

# Immunolocalization of CD1d in human intestinal epithelial cells and identification of a $\beta_2$ -microglobulin-associated form

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## Abstract

In order to better understand the role of intestinal CD1d, we sought to define the cellular localization and further characterize the biochemical structure of CD1d in human intestinal epithelial cells (IEC). Using a CD1d-specific rabbit anti-gst-CD1d antibody, immunoprecipitation of radiolabeled cell surface proteins detected a previously identified 37 kDa protein as well as a 48–50 kDa protein which were confirmed by Western blotting with a CD1d-specific mAb, D5. Immunoprecipitation of protein lysates with the CD1d-specific mAb, D5 and 51.1.3, and the  $\beta_2$ -microglobulin ( $\beta_2m$ )-specific mAb, BBM.1, followed by *N*-glycanase digestion and Western blotting with the D5 mAb showed that the 48–50 kDa protein was a  $\beta_2m$ -associated, CD1d glycoprotein. CD1d was immunolocalized to the apical and lateral regions of native small and large intestinal IEC as defined by confocal laser microscopy using the D5 mAb and the rabbit anti-gst-CD1d antibody. In addition, a large apical intracellular pool of CD1d was identified. Identical observations were made with polarized T84 cells. Selective biotin labeling of apical and basolateral cell surfaces followed by immunoprecipitation with the D5 mAb, *N*-glycanase digestion and avidin blotting confirmed the presence of glycosylated CD1d on both cell surfaces and immunolocalization of the 37 kDa non-glycosylated form of CD1d to the apical cell surface. These studies show that CD1d is located in an ideal position for luminal antigen sampling and presentation to subjacent intraepithelial lymphocytes.

## Introduction

The human CD1 gene family is composed of five members, four of which are known to be expressed *in vivo*: CD1A–D (1). These molecules are most closely related to MHC class I on the basis of exon–intron organization and the ability to associate with  $\beta_2$ -microglobulin ( $\beta_2m$ ). The homology to MHC class I, however, is quite low and suggests a marked divergence in function. In addition, the CD1 family members map outside the HLA locus on chromosome 6, and exhibit limited polymorphism in the  $\alpha 1$  and  $\alpha 2$  domains, suggesting that CD1 molecules may function as unique antigen-presenting molecules for distinct classes of T cells and/or antigens (2). Recent data have confirmed such a hypothesis, in which

CD1b has been reported to present non-peptide lipoglycan mycobacterial antigens to human CD4<sup>+</sup>CD8<sup>-</sup> T cells (3–5) and CD1d may present relatively hydrophobic molecules based on studies with the mouse homologue of this molecule (6–8). Indeed, the recent X-ray crystallographic analysis of mouse CD1d has revealed the presence of a deep hydrophobic pocket which is predicted to bind highly hydrophobic lipids such as those derived from mycobacteria (9). Since structurally similar forms of these lipid moieties likely exist for other bacteria as well as mammalian cells, it is intriguing to speculate on a more general role for CD1d in regulating lymphocyte responses to microbial and host antigens (8).

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One potential site for such a lymphoregulatory function is the epithelial surfaces since CD1d expression has been observed in this location in a variety of human tissues including the intestine (10). CD1d has been previously identified on the intestinal epithelial cells (IEC) in rat (11,12), humans (10,13) and mouse (14) although recent observations by Brosssay *et al.* (15) have drawn into question significant CD1d expression by intestinal epithelia in this species. In addition, the function of CD1d on IEC is poorly understood. On epithelia, CD1d is potentially placed in a critical location for interaction with intestinal intraepithelial lymphocytes (iIEL), CD8<sup>+</sup> T cells which localize to the lateral surface of intestinal epithelia (16). iIEL are, at least in part, cytolytic effector cells which express a limited number of TCR  $\alpha\beta$  and  $\gamma\delta$  chains predicting that they recognize a limited range of antigens in the context of an MHC class I-related ligand on the intestinal epithelium (17–20). The relatively non-polymorphic character, anatomic context and the class I-like structure make intestinal CD1d an excellent candidate ligand for TCR–CD3 complex-mediated activation of at least a subset of iIEL.

Direct evidence in support of a functional role of CD1d in this location is, however, limited. Blockade of IEC surface CD1d with anti-CD1d mAb has been shown to abrogate the proliferation of CD8-bearing peripheral blood lymphocytes to fresh IEC (21). Interestingly, the structural isoform of CD1d that was identified on the cell surface of human IEC was previously reported to be a non-glycosylated form of CD1d which was expressed on the cell surface without an obvious  $\beta_2m$  association (22). These observations were generated by immunoprecipitation with several different mAb and a comparison of the peptide map of this 37 kDa molecule with bacterially synthesized CD1d. However, these earlier biochemical studies were primarily performed with two rat anti-mouse CD1d mAb raised in rat against mouse CD1d which cross-react with human CD1d (13,14). In addition, recent studies in mouse models has shown that the major selecting ligand for CD8  $\alpha\beta^+$  TCR  $\alpha\beta^+$  iIEL is a transporter associated with antigen presentation (TAP)-independent,  $\beta_2m$ -dependent ligand (23–24). Although, CD1d had been shown to exhibit these features, these observations on iIEL selection in mouse models cannot be reconciled with the previous biochemical studies on human IEC which described an apparent absence of  $\beta_2m$  association with CD1d (22).

Unlike many cell types which express CD1d, the intact epithelial plasma membrane bears strictly polarized apical and basolateral domains (25). It is not presently known, however, whether CD1d expression on IEC is polarized. If it is to be hypothesized that CD1d fulfills an important function of presentation of luminal antigen by the intestinal epithelium (26) it would be important to demonstrate that CD1d is oriented in a fashion potentially appropriate for such a role. This study, therefore, had two parallel goals. Using newly generated CD1d-specific antibodies we sought to examine the cellular localization of CD1d on native intestinal tissues and an IEC line (T84 cells), and to re-examine the biochemical structure of CD1d in association with human epithelia. We report that intestinal epithelial CD1d localizes to the apical and lateral membrane domains of polarized epithelia, and data are provided for a substantial subapical intracellular pool of CD1d. Such localized expression of CD1d lends

credence to the hypothesis that CD1d is ideally positioned for an involvement in presentation of luminal antigens to the laterally positioned iIEL. In addition, we have confirmed previous studies on the existence of a 37 kDa isoform of CD1d in IEC and provide new evidence for the presence of a fully glycosylated,  $\beta_2m$ -associated isoform which has important implications for understanding CD1d biosynthesis and function in this cellular location.

## Methods

### *Tissue specimens*

Post-surgical specimens of normal human small bowel and colon were frozen in OCT compound (Tissue-Tek, Miles, IL), sectioned to 3  $\mu$ m thick sections and mounted on to gelatin-coated slides. For gelatin coating, slides were cleaned in 95% ethanol, rinsed in distilled water, dipped into 0.5% gelatin (Fisher Scientific, Pittsburgh, PA) and air dried. Slides then were dipped into 10% formalin for 2 min, rinsed in distilled water and air dried. Gelatin coating of slides obviated the need for fixing tissue sections by paraformaldehyde, which in pilot experiments revealed interference with antibody staining. Intestinal sections were then mounted on gelatin-coated slides that were pretreated with formalin and processed for antibody staining as described below. Tissue specimens were approved by the human studies committee of the Brigham & Women's Hospital.

### *Antibody staining for confocal laser microscopy (CLM) of specimens*

Tissue sections on gelatin-coated slides were washed with cold HBSS containing Ca<sup>2+</sup> and Mg<sup>2+</sup> (Sigma, St Louis, MO), permeabilized with 0.5% Triton for 20 min and incubated with 5% normal goat serum in HBSS for 30 min at 4°C to block the non-specific binding. Subsequently the tissue sections were incubated for 1 h with the primary antibody used at a concentration of 1:100 of ascitic fluid antibody prepared in 5% normal goat serum. After washing 3 times with HBSS the sections were incubated with FITC-conjugated secondary antibody (Cappel, Durham, NC) for 1 h at a concentration of 1  $\mu$ g/ml. The sections were mounted in PBS-glycerol-*p*-phenylenediamine and viewed with a BioRad MRC confocal fluorescence microscope (BioRad, Richmond, CA.). VCAM-1 (clone 6G10 obtained from the Developmental Studies Hybridoma Bank, University of Iowa) or normal preimmune rabbit serum were used as controls.

### *Isolation of native IEC*

Resected tissue specimens were collected and washed with prewarmed RPMI (Gibco/BRL, Gaithersburg, MD). The surface mucosa was stripped from the underlying submucosa by dissection and cut into small square pieces about 1 cm<sup>2</sup> in size. These were then incubated at 37°C on an orbital shaker with HBSS containing 150 mM DTT (Fisher Scientific) followed by HBSS containing EDTA (Fisher Scientific) and FCS (Sigma) for 20 min. IEC were then isolated by scraping gently across a steel mesh with the mucosal surface of the tissue pieces facing down. The steel mesh was then immersed in RPMI and cells which remained adherent to the mesh were

recovered by gentle scraping off with a rubber policeman. Cells were then pelleted and resuspended in 15 ml fresh RPMI.

#### Antibodies

Newly developed anti-CD1d mAb, D5 and 51.1, have been described previously (27). Briefly, the D5 mAb (IgG2a) is a mouse anti-mouse CD1d mAb raised against a glutathione-S-transferase-CD1d fusion (gst-CD1d) protein containing the  $\alpha$ 1- $\alpha$ 3 domains of CD1d. The D5 mAb is specific for the  $\alpha$ 1 domain of CD1d by immunoblotting (S. P. Balk, unpublished observations). The 51.1 mAb (IgG2b) is a mouse anti-human CD1d mAb, (a kind gift by Dr Steven Porcelli, Brigham & Women's Hospital) raised against a Fc-fusion protein of CD1d expressed in mouse NSO cells (27). Rabbit anti gst-CD1d is a polyclonal antibody raised against the gst-CD1d fusion protein (28). The 3C11 mAb (IgM) is a rat anti-mouse CD1d mAb that cross-reacts with human CD1d (13,14). BBM.1 (IgG1) is a mouse anti-human  $\beta$ <sub>2</sub>m antibody (kindly provided by Dr Steven Porcelli, Brigham & Women's Hospital). The 1E9 (IgG3) mAb (29) is a mouse anti-human CD73 mAb (kindly provided by Dr Linda Thompson, Oklahoma Medical Research Foundation).

#### Western blotting

Approximately 10<sup>6</sup> freshly isolated epithelial cells were resuspended in ice-cold 2% lysis buffer (prepared from 1% Triton and 1% NP-40) with protease inhibitors including 1.25 mM PMSF, 5  $\mu$ g/ml chymostatin, and 1  $\mu$ g/ml each of aprotinin, leupeptin, pepstatin, bestatin and DFP, and incubated on a rocker at 4°C for 30 min with gentle vortexing every 5 min. Cell lysates were then centrifuged at 13,000 r.p.m. for 10 min in a microfuge (Eppendorf) at 4°C to remove cell debris and supernatants resuspended in Laemmli buffer with reducing agents. The IEC lysates were resolved on a 12% SDS-PAGE and transferred onto a nitrocellulose membrane for 1 h at 100 V in transfer buffer (20% methanol, 150 mM glycine and 25 mM Tris, pH 8.3). After transfer, the nitrocellulose sheet was blocked with 50 ml of 5% non-fat milk in PBS overnight at 4°C. The nitrocellulose membrane was then incubated with the D5 mAb (ascites diluted 1:1000 overnight at 4°C). The nitrocellulose membrane was washed with PBS containing 0.01% Tween (PBST) for five washes of 15 min each and then incubated with 0.5  $\mu$ g/ml of goat anti-mouse IgG conjugated with horseradish peroxidase (Cappel, Malvern, PA.) This incubation was performed at room temperature for 1 h. The nitrocellulose membrane was then washed with PBST for five washes of 15 min each and finally incubated with 10 ml of chemiluminescence reagent (DuPont, Wilmington, DE) at room temperature for 1 min. A sheet of plastic wrap was placed onto the nitrocellulose membrane, and exposed and developed (XAR-5 film; Eastman Kodak, Rochester, NY).

#### Immunoprecipitation and Western blotting with alkylation, reduction and deglycosylation

In subsets of experiments, cell lysates were immunoprecipitated with mAb cross-linked to Sepharose beads with dimethyl pimelimidate (Sigma). Briefly, cell lysates were precleared with Protein A-Sepharose beads (Pharmacia, Piscataway, NJ), followed by beads adsorbed with irrelevant antibodies which were saved to serve as controls for the immunoprecipitation.

Preleared lysates were then divided equally into three Eppendorf tubes for immunoprecipitation with mAb D5 (anti-CD1d), mAb 51.1 (anti-CD1d) or mAb BBM.1 (anti- $\beta$ <sub>2</sub>m). Immunoprecipitation was performed by rocking lysates and mAb cross-linked beads overnight at 4°C. Beads were pelleted, and simultaneously with pre-clear control beads, washed 5 times with 1% NP-40 in PBS. Then 10  $\mu$ l of 10% SDS solution in water was added to the eluted supernatants to make a final concentration of 1.5% SDS and 20 mM DTT was added. The samples were heated to 85°C for 10 min and then 10  $\mu$ l of freshly prepared 0.2 M *N*-ethylmaleimide solution in water was added to each sample with incubation on ice for 1 h. For deglycosylation of CD1d, an equal volume (30  $\mu$ l) of 0.5%  $\beta$ -mercaptoethanol was added, boiled for 5 min, and 2 volumes (60  $\mu$ l) of a solution consisting of 0.25 M NaHPO<sub>4</sub>, 1 mM EDTA and 1% NP-40 added. Each sample was divided into two equal parts for treatment with [2  $\mu$ l of *N*-glycanase (PNGase; New England Biolabs, Boston, MA)] or without *N*-glycanase and the samples placed into a 37°C water bath for an 18 h incubation. Subsequently, reducing sample buffer was added and the proteins separated on a 12% SDS-PAGE gel and electrotransferred to a nitrocellulose (BioRad) membrane. Following transfer, blots were blocked with 5% non-fat milk in PBS and probed with mAb D5 from ascites (concentration 1:1000) overnight at 4°C. Blots were subsequently washed with PBS with 0.1% Tween and incubated with horseradish peroxidase-conjugated goat anti-mouse IgG (0.8  $\mu$ g/ml; Immunopure, Pierce, Rockford, IL) at room temperature for 1 h. Blots were washed with PBS with 0.1% Tween and proteins visualized by enhanced chemiluminescence (DuPont) at room temperature for 1 min.

#### Iodination of IEC

For <sup>125</sup>I cell surface protein labeling, normal human small bowel epithelial cells were freshly isolated and ~10<sup>6</sup> cells were used. Cells were washed twice in PBS at room temperature and were resuspended in 150  $\mu$ l of PBS. <sup>125</sup>I (1 mCi) was added along with 30  $\mu$ l of lactoperoxidase (2.7 mg/ml; Sigma) and 10  $\mu$ l of diluted H<sub>2</sub>O<sub>2</sub> (Sigma) to 0.03%. The reaction was allowed to take place for 5 min at room temperature and 10  $\mu$ l H<sub>2</sub>O<sub>2</sub> was added consecutively 3 times, each after 5 min of incubation. The labeling was terminated by adding 0.02 M potassium iodide in PBS containing 200  $\mu$ g/ml BSA. Cells were centrifuged at 1500 r.p.m. for 10 min at 4°C and washed once with cold PBS. The pelleted cells were lysed using 1% NP-40 with protease inhibitors at 4°C for 30 min and were transferred to Protein A-Sepharose beads overnight with rocking at 4°C. Three preclears were performed with normal serum prior to specific immunoprecipitations. The beads were washed 10 times with 1% NP-40 lysis buffer. The proteins were eluted from the beads following the alkylation and reduction protocol as described above, and resolved by SDS-PAGE on 13% gels. The gel was exposed for autoradiography using an intensifying screen.

#### Cell lines

T84 cells, a human colonic epithelial cell line (25) with documented expression of surface CD1d (28), was used as an *in vitro* model to study localization of CD1d on human IEC. T84 cells were grown on collagen-coated 75 cm<sup>2</sup> tissue

culture flasks in a 1:1 mixture of DMEM and Ham's F12 medium supplemented with 15 mM HEPES buffer (pH 7.5), 14 mM NaHCO<sub>3</sub>, 40 mg/l penicillin, 8 mg/l ampicillin, 90 mg/l streptomycin and 5% newborn FCS as previously described (28). Subculturing was performed every 6–8 days by treatment with 0.1% trypsin and plating on collagen-coated flasks. C1Rd is a stable transfectant of the HLA-A, -B-negative cell line C1R as previously described (27).

#### Localization of CD1d in T84 monolayers by confocal laser microscopy

The T84 monolayers were grown on collagen-coated, polycarbonate permeable supports (inserts) with a surface area of 0.33 cm<sup>2</sup> (Costar, Cambridge, MA) in order to preserve polarity of their apical versus basolateral surfaces. The cells were used once they had achieved electrical confluency (intercellular resistances >1000 ohm·cm<sup>2</sup>). The inserts with the cell monolayers were removed, gently washed by dipping into cold HBSS containing Ca<sup>2+</sup> and Mg<sup>2+</sup> and maintained on ice at 4°C throughout the staining procedure. Filtered 5% normal goat serum was used to block non-specific binding sites for 30 min. After blocking, the monolayers were processed for staining like the intestinal tissue sections described above and then visualized by confocal microscopy using a BioRad MRC confocal fluorescence microscope (BioRad)

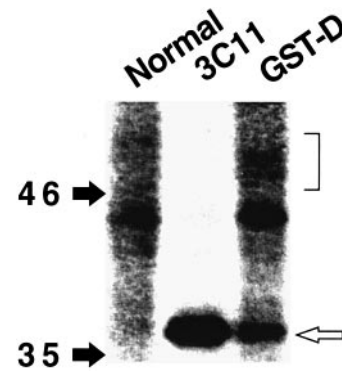
#### Selective surface biotinylation of T84 cells

Western blot experiments were performed on biotinylated T84 monolayers cultured on 5 cm<sup>2</sup> permeable supports. Confluent, electrically resistant T84 monolayers were washed free of media and surface labeled either apically or basolaterally with a solution of 1 mM sulfo-NHS biotin (Pierce) in HBSS for 20 min at 4°C. After quenching with 150 mM NH<sub>4</sub>Cl, each 5 cm<sup>2</sup> monolayer was solubilized in 1 ml of buffer containing 100 mM KCl, 30 mM NaCl, 2 mM EDTA, 10 mM HEPES pH 7.4, 1% Triton X-100, 1% NP-40, and protease inhibitors including 1.25 mM PMSF, 5 µg/ml chymostatin, and 1 µg/ml each of leupeptin, pepstatin and bestatin. Solubilized proteins were separated from the insoluble material by centrifugation in a microcentrifuge for 6 min at 1400 r.p.m. Biotinylated, solubilized proteins were immunoprecipitated with the D5 mAb bound to Protein A–Sepharose beads (Pierce) overnight at 4°C with gentle end-over-end rotation. As a control, biotinylated T84 cell lysates were examined for apical and basolateral expression of CD73, a molecule primarily expressed on the apical cell surface of IEC (30). Beads were washed 3 times with high salt (500 mM NaCl) and 3 times with low salt (50 mM NaCl) buffer. The immunoprecipitates were subjected to alkylation, reduction and treatment with *N*-glycanase as described above prior to addition of Laemmli buffer containing reducing agents. The immunoprecipitates were boiled, resolved by SDS–PAGE on 10% gels, transferred to a nitrocellulose membrane, blotted with horseradish peroxidase-conjugated avidin (Pierce) at a concentration of 0.3 µg/ml and developed with ECL as described above.

## Results

#### Biochemical characterization of CD1d on isolated native intestinal epithelia

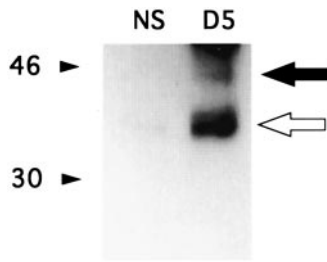
We have utilized rat anti-mouse CD1d mAb which cross-react with human CD1d to report that human CD1d on IEC is



**Fig. 1.** Immunoprecipitation of radiolabeled cell surface proteins from native IEC. Cell surface proteins of normal human IEC from the small intestine were radiolabeled with <sup>125</sup>I and immunoprecipitates prepared with anti-CD1d antibodies, 3C11 and gst-CD1d. The immunoprecipitates were resolved by SDS–PAGE on 12.5% gels under reducing conditions and autoradiography performed. NS represents the immunoprecipitates with the control serum representing a mixture of normal rat and rabbit serums. The bracket indicates the 48 kDa smear consistent with a glycoprotein and the open arrow, the location of a 37 kDa band. The mol. wt markers in kDa are indicated on the left.

primarily present as a 37 kDa non-glycosylated protein consistent with the size of the CD1d polypeptide backbone without any detectable β<sub>2</sub>m association (13,14,22). To confirm and further extend this result, we re-examined native IEC cell surface proteins with a rabbit anti-gst-CD1d antibody as well as the previously applied rat anti-mouse/human mAb, 3C11 (13,14,22). Normal human IEC were isolated from small intestine and cell surface proteins radiolabeled with <sup>125</sup>I using the lactoperoxidase catalyzed method. The protein lysates prepared from the radiolabeled IEC were immunoprecipitated with the affinity-purified rabbit anti-CD1d antibody raised against a gst-CD1d fusion protein and mAb 3C11. When the immunoprecipitates were resolved under conditions of alkylation and reduction, it could be seen that the anti-CD1d-specific antibodies, anti-gst-CD1d and 3C11, but not the irrelevant antibody, immunoprecipitated a major band of 37 kDa, as previously reported (22) (Fig. 1, open arrow). In addition, a band which resolved as a smear, consistent with a glycosylated protein, was also identifiable in the lane associated with the rabbit gst-CD1d antibody (Fig. 1, bracket). To confirm this result, Western blot analysis of protein lysates prepared from purified IEC obtained from normal human colon was performed using mAb D5. This analysis confirmed the results with cell surface iodination and revealed a prominent 37 kDa band when proteins were resolved under conditions of alkylation and reduction (Fig. 2, open arrow). In addition, a prominent band which appeared as a smear at ~48 kDa (Fig. 2, closed arrow) was observed. Importantly, none of these bands were observed when identical protein lysates were Western blotted with similarly diluted normal mouse serum (Fig. 2, lane NS).

Although the 37 kDa band observed by cell surface iodination and Western blotting was consistent with the previously reported non-glycosylated, β<sub>2</sub>m-independent form of CD1d (22), the smear observed at 48 kDa was consistent with a

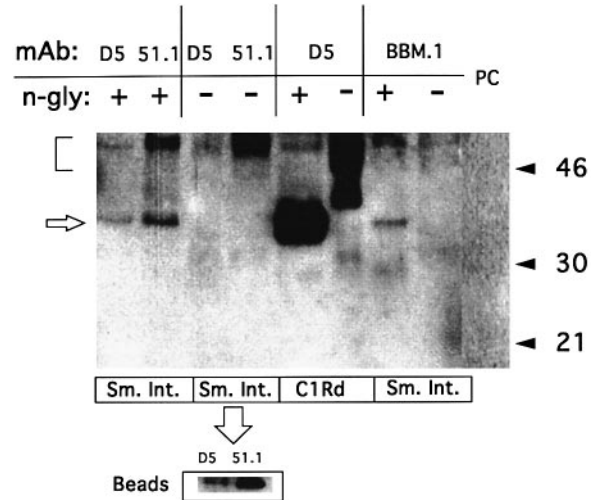


**Fig. 2.** Western blotting of native IEC with a CD1d-specific mAb. Cold protein lysates prepared from normal human IEC obtained from normal small intestine were resolved by SDS-PAGE under conditions of alkylation and reduction, and Western blotting performed with either normal mouse serum (NS) or the CD1d-specific mAb, D5. The closed arrow indicates the location of a 48 kDa smear and the open arrow the location of a 37 kDa band. The mol. wt in kDa are indicated on the left.

glycosylated form of CD1d. To further analyze the glycosylation patterns of this larger form of CD1d in native IEC, tandem immunoprecipitation/Western blotting studies were performed in the presence and absence of *N*-glycanase, an enzyme which cleaves *N*-linked carbohydrate side chain modifications. In these studies, immunoprecipitates were obtained from total protein lysates prepared from purified human IEC derived from normal human small intestine using the CD1d-specific antibodies D5 and 51.1 cross-linked to Protein A-sepharose beads. When the proteins precipitated by these antibodies were eluted from the Sepharose beads by boiling in 0.75% SDS and the eluted proteins were Western blotted with the D5 mAb without prior *N*-glycanase treatment, the dominant band identified was a 48–50 kDa smear which co-resolved with the fully glycosylated CD1d expressed by a stable transfectant of CD1d treated similarly (Fig. 3, bracket). However, when the elutes were treated with *N*-glycanase and Western blotted with the D5 mAb, a significant fraction of the 48 kDa smear was observed to be reduced to a band of 37 kDa consistent with the size of the CD1d polypeptide chain (Fig. 3, open arrow). Identical observations were made when immunoprecipitates were prepared with the anti- $\beta_2m$  specific mAb, BBM.1, indicating that CD1d is present in native IEC as a 48 kDa,  $\beta_2m$ -associated glycoprotein (Fig. 3). This association of the 48 kDa form of CD1d with  $\beta_2m$  was confirmed by immunoblotting mAb 51.1 immunoprecipitates with the BBM.1 mAb which revealed a 12 kDa band consistent with  $\beta_2m$  (data not shown). When the immunoprecipitates prepared with the D5 and 51.1 mAb were examined directly on the Protein A-Sepharose beads without prior elution, the D5 mAb immunoblotted a prominent 37 kDa band (Fig. 3, insert) indicating that the 37 kDa form of CD1d eluted poorly with 0.75% SDS from the antibody-linked Sepharose beads.

#### Localization of epithelial CD1d in native intestinal tissue specimens

The results described above indicated that IEC express biochemically distinct forms of CD1d. To further confirm these results and define the cellular localization of CD1d, antibody staining of native IEC was defined by CLM. Figure 4 demonstrates CD1d distribution in cross section views of surface

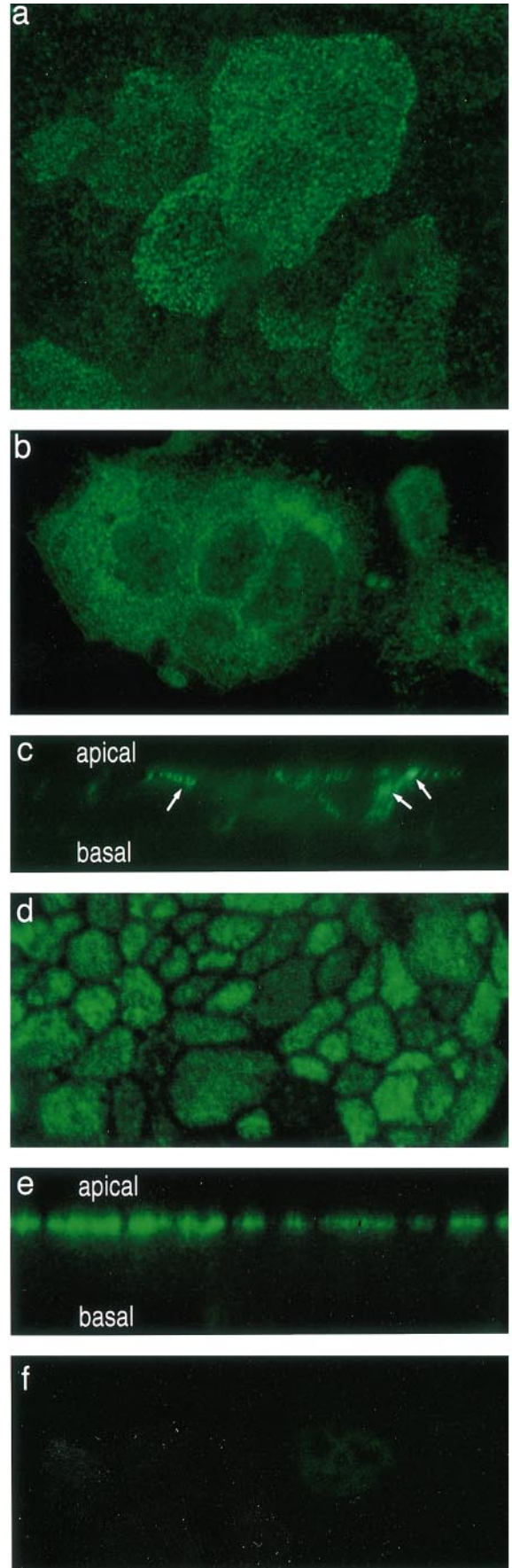
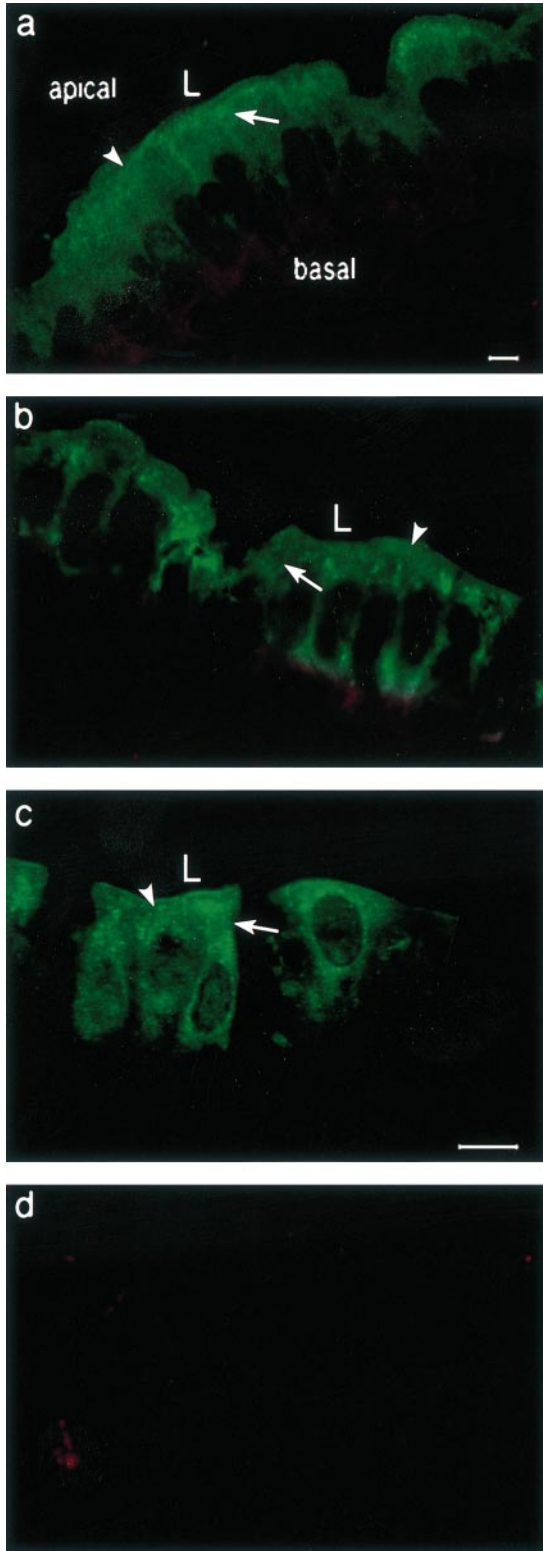


**Fig. 3.** Characterization of IEC CD1d by immunoprecipitation and Western blotting. Protein lysates derived from normal human small intestine (Sm. Int.) and a CD1d-transfected cell line, C1Rd, were pre-cleared with normal mouse serum and subsequently immunoprecipitated with either the CD1d-specific mAb (D5 and 51.1) or the  $\beta_2m$ -specific mAb (BBM.1) cross-linked to Protein A-Sepharose beads. The immunoprecipitates were eluted with SDS alone and the eluates subjected (+) or not subjected (-) to digestion with *N*-glycanase (n-gly). An immunoprecipitation pre-clear is also indicated (PC). After resolving by SDS-PAGE, and conditions of alkylation and reduction, the immunoprecipitates were immunoblotted with the CD1d-specific mAb, D5. The mol. wt markers in kDa are indicated on the right. The insert shows results of the immunoblot with the D5 mAb of the beads containing the immunoprecipitates which resisted elution with SDS.

epithelial cells. CD1d localized in the apical (small arrowhead) and lateral (large arrow) membranes of small intestine (Fig. 4a) and colonic (Fig. 4b and c) surface epithelial cells. In addition, higher magnification revealed a significant subapical and faint perinuclear pool of CD1d (Fig. 4c). A fine punctate staining was observed in these intracellular sites. Staining for VCAM-1 (Fig. 4d), a protein not normally expressed on intestinal epithelia (31), served as a negative control for these experiments. Similar results were obtained with a polyclonal rabbit anti-human CD1d anti-serum raised against the *gst*-CD1d fusion protein (data not shown).

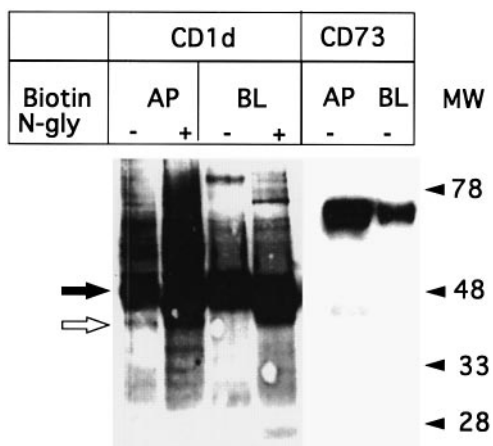
#### Localization of T84 cell CD1d expression

In order to confirm these observations the following experiments were performed on confluent epithelia *in vitro*. For such studies, CD1d was localized in a model human IEC line, T84. While T84 cell represent a transformed cell line, we and others have shown that T84 cells are functionally, phenotypically and structurally similar to native human intestinal crypt epithelium (22,32–34) and that these cells express surface CD1d (28). As shown in Fig. 5, CLM sectioning of T84 cells grown on permeable supports revealed that expression of CD1d paralleled that of native epithelia. The *en face* confocal photomicrographs revealed a distribution of CD1d in the apical membrane (Fig. 5a and d). Moderate staining was observed on the lateral membrane domain on a lower cross-sectional image with the anti-*gst*-CD1d fusion protein antibody



**Fig. 4.** Immunolocalization of CD1d on native IEC examined by CLM using mAb, D5. Native IEC from the normal human small intestine (a and d) and large intestine (b and c) were stained with the D5 mAb (a–c) or an irrelevant isotype control mAb VCAM-1 (d) as a negative control. L indicates the apical surface facing the lumen. Specific fluorescence is shown in green and counterstain in red. The small arrowhead indicates apical staining and the large arrow indicates lateral membrane staining. (c) A high power view of the colon staining shown in (b). Microbar in (a) is 4 mm which equals 5 micron and in (c) is 8 mm which also equals 5 micron.

(Fig. 5b). In addition, similar to native tissue, an intense subapical, intracellular pool of CD1d was evident in permeabilized monolayers (Fig. 5b). Figure 5(c) is a reconstructed confocal image in the *xz*-plane of the staining with the *gst*-CD1d fusion protein antibody which shows a fine punctate pattern of staining in the apical and subapical region of T84 epithelial cells (arrow) as well as staining of the lateral membrane regions (double arrow). A similarly reconstructed confocal image of the staining with the 3C11 mAb (Fig. 5e) revealed staining of only the apical, but not the lateral, regions of the T84 epithelial cells. No CD1d was observed in the basal poles of T84 epithelial cells (Fig. 5c). As a negative control, no staining of T84 cells was observed with preimmune rabbit serum (Fig. 5d). Such data reveal that the T84 cell line accurately reflects the distribution of CD1d in native intestinal tissue.



**Fig. 6.** Analysis of apical and basolateral distribution of CD1d by selective biotin labeling of proteins in T84 cells. Polarized T84 cells were selectively labeled with biotin on either the apical or basolateral cell surfaces and lysates prepared which were immunoprecipitated with the CD1d-specific mAb, D5, after extensive preclearing with normal mouse serum as a negative control. The immunoprecipitates were either subjected (+) or not subjected (-) to *N*-glycanase (N-gly) digestion after prior alkylation and reduction, resolved by SDS-PAGE and Western blotted with horseradish peroxidase coupled to avidin. The closed arrow indicates glycosylated, 48 kDa CD1d and the open arrow indicates deglycosylated, 37 kDa CD1d. CD73 was similarly analyzed as a control. The mol. wt markers in kDa are indicated on the right.

**Fig. 5.** Immunolocalization of CD1d on polarized T84 monolayers. T84 cells, polarized on semipermeable supports, were stained with either the rabbit anti-*gst*-CD1d antibody (a-c) or the 3C11 mAb (d and e). Panels (a) and (d) are *en face* images, panel (b) a cross-section of T84 monolayer, panels (c) and (e) are computer reconstructed *xz* images, and panel (f) is a negative control staining with preimmune rabbit serum. Normal rat serum as negative control for the 3C11 mAb was also negative (data not shown). Specific fluorescence is shown in green. Apical and basal surfaces are indicated. The single arrow indicates fine punctate apical and subapical staining, and the double arrow staining of the lateral membrane region in (c).

#### Biochemical localization of CD1d on the cell surface of T84 cells

To biochemically confirm this localization of cell surface CD1d, selective cell surface labeling with biotin was performed. Apical and basolateral surface proteins of T84 monolayers were selectively labeled with biotin. Cellular lysates prepared from the labeled cells were then immunoprecipitated with the D5 mAb. The immunoprecipitates were subjected to digestion with *N*-glycanase after alkylation and reduction. After resolution by SDS-PAGE, the treated D5 immunoprecipitates were blotted with avidin coupled to horseradish peroxidase. As can be seen in Fig. 6, the D5 mAb immunoprecipitated a 48 kDa protein (closed arrow) consistent with fully glycosylated CD1d from the apical and basolaterally labeled cell surfaces (lanes -) that was reduced to 37 kDa (open arrow) consistent with fully deglycosylated CD1d after digestion with *N*-glycanase (lanes +). In addition, the 37 kDa non-glycosylated isoform was identifiable from the apical but not the basolaterally labeled lysate (apical, lane -). As previously described (30), CD73 was expressed primarily apical on confluent T84 monolayers. These studies confirmed the biochemical studies performed with native intestinal epithelia, and further showed that the 37 and 48 kDa forms of CD1d exist on the cell surface and that the 37 kDa isoform immunolocalized to the apical cell surface.

#### Discussion

We demonstrate here that IEC CD1d is expressed in a polarized fashion on both native and transformed human cells with an unusual pattern characterized by restriction to the apical and lateral membrane domains. Additionally, these studies confirm previous descriptions of a non-glycosylated  $\beta_2$ m-associated, fully glycosylated epithelial cell CD1d molecule. Such results define the cell surface parameters necessary to begin dissecting the functional role of CD1d *in vivo* in this location.

Proteins expressed on epithelia exist in a polarized fashion; constituted by apical, lateral and basolateral membrane domains. Such compartmentalization of specific proteins serves as the basis for vectorial processes such as transcellular and paracellular transport, leukocyte trafficking, and barrier function (35,36). Since both homotypic and heterotypic cell-cell interactions occur in the intestine, it is imperative to understand how cell surface proteins are distributed. To date, such a road map for epithelial CD1d has not come to fruition. The results of the present study extend previous work defining a potential role for CD1d in the human intestine and several points are noteworthy.

First, CD1d localization to the apical and lateral membrane domains places CD1d in an ideal location to function in the processing and presentation of luminal antigens to laterally positioned iIEL. Such an hypothesis has been proposed previously for CD1d (26) and CD1d on IEC has been demonstrated to interact directly with peripheral blood T cells (21). However, localization studies of CD1d in epithelia have not been previously accomplished. In addition to localization on the apical and lateral membranes, we confirmed CD1d

localization to a prominent subapical pool. This is consistent with the hypothesis that CD1d may be cycling between the apical and lateral membranes, and perhaps for a role in sampling luminal antigens for presentation to laterally positioned iIEL. Such observations are consistent with previous studies demonstrating expression of iIEL counter-receptors on the lateral membrane domain such as E-cadherin (37). CD1d exhibits an endosomal sorting motif in the cytoplasmic tail (38), which has been previously shown to be important in the localization of CD1b to an MHC class II compartment (39). Whether CD1d co-localizes to an MHC class II compartment in IEC remains to be determined. Nonetheless, it is increasingly evident that IEC can internalize, process and present soluble antigens to adjacent T cells and that the antigen presentation process is polarized (40). Since digestive processes may generate antigen fragments that may potentially be able to directly load into an antigen-presenting molecule such as CD1d, it is possible that apically oriented CD1d may directly couple luminal antigen. It is thus possible that CD1d in human IEC may share structural and functional features of the MHC class II processing pathway and, as such, concomitantly function in the presentation of distinct types of luminal antigens such as those derived from microbial antigens.

In this report, we have also confirmed previous observations that a major form of CD1d on the cell surface of IEC is a 37 kDa isoform which we have previously shown is non-glycosylated and  $\beta_2m$  independent (22). In addition, this 37 kDa form, which had a strong tendency to form trimers as previously described (22 and data not shown), immunolocalized solely apically based upon selective biotin labeling studies and CLM examination of staining with the 3C11 mAb which seems to preferentially recognize this isoform of CD1d (Figs 1 and 5). The functional implications of this immunolocalization remains to be established. In addition, as predicted from studies in transfected model systems (13), a fully glycosylated,  $\beta_2m$ -associated form of CD1d is also present in native IEC and likely on model IEC lines. The relationship between the 37 kDa non-glycosylated isoform and fully glycosylated 48 kDa isoform is unknown; in particular, whether a precursor-product relationship exists between these two isoforms. One hypothetical model is that CD1d is synthesized as a fully glycosylated, 48 kDa,  $\beta_2m$ -associated molecule in intestinal epithelia but subsequently becomes deglycosylated during recycling between the plasma membrane and intracellular organelles, such as an endosome, generating the 37 kDa isoform. This model would require the presence of cellular *N*-glycanases which do indeed exist but have not yet been documented in human IEC and, in particular, in endosomes (41). Alternatively, the 37 kDa form of CD1d may be characterized by a post-translational modification other than carbohydrate side chain modifications. Whether this biochemically distinct 37 kDa isoform represents only a by-product of the CD1d biosynthetic pathway in epithelia and/or has distinct functional consequences for iIEL-IEC interactions must await further investigation.

The fact that a  $\beta_2m$ -associated isoform is identifiable on epithelia is also of significant importance to understanding iIEL development. Studies by Sydora and colleagues have shown that a TAP-independent,  $\beta_2m$ -dependent molecule is responsible for the development of most CD8  $\alpha\beta^+$ , TCR  $\alpha\beta^+$

iIEL in mouse (23,42). Although these characteristics are exhibited by CD1d, our previous inability to identify a  $\beta_2m$  association for CD1d in epithelia caused some concern for considering this molecule as a candidate selecting ligand for iIEL. An additional concern has been the inability of several investigators to corroborate CD1d expression in mouse intestinal epithelia (15). The reasons for this difference between mouse and human CD1d expression in IEC are unclear but may relate to quantitative differences in protein expression among other potential factors (26). Indeed, another MHC class I-related molecule, FcRn, is not evident in adult epithelial surfaces of the mouse intestine (43) but is readily demonstrable in adult human IEC (44). Whether this reflects differences in FcRn promoter structure or some other property remains to be established but is certainly illustrative in considering the expression of CD1d in epithelia.

Identification of a  $\beta_2m$ -associated isoform of CD1d in human intestinal epithelia makes CD1d an excellent candidate as a cognate ligand for CD8<sup>+</sup> TCR  $\alpha\beta^+$  iIEL. Since TCR  $\gamma\delta^+$  iIEL are present in  $\beta_2m$ -deficient animals (42), it remains possible that  $\beta_2m$ -independent forms of non-classical MHC class I molecules may be important in the selection of other iIEL subsets. Indeed,  $\beta_2m$ -dependent and -independent forms of CD1d have been identified in mouse B cells which are capable of activating T cells (45). The only  $\beta_2m$ -independent MHC class I like molecules that have been identified to date on native IEC are: (i) CD1d which has been identified as a  $\beta_2m$ -independent, non-glycosylated molecule on native intestinal epithelia (22) and (ii) the MHC class I-related chain A gene product which has been reported as a glycosylated,  $\beta_2m$ -independent protein, at least in transfected model systems (46) which is recognized by  $V\delta 1^+$  iIEL (47). In addition, we have recently identified glycosylated forms of CD1d on the cell surface of a melanoma cell line transfected with CD1d in the absence of  $\beta_2m$ . However, in the latter case, the carbohydrate side chain modifications decorating the CD1d molecule were immature, as defined by Endoglycosidase-H sensitivity, indicating the absence of processing beyond the initial *N*-glycanase modification within the endoplasmic reticulum (48). Interestingly, this  $\beta_2m$ -independent, CD1d glycoprotein can activate p59<sup>l<sup>y</sup>n</sup> on peripheral T cells, providing a potential functional role for this molecule (N. Campbell and L. Mayer, pers. commun.). These studies suggest, therefore, that biochemically distinct, yet functionally relevant isoforms, of CD1d and other MHC class I-related molecules may decorate the cell surface of native intestinal epithelia.

In summary, we have used several newly generated human CD1d-specific antibodies to characterize CD1d expression by intestinal epithelia. These studies confirm previous biochemical and immunohistologic studies in humans, indicate that CD1d exhibits an unusual localization to the apical and lateral, but not basal, membrane domains and have further shown that, as would be necessary for selection of a significant fraction of iIEL, CD1d on IEC is present as a fully glycosylated,  $\beta_2m$ -associated molecule.

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## Abbreviations

IEC	intestinal epithelial cell
$\beta_2m$	$\beta_2$ -microglobulin
CLM	confocal laser microscopy
iIEL	intestinal intraepithelial lymphocyte
TAP	transporter associated with antigen presentation

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