linked carbohydrates were removed by digestion with *N*-glycanase (New England Biolabs, Beverly, MA), as described previously (26). Endoglycosidase-H (New England Biolabs) digestion was carried out by denaturing glycoproteins in 0.5% SDS, 1% β -mercaptoethanol, boiling at 100 °C for 5 min, and incubating with 1 μ l of endoglycosidase-H overnight at 37 °C in reaction buffer as defined by the manufacturer containing 50 mM sodium citrate, pH 5.5.

Pulse-Chase Metabolic Labeling—Semi-confluent $1-2 \times 10^7$ FO-1 CD1d and FO-1 CD1d/ β_2 m-transfected cells grown in 60- \times 15-mm tissue culture dish (Becton Dickinson; Falcon 3002) were used for metabolic labelings. After washing cells three times with Hanks' balanced salt solution containing divalent cations, Dulbecco's modified Eagle's medium without cysteine, and methionine supplemented with 0.15% dialyzed fetal calf serum and penicillin/streptomycin was added to the cells and incubated at 37 °C for 30 min. Tran³⁵S-labeled cysteine/methionine (ICN, Costa Mesa, CA) 0.75–1 mCi was added for 10 min at 37 °C to label the cells. After discarding the radioactive medium, cold RPMI supplemented with 10% dialyzed fetal calf serum was added, and cells were lysed at 0, 2, 4, and 24 h of the chase period with 1% Nonidet P-40 lysis buffer containing protease inhibitors. Immunoprecipitations of the lysates with the D5 and the 51.1.3 mAb were performed followed by the *N*-glycanase and endoglycosidase-H digestion protocols as mentioned above.

RESULTS

CD1d Is Expressed on the Cell Surface in the Absence of β_2 m—Cell surface expression of CD1d by transfected, β_2 m-deficient FO-1 human melanoma cells has been shown previously using flow cytometry with a cross-reactive rat anti-mouse CD1d mAb (26). Expression of human CD1d by these cells was confirmed by flow cytometry using a polyclonal affinity purified rabbit anti-human CD1d antibody and two murine anti-human CD1d mAbs, 42.1 and 51.1 (Fig. 1; wild type *versus* CD1d). As expected, there was no binding by the anti- β_2 m mAb, BBM.1, and wild type FO-1 cells did not react with any of the CD1d-specific antibodies. FO-1 cells co-transfected with CD1d and human β_2 m were also examined (Fig. 1; CD1d/ β_2 m). These cells stained with each of the anti-CD1d antibodies and with the BBM.1 mAb. It should be noted that staining of each transfected line with the murine mAbs suggested two populations of cells, although this was not apparent with the rabbit antibody.

Structure of CD1d Expressed by FO-1 Cells in the Absence of

² S. P. Balk, unpublished observation.

³ S. P. Balk and R. S. Blumberg, unpublished observation.

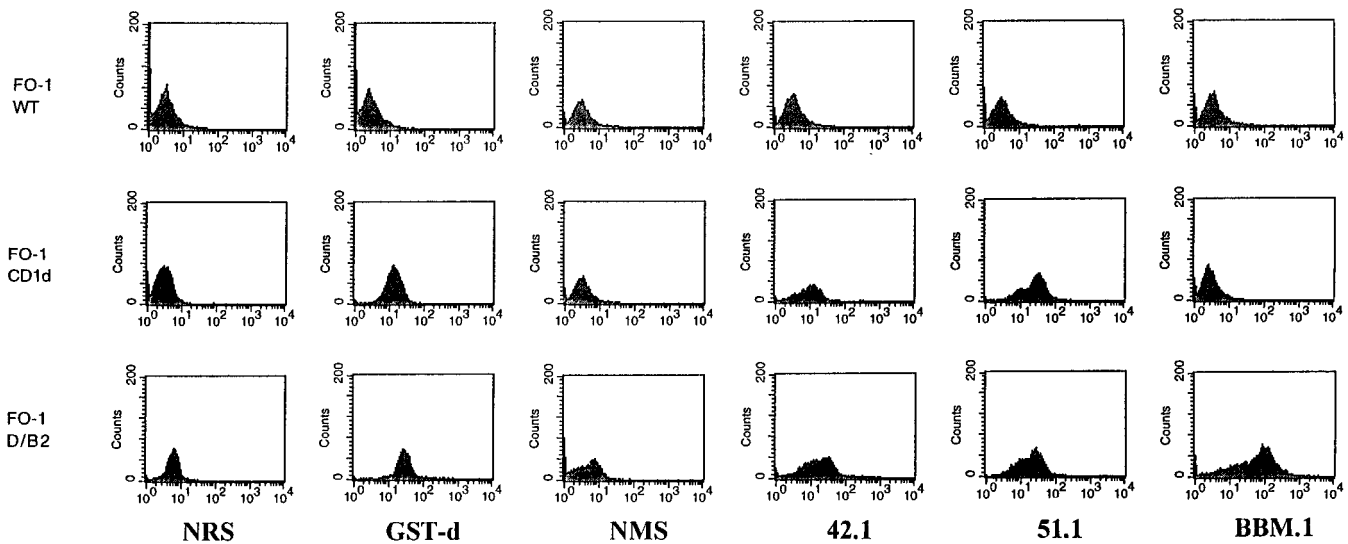


FIG. 1. Analysis of FO-1 transfectants by flow cytometry. FO-1 wild type (WT), CD1d-transfected FO-1, and CD1d plus β_2 m-transfected (D/B2) FO-1 cells were examined by flow cytometry using the indicated primary antibodies. NRS, normal rabbit serum; GST-d, affinity purified rabbit anti-CD1d; NMS, normal mouse serum; 42.1 and 51.1, mouse anti-CD1d mAbs; BBM.1, mouse anti- β_2 m mAb.

β_2 m—A previous report showed that CD1d expressed on the surface of intestinal epithelial cells lacked β_2 m and N-linked carbohydrates (26). To assess the structure of CD1d expressed by FO-1 cells in the absence of β_2 m, a series of immunoprecipitations were carried out. FO-1 wild type and CD1d-transfected cells were surface iodinated and immunoprecipitated with the affinity purified rabbit anti-CD1d antibody (Fig. 2A) or the mouse anti-human CD1d mAb, D5 (Fig. 2B). Both antibodies immunoprecipitated heavy chains of approximately 45 kDa from the CD1d-transfected cells but not the wild type cells (Fig. 2, A and B, lanes -). There were no bands detected at 37 kDa, indicating that most or all of the CD1d expressed by these cells was glycosylated. To confirm that the CD1d transfected FO-1 cells were expressing glycosylated CD1d, the CD1d immunoprecipitates were treated with N-glycanase to remove N-linked carbohydrates. Subsequent analysis of these proteins showed that they were reduced to about 37 kDa, consistent with the CD1d polypeptide backbone (Fig. 2, A and B, lanes +). These results demonstrated that CD1d expressed by FO-1 cells in the absence of β_2 m contained N-linked carbohydrate, in contrast to the CD1d protein expressed by intestinal epithelial cells (26).

The double transfected, CD1d plus β_2 m FO-1 cells were examined similarly by immunoprecipitation. The rabbit polyclonal antibody (Fig. 3A) immunoprecipitated from these cells a heavy chain of about 48 kDa (lane -, thick arrow), which was associated with a 12-kDa protein (lane -, open arrow), consistent with β_2 m. The D5 mAb (Fig. 3B) immunoprecipitated the 48-kDa CD1d heavy chain (lane -, thick arrow). Note the absence of the 12-kDa band in the D5 immunoprecipitate of the FO-1d/ β_2 m transfectant (Fig. 3B, lane -, open arrow). The lack of β_2 m in the D5 mAb immunoprecipitates was similarly observed in cells that expressed endogenous β_2 m and appeared to reflect disruption of β_2 m binding by this mAb (see below).

The size of the CD1d heavy chain expressed by the FO-1d/ β_2 m transfectant appeared to be about 3-kDa larger than the FO-1d transfectant expressing only CD1d. This difference could be clearly appreciated when either rabbit polyclonal anti-CD1d Ab (Fig. 3A) or D5 mAb (Fig. 3B) immunoprecipitates were prepared from both cell types and analyzed on the same gel (Fig. 3, A and B, respectively, lanes -, thin arrow). In contrast, the CD1d bands immunoprecipitated from both cell types of transfectants migrated similarly at 37 kDa after N-glycanase digestion (Fig. 3, A and B, lanes +, arrowhead),

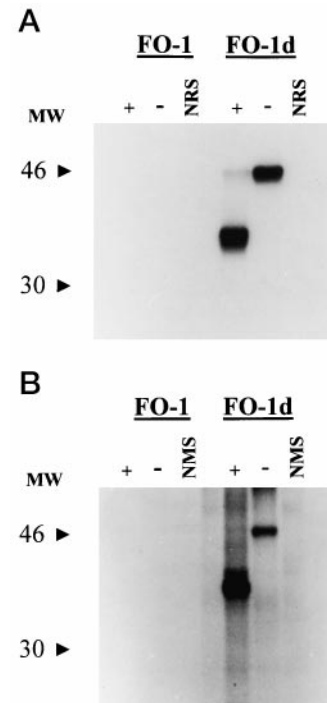


FIG. 2. Immunoprecipitation of cell surface CD1d from CD1d-transfected FO-1 cells. Wild type (FO-1) or transfected (FO-1d) cells were radiolabeled with 125 I and lysates were immunoprecipitated with either GST-d, a rabbit anti-CD1d mAb, and NRS (normal rabbit serum) as a control (A) or D5, an anti-CD1d mAb, and NMS (normal mouse serum) as a control (B). The immunoprecipitates were analyzed by SDS-PAGE on 12% gels under reducing conditions. (+) and (-) indicate either treatment or lack of treatment with N-glycanase. The molecular weight markers in kilodaltons are shown on the left.

indicating that differences in N-linked carbohydrate accounted for the smaller apparent size of CD1d expressed in the absence of β_2 m.

CD1d Structure in Cells Expressing Endogenous β_2 m—Additional CD1d-transfected cell lines that expressed endogenous β_2 m were analyzed similarly by iodination and immunoprecipitation. Both C1R cells, a human B cell line, and CHO cells yielded CD1d heavy chains of approximately 48 kDa that migrated identically to the CD1d heavy chain identified from the

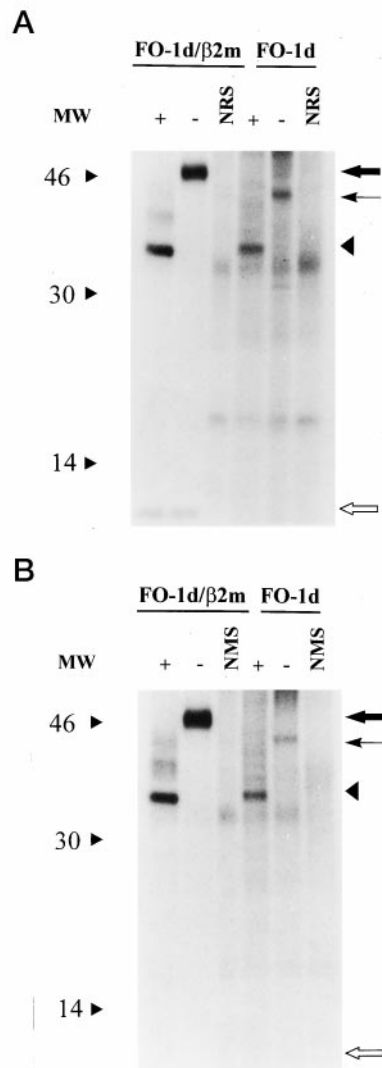


FIG. 3. Altered glycosylation pattern of CD1d on cell surface of CD1d-transfected FO-1 cells. FO-1d cells and FO-1 cells transfected with CD1d plus β_2m (FO-1d/ β_2m) were radiolabeled with ^{125}I , and lysates were immunoprecipitated with either GST-d (rabbit anti-CD1d Ab) and NRS (normal rabbit serum) as a control (A) or D5, an anti-CD1d mAb, and NMS (normal mouse serum) as a control (B). (+) and (-) indicate either treatment or lack of treatment with *N*-glycanase. Molecular weight markers in kilodaltons are shown on the left. *Thick arrow*, 48-kDa glycosylated CD1d; *thin arrow*, 45-kDa glycosylated CD1d; *arrowhead*, 37-kDa deglycosylated CD1d; *open arrow*, 12-kDa β_2m .

FO-1 CD1d/ β_2m double transfectant (Fig. 4). The rabbit polyclonal antibody immunoprecipitates from these cells again demonstrated an associated 12-kDa protein consistent with β_2m . This 12-kDa protein was not detected in the D5 precipitates, again indicating that D5 may disrupt CD1d- β_2m binding.

Immunoblotting with an anti- β_2m mAb, BBM.1, was used to confirm the identity of the 12-kDa protein as β_2m . CD1d-transfected C1R cells were immunoprecipitated with a series of CD1d mAbs and immunoblotted with the β_2m -specific mAb, BBM.1. Immunoblotting confirmed a CD1d association with β_2m when lysates were precipitated with the 42.1, 51.1, or 55.3 anti-CD1d mAbs (Fig. 5, top), but not with the D5 mAb. The precipitation of CD1d by each of the anti-CD1d mAbs was confirmed by subsequent immunoblotting with the rabbit anti-CD1d antibody (Fig. 5, bottom).

Interestingly, the BBM.1 mAb in these experiments did not

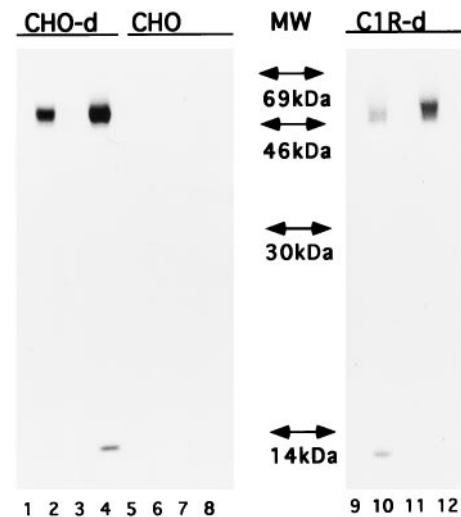


FIG. 4. Immunoprecipitation of cell surface CD1d from C1R and CHO cells transfected with CD1d. Immunoprecipitates from lysates prepared from surface iodinated CD1d-transfected CHO cells (CHO-d), wild-type CHO cells (CHO), and CD1d transfected C1R cells (C1R-d) are shown. Lanes 1, 5, and 11, normal mouse serum; lanes 2, 6, and 12, D5 anti-CD1d mAb; lanes 3, 7, and 9, normal rabbit serum; lanes 4, 8, and 10, rabbit anti-CD1d Ab. Samples were analyzed by SDS-PAGE on a 13% gel under reducing conditions. Molecular weight markers in kilodaltons are shown in the middle of the figure.

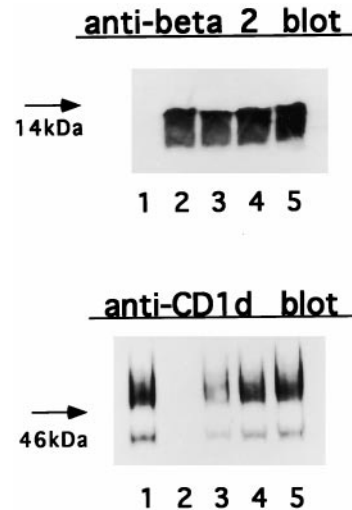


FIG. 5. Western blotting of CD1d-transfected C1R cells. Unlabeled lysates of CD1d transfected C1R cells were immunoprecipitated with various Abs and resolved by SDS-PAGE in 15% gels under reducing conditions and Western blotted with either mouse anti-human β_2m mAb, BBM.1 (*anti-beta 2 blot*), or rabbit anti-CD1d antibody (*anti-CD1d blot*). Lane 1, D5 anti-CD1d mAb; lane 2, BBM.1 anti- β_2m mAb; lane 3, 55.1 anti-CD1d mAb; lane 4, 51.1 anti-CD1d mAb; lane 5, 42.1 anti-CD1d mAb. The location of the 14- and 46-kDa markers are shown on the left.

co-precipitate CD1d (Fig. 5, bottom), a result which indicates that β_2m interacts differently with CD1d versus HLA class I proteins, which are co-precipitated by this mAb (data not shown). However, we have observed BBM.1 co-precipitation of CD1d when cells were iodinated (data not shown), indicating that the association may be stabilized by oxidation. This is consistent with a previous report showing BBM.1 precipitation of CD1d from iodinated tumor cells (39).

CD1d Glycosylation Pattern in the Absence of β_2m —The previous studies described above showed that CD1d is glycosylated differently in the absence of β_2m . One possible explanation is that in the absence of β_2m , the CD1d on the cell surface represented an immature form of CD1d that was not processed

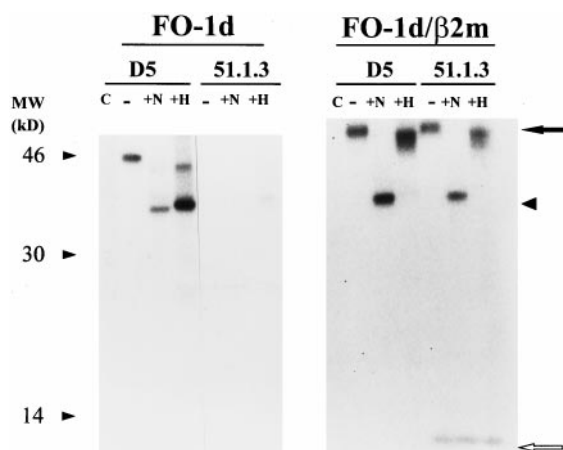


FIG. 6. Characterization of endoglycosidase-H sensitivity of cell surface CD1d in either the presence or absence of β_2 m. FO-1 cells transfected either CD1d alone (FO-1d) or CD1d plus β_2 m (FO-1d/ β_2 m) were radiolabeled with 125 I, and the lysates were immunoprecipitated with the D5 and 51.1.3 mAbs with normal mouse serum serving as a control (lanes marked C). The immunoprecipitates were subjected to digestion with either *N*-glycanase (lanes marked +N) or endoglycosidase-H (lanes marked +H) and resolved by SDS-PAGE on 13% gels under reducing conditions. The untreated (lanes marked -) immunoprecipitates were resolved similarly. Autoradiography of the immunoprecipitates is shown. The open arrow indicates 12-kDa β_2 m. The molecular weight (MW) markers in kilodaltons are indicated on the left.

to maturity after initial modification by *N*-glycosyltransferase. This was raised as a possibility because preliminary partial *N*-glycanase experiments suggested that all four potential sites for *N*-linked carbohydrate side chains were modified, yet the protein migrated faster in SDS-PAGE (data not shown). To test for this possibility, the sensitivity of CD1d to endoglycosidase-H (Endo-H) on the cell surface of the FO-1d single transfectant in the absence of β_2 m was evaluated. Radiolabeled cell surface proteins of the FO-1d transfectant were immunoprecipitated with either the D5 or 51.1 anti-CD1d mAbs, and the immunoprecipitates subjected to either digestion with *N*-glycanase (+N) to remove all *N*-linked modifications or endoglycosidase-H (+H) to remove immature *N*-linked carbohydrate side-chain modifications that had not been further modified beyond the initial glycosyltransferase transfer. As can be seen in Fig. 6, the CD1d protein on the cell surface of the FO-1d single transfectant migrated as a 45-kDa endoglycosidase-H-sensitive protein (arrowhead), whereas the CD1d protein on the cell surface of the FO-1d/ β_2 m double transfectant migrated as a 48-kDa endoglycosidase-H-resistant protein (arrow). This indicates that, in the absence of β_2 m, transport of an immature CD1d glycoprotein had occurred to the cell surface.

Examination of CD1d Biosynthetic Pathway by Pulse-Chase Metabolic Labeling—To confirm these observations on cell surface expression of CD1d in the absence and presence of β_2 m, metabolic labeling studies were performed. Semi-confluent FO-1 CD1d and FO-1 CD1d/ β_2 m cells were labeled with 35 S for 10 min and subsequently chased for 0, 2, 4, and 24 h. The pulse-chase studies of the FO-1 cells transfected with CD1d alone are shown in Fig. 7 after immunoprecipitation with the D5 and 51.1.3 mAbs. Throughout the 24-h chase, CD1d in the β_2 m negative FO-1 cells remained completely sensitive to Endo-H, consistent with the predicted molecular weight of the polypeptide backbone, resolving as 37 kDa after this type of digestion (open arrow; lanes +H). At 0 h, the glycosylated 45-kDa CD1d (open arrowhead; lanes -N) was observed to be sensitive to Endo-H (lanes +H) and *N*-glycanase (lanes +N). At 2, 4, and 24 h, the 45-kDa form of CD1d (open arrowhead) chased into a 48-kDa form of CD1d (closed arrowhead). Al-

though the nature of the carbohydrate side-chain modification of this 48 kDa form is presently unknown, these post-translational changes exhibited a complete sensitivity to Endo-H. Interestingly, because only the 45-kDa glycoprotein was identifiable on the cell surface (see Fig. 6) it is likely that this 48-kDa glycoprotein was retained intracellularly. Of additional note, the D5 mAb, which was raised against a GST-CD1d fusion protein, but not the 51.1.3 mAb, which was raised against a β_2 m-associated Fc-fusion protein of CD1d, readily detected the β_2 m-independent form of CD1d. Although the 51.1.3 mAb was able to immunoprecipitate the 48-kDa form of CD1d in the absence of β_2 m, it did so very late in the chase and with significantly less avidity than the D5 mAb.

The examination of CD1d biosynthesis in the FO-1 CD1d/ β_2 m double transfectants is shown in Fig. 8. In this cell line, at 0 h of the chase, a 45-kDa band (open arrowhead) was identifiable that was completely sensitive to Endo-H digestion (open arrow) but not recognized by the 51.1.3 mAb. In contrast to the single transfectant (see Fig. 7), this 45-kDa band, rapidly chased into a 48-kDa band (closed arrowhead) that was recognized by the 51.1.3 mAb and was resistant to digestion with Endo-H. A slight reduction of the molecular weight of the 51.1.3 immunoprecipitable material was observed, which we speculate is related to either contamination of the endoglycosidase-H enzyme with neuraminidase or incomplete maturation of one of the four carbohydrate side-chain modifications on CD1d. This observation needs clarification in future studies. By 4 h, the majority of CD1d had reached maturity based on Endo-H sensitivity, which was complete at 24 h in contrast to the single transfectant. The 51.1.3 mAb also notably preserved the interaction of CD1d with β_2 m (closed arrow), which, taken together with the other observations described above, suggest that the 51.1.3 mAb recognizes a conformational epitope associated with CD1d maturity. These metabolic-labeling studies of CD1d in the FO-1 CD1d and FO-1 CD1d/ β_2 m transfectants thus confirm the cell surface radiolabeling studies.

DISCUSSION

Several studies have demonstrated expression of human CD1d and its murine homologue in association with β_2 m (22) and some have suggested that β_2 m may be required for murine CD1d expression (40). In contrast, we reported previously that endogenous CD1d could be detected on the cell surface of human intestinal epithelial cells in the absence of β_2 m and that transfected human and mouse CD1d could be expressed at the cell surface in a β_2 m-deficient cell line as defined by cell surface staining with a cross-reactive rat anti-mouse CD1d mAb (26). However, this initial report could not be corroborated by a second group working with mouse CD1d (40), perhaps because of the use of a transient transfection system. Using the original human CD1d transfectant of FO-1 plus a variety of other newly generated transfectants and newly generated CD1d-specific antibodies, we now provide definitive data that prove the ability of CD1d to be displayed on the cell surface in the absence of β_2 m. Cell surface expression of transfected human CD1d by the β_2 m deficient FO-1 human melanoma cell line was confirmed by flow cytometry with several CD1d-specific antibodies. Moreover, confirmation of this staining data was provided by immunoprecipitation and autoradiography of cell surface labeled proteins. The immunoprecipitations also showed that CD1d expressed by β_2 m-deficient FO-1 cells contained *N*-linked carbohydrate. This is in contrast to the non- β_2 m-associated form of CD1d expressed by intestinal epithelial cells, which does not carry *N*-linked carbohydrate (26). These results confirm that CD1d expression is not strictly β_2 m-dependent and is distinct from human CD1a, -b, and -c (41). In addition, these studies also indicate that the absence of β_2 m binding is probably not

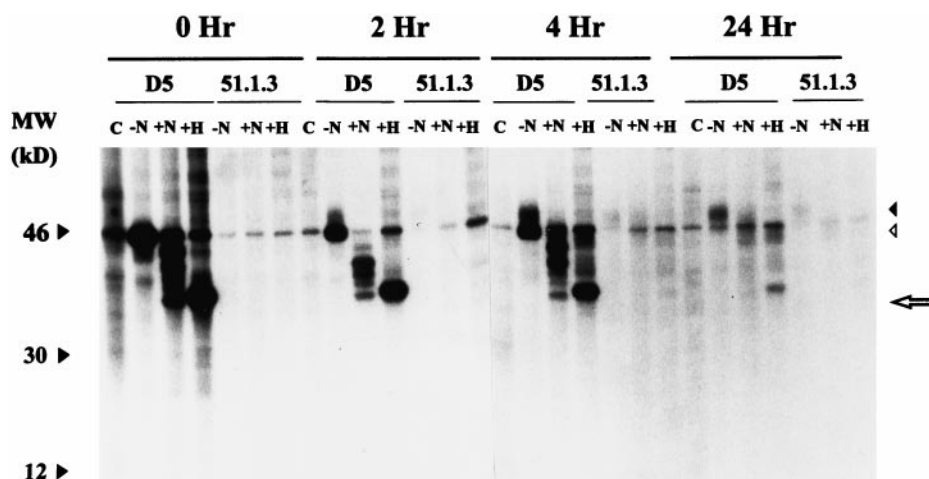


FIG. 7. **Pulse-chase metabolic labeling of CD1d in the FO-1 CD1d cell line.** FO-1 CD1d cells were radiolabeled with 1 mCi of Tran^{35}S -labeled cysteine and methionine for 10 min in Dulbecco's modified Eagle's medium in the absence of cysteine and methionine and chased for 0, 2, 4, and 24 h. Cells were lysed in 1% Nonidet P-40, and immunoprecipitations were performed using the D5 and 51.1.3 mAbs with normal mouse serum serving as a negative control (lanes marked C). The immunoprecipitates untreated with glycanases (-N), or treated with either N-glycanase (lanes marked +N) or endoglycosidase-H (lanes marked +H) digestions, and the proteins were resolved by SDS-PAGE on 13% gels under reducing conditions. Closed arrowhead, 48-kDa form of CD1d; open arrowhead, 45-kDa form of CD1d; and open arrow, deglycosylated CD1d. The molecular weight (MW) markers in kilodaltons are shown on the left.

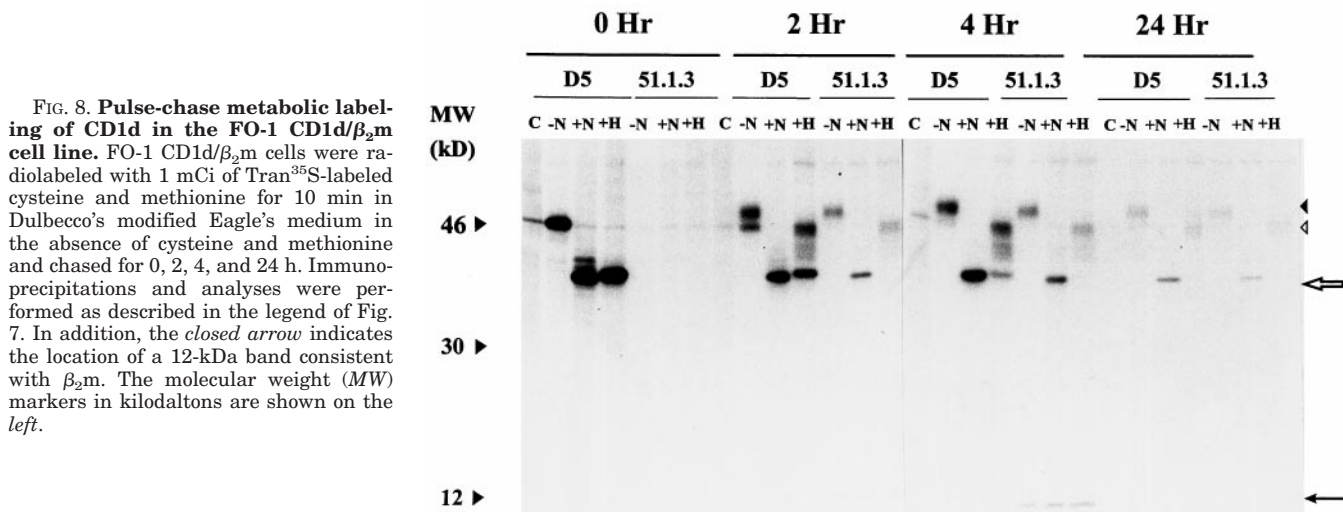


FIG. 8. **Pulse-chase metabolic labeling of CD1d in the FO-1 CD1d/ β_2 m cell line.** FO-1 CD1d/ β_2 m cells were radiolabeled with 1 mCi of Tran^{35}S -labeled cysteine and methionine for 10 min in Dulbecco's modified Eagle's medium in the absence of cysteine and methionine and chased for 0, 2, 4, and 24 h. Immunoprecipitations and analyses were performed as described in the legend of Fig. 7. In addition, the closed arrow indicates the location of a 12-kDa band consistent with β_2 m. The molecular weight (MW) markers in kilodaltons are shown on the left.

the primary event directing the expression of CD1d without N-linked carbohydrate in intestinal epithelial cells as previously reported (26, 27).

Although CD1d expressed by the β_2 m-deficient FO-1 cells was glycosylated, it was not identical to CD1d expressed by FO-1 cells transfected with both CD1d and β_2 m. CD1d expressed by this double transfectant was β_2 m associated and migrated at about 48 kDa, similar to CD1d in other cells, which express β_2 m endogenously such as C1R and CHO. In contrast, cell surface CD1d expressed by the β_2 m deficient FO-1 cells migrated at about 45 kDa. This difference in mobility was due to N-linked carbohydrate as CD1d from both the β_2 m-deficient and β_2 m-transfected FO-1 cells migrated to 37 kDa after N-glycanase digestion. However, interestingly, the N-linked carbohydrate associated with CD1d on the cell surface of the β_2 m-negative transfectant exhibited complete sensitivity to Endo-H. This indicates that all of the CD1d expressed on the cell surface in the absence of β_2 m was immature and thus not subjected to further processing beyond that which occurred in the endoplasmic reticulum. Whether this altered form of CD1d is able to present processed antigen or form multimers with similarly processed CD1d or other molecules is not known. However, preliminary data suggest that the abnormally glyco-

sylated CD1d expressed by the β_2 m-deficient FO-1 cells may be biologically relevant. Co-cultivation of the FO-1d cell line, but not the untransfected FO-1 cell line, leads to the phosphorylation of the T cell receptor-associated kinase p59^{lck}.⁴ In addition, a recent study of CD1d expression on β_2 m^{-/-} mice splenic B cells indicate that CD1 is expressed at low levels independently of β_2 m and this form of CD1 is capable of inducing T cell proliferation, which can be blocked by a rat anti-mouse CD1d mAb, 3C11 (42). These observations by others provide strong support to our previous report of β_2 m independence of CD1d expression on human intestinal epithelial cell surface (26).

The importance of β_2 m association with CD1d with respect to the CD1d biosynthetic pathway has not been adequately explored. As demonstrated by surface labeling and pulse-chase metabolic labeling of CD1d, the association of β_2 m with CD1d appears to have an important role in regulating the extent of CD1d glycosylation and the maturity of the attached carbohydrate side chains. It is possible that in the presence of β_2 m, CD1d may be associating with chaperoning protein(s) in the endoplasmic reticulum that recognize both CD1d and β_2 m as a

⁴ N. A. Campbell, H. S. Kim, K. N. Evans, R. S. Blumberg, L. Mayer, submitted for publication.

heterodimer and which function to delay the biosynthetic process until CD1d becomes fully glycosylated and reaches its maturity. Given the differences in the glycosylation pattern and Endo-H sensitivity in the presence and the absence of β_2m in FO-1 CD1d cells, it is likely that CD1d synthesized in these two cell lines may have different chaperoning proteins involved in its assembly/biosynthesis.

In summary, using cell lines that expressed either endogenous (C1R and CHO) or exogenous (FO-1) β_2m , human CD1d was displayed on the cell surface as a β_2m -associated heavy chain that was modified with *N*-linked carbohydrates that were processed to maturity. In contrast, in the absence of β_2m , although CD1d transport to the cell surface was not significantly disrupted, CD1d associated *N*-linked carbohydrate modifications remained immature. These data provide another example of how CD1d biosynthesis is distinct from classical MHC class I (43) and suggest that β_2m may regulate the expression of structurally distinct forms of CD1d that may deliver unique signals to CD1d reactive lymphocytes as may occur in intestinal neoplasia wherein β_2m expression is often disrupted (44).

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