The Nonclassical Class I Molecule CD1d Associates with the Novel CD8 Ligand gp180 on Intestinal Epithelial Cells*

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The goal of the systemic immune system is to generate specific responses against foreign pathogens that result in their elimination. In contrast, whereas antigen exposure in the gastrointestinal tract appears to be magnified due to dietary and microbial antigen load, the immune response is limited or suppressed. This state of nonresponsiveness to orally administered antigens is referred to as oral tolerance (1–12). Although there have been several mechanisms invoked to explain the development of this state, many studies have shown that the activation of suppressor T cells is crucial for the existence of oral tolerance (5, 6). Unfortunately, the mechanisms responsible for the activation of this subset of T cells have not been completely defined.

Previous studies have demonstrated that intestinal epithelial cells can act as antigen-presenting cells capable of stimulating primed T cells (13–16). Interestingly, despite the constitutive expression of class II MHC1 molecules on these cells, the T cells proliferating in these co-cultures are CD8+ (17, 18). The subset of CD8+ T cells that proliferate when co-cultured with IECs are phenotypically similar to suppressor T cells (CD8+CD28−) (19). These T cells inhibit primary, secondary, and unrelated mixed lymphocyte reactions, as well as B cell responses in vitro in an antigen-nonspecific manner. The addition of anti-CD8 monoclonal antibodies to these co-cultures have supported the contention that the CD8 molecule itself is important in the activation of CD8+ T cells by intestinal epithelial cells (IECs) with the activation of the CD8-associated protein tyrosine kinase p56lck being a necessary but not sufficient event (20). Interestingly, the addition of monoclonal antibodies against classical restriction elements, class I and class II MHC, do not inhibit the activation of these CD8+ T cells, thus suggesting some novel form of interaction.

It is known that IECs express the nonclassical restriction elements CD1d in humans and CD1 and TL in mouse (21–24). CD1d transcripts are only observed within intestinal crypt cells, whereas protein expression appears to be localized predominantly in the intestinal villus (25). On the IEC, CD1d is restricted to the apical and lateral surfaces, placing it in a potential location for interaction with local T cells (24). Previous studies have documented that cytolytic intraepithelial lymphocytes can be restricted by these class Ib molecules (26) and that the proliferation of T cells in IEC-T cell co-cultures can be inhibited by mAbs to CD1d in a human system (27). This molecule may, therefore, serve as a restriction element for IEC-T cell interactions. However, in contrast to the conventional restriction element class I MHC, CD1d fails to bind CD8.2 Thus, in order for a TCR-CD8 co-receptor complex to form in this system, another surface molecule must exist.

Studies in our laboratory have reported on two monoclonal antibodies generated against intestinal epithelial cells that were capable of inhibiting the IEC-induced proliferation of CD8+ T cells and activation of the CD8-associated kinase p56lck (20). Both of these mAbs, B9 and L12, recognize a 180-kDa glycoprotein, termed gp180, which is expressed on epithelial cells in various organs and, comparable to CD1d (28), the predominant expression pattern is on surface rather than crypt epithelium. The monoclonal antibody B9 recognizes gp180 expressed by gastrointestinal epithelium, cortical thymic epithelium, and placental syncytiotrophoblasts, whereas L12 recognizes gp180 expressed only by the epithelium of the gastrointestinal tract. The 180-kDa intestinal

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1 The abbreviations used are: MHC, major histocompatibility complex; IEC, intestinal epithelial cell; mAb, monoclonal antibody; PBT, peripheral blood T cell; ELISA, enzyme-linked immunosorbent assay; HRP, horseradish peroxidase; GST, glutathione S-transferase; PBS, phosphate-buffered saline; TCR, T cell receptor; PAGE, polyacrylamide gel electrophoresis; GAM, goat anti-mouse Ig; RAM, rabbit anti-mouse IgG.

2 N. A. Campbell and L. Mayer, unpublished data.
epithelial cell surface glycoprotein is a novel molecule capable of binding to CD6 and activating the CD8-associated protein tyrosine kinase p56lck, but not the TCR-associated kinase p59fyn (29). This is in contrast to studies using intact intestinal epithelial cells, in which activation of p59fyn is also seen. These data suggest that gp180 may be associated with a molecule that is capable of interacting with the T cell receptor and activating TCR-associated p59fyn.

Given the findings reported above, CD1d might be an eligible candidate to aid gp180 in the activation and proliferation of CD8+ T cells, including those with suppressor activity. In the present study, we demonstrate that gp180 associates with CD1d and that this gp180-CD1d complex is involved in the activation of CD8+ T cells.

MATERIALS AND METHODS

Cell Isolation, Cell Lines, and Culture Conditions—FO-1 cells transfected with human CD1d cDNA (FO-1 D5) have been previously described (30). These transfected cells were maintained in RPMI medium supplemented with 10% fetal calf serum, 20 mM HEPES, 1% nonessential amino acids, 1% penicillin/streptomycin, and 3 mM/ml G418 (Sigma). Untransfected FO-1 cells were cultured in similar media without G418 and served as a negative control.

Peripheral blood mononuclear cells were isolated from leukocyte concentrate packs and separated into T and non-T cells using rosetting and density gradient centrifugation as described previously (27). Brieﬂy, heparinized venous blood was collected from normal donors and separated by Ficoll-Paque (Amersham Pharmacia Biotech) density gradient centrifugation. T cells and non-T cells were isolated from PBMCs by a rosetting method using neuraminidase-treated sheep red blood cells followed by Ficoll-Paque density gradient centrifugation.

Enterocytes were isolated by a method described previously (17, 27). Surgical specimens were obtained from the operating room. Specimens were washed extensively with PBS containing 1% penicillin/streptomycin and 1% Fow Laboratory Inc., McLean, VA). The muccosa was stripped from the submuccosa, cut into small pieces, and placed in 1 mM dithiothreitol (Sigma) for 5 min at room temperature to remove the mucus. The pieces were then washed in PBS and incubated in dispase (3 mg/ml in RPMI 1640 medium, Roche Molecular Biochemicals) for 30 min in a 37 °C shaking incubator. This was repeated four times. The tissue pieces were removed and the cell suspension collected, pooled, and centrifuged on a Percoll density gradient (Amersham Pharmacia Biotech). Enterocytes located at the 0–30% interface were harvested and cell-wall breaks were made using a tissue homogenizer for 15 min, and the epithelial cell lysate was rotated with the coated beads overnight. The beads were washed five times with PBS and resuspended in RPMI medium with 1% bovine serum albumin for co-culture experiments. Preparations of purified enterocytes were >90% viable, and free of macrophages and B cell contamination as determined by staining with anti-CD14 and anti-CD20 mAbs (Coulter Corp., Hialeah, FL), and contaminated with only 2–4% intraepithelial lymphocytes (CD3+ cells).

Antibodies—B9 is a murine IgG1 anti-human IEC monoclonal antibody that appears to recognize a carbohydrate epitope on the novel intestinal epithelial cell surface molecule gp180. Ascites was generated at a concentration of 1 mg/ml and used at a 1:1000 dilution for Western blotting, whereas 10 μg was used for immunoprecipitation studies. An irrelevant murine IgG1 antibody was used as a negative control whenever necessary.

Four monoclonal antibodies were used to detect CD1d. 3C11 and 1H1 are rat IgM anti-mouse CD1d monoclonal antibodies, which have been previously shown to cross-react with human CD1d (21, 22, 30). Supernatants were harvested from the 1H1 or 3C11 hybridomas, and antibody concentrations were adjusted to 10 μg/ml. D5 is a murine anti-human CD1d monoclonal antibody generated against glutathione S-transferase fusion protein of CD1d (31) and was a kind gift of Dr. Steven Balk (Beth Israel Medical Center, Boston, MA). The D5 antibody was used at a concentration of 5 μg/ml. 5.1.3 is a mouse anti-human CD1d mAb raised against an Fc-fusion protein of CD1d (32) and was the kind gift of Dr. Steve Porcelli (Brigham and Women’s Hospital, Boston, MA).

OKT3, OKT8, and W6/32 are hybridomas obtained from the American Type Culture Collection (ATCC, Manassas, VA) that produce monoclonal antibodies against CD3, CD8, and a nonpolymorphic domain of class I HMC, respectively.

Isolation of Purified gp180—2 × 107 intestinal epithelial cells were washed, resuspended in 1 ml of RPMI medium with 1 unit of phosphoinositol phospholipase C (Sigma) and incubated for 45 min at 37 °C. gp180 has previously been shown to exist in two forms: a GPI-anchored, apically sorted form and a transmembrane basalolateral form (29). The cell suspension was centrifuged for 10 min at 500 × g, and the cells and supernatant were analyzed for released gp180 (GPI-anchored form) and total cell blot.

Construction of CD1d-GST Fusion Proteins—The CD1d-GST fusion protein was constructed using the α1-α3 domains of CD1d in a pGEX vector (Amersham Pharmacia Biotech) (33). For large scale preparations of CD1d-GST fusion protein, 10 ml of transformed bacteria grown freshly overnight was inoculated into 500 ml of LB medium with ampicillin selection. This was incubated for 90 min at 37 °C. After adding 0.5% isopropyl-1-thio-β-D-galacopyranoside, the bacterial culture was grown for an additional 4 h. The culture was spun in a Sorvall centrifuge for 10 min at 3000 × g at room temperature and resuspended in 6 ml of STE buffer (10 mM Tris, pH 8.0, 150 mM NaCl, 1 mM EDTA). It was then spun for 10 min at 1500 × g. The pellet was resuspended in 24 ml of 100 μg/ml lysozyme in STE along with protease inhibitors (0.1 mg/ml PMSF and 20 μg/ml aprotinin) and incubated on ice for 15 min prior to adding 5 mM dithiothreitol and 1.5% Sarkosyl in STE. Bacterial cell walls were broken using a tissue homogenizer for 15 min, and pellets were obtained by spinning in a Sorvall SS-34 rotor at 23,500 × g for 30 min. After adding 4% Triton X-100 (v/v), 2.5 ml of glutathioneagarose beads (Sigma) were incubated with the lysate for 2 h at 4 °C on an orbital rocker. The beads were spun down, transferred to microcentrifuge tubes, and washed with 5 ml of STE containing 0.5% Triton X-100 (v/v). The final wash was with PBS alone, and CD1d-GST protein was eluted by rocking at 4 °C for 10 min in 0.75 ml of elution buffer (75 mM HEPES, pH 7.4, 150 mM NaCl, 5 mM dithiothreitol, 0.1% Triton X-100 (v/v)) containing 100 μM reduced glutathione. The supernatant (eluant) was saved, and another 200 μl of elution buffer was added to elute the remaining fusion protein. When the eluate was resolved by SDS-PAGE and stained with Coomassie, a single band at 65 kDa was seen, the approximate mass of the CD1d-GST fusion protein.

Co-culture, Immunoprecipitation, and Kinase Assays—T cells and intestinal epithelial cells were resuspended to achieve a concentration of 1 × 106 cells/ml in 0.1% bovine serum albumin-RPMI 1620 medium (v/v). Cells were prewarmed in a 37 °C water bath. For each reaction, one million T cells were plated in a 1.5-ml Eppendorf tube and centrifuged for 5 s. The supernatant was removed, and the pellet was resuspended. One million intestinal epithelial, FO-1 D5, or untransfected FO-1 cells were mixed with the T cells, spun quickly for 20 s, and placed in a 37 °C water bath. After 1, 2, or 5 min, 1 ml of ice-cold stop buffer (PBS with 10 mM sodium orthovanadate (Sigma) was added. The pellet was resuspended in 100 μl PBS containing 100 μl 0.5% Triton X-100, 1% sodium deoxycholate, 1% SDS, 0.1% protease inhibitors (Sigma) and vortexed 20 times at the start and end of a 30-min incubation on ice. As a positive control, one million T cells were incubated with 10 μg/ml anti-CD8 antibody (OKT8) for 30 min at 4 °C and cross-linked with 10 μg/ml rabbit anti-mouse IgG antibody for 2 min at 37 °C. Time zero tubes were prepared by adding stop buffer to the tubes containing T cells and either epithelial, FO-1 D5, or untransfected cells. The lysate from these two tubes were then combined into one tube to account for all constitutive kinases and substrates in both cell types. In some reactions, the lysates from these co-culture conditions were immunoprecipitated with a rabbit anti-human p59fyn or rabbit anti-human p56lck antibody, which was covalently bound to Sepharose beads (Santa Cruz Biotechnology, Santa Cruz, CA). These conjugated antibodies were rotated with co-culture lysates (pre-cleared with rabbit serum-coated Sepharose 4B beads) for 1 h at 4 °C. The beads were washed four times with PBS, and 75 μl of reducing buffer (50 mM Tris-Cl, pH 6.8, 5% 2-ME, 10% glycerol, 1% SDS) was added prior to boiling for 5 min. The resulting samples were then run on a 10% SDS-PAGE gel and transferred to nitrocellulose paper (Schleicher & Schuell Inc., Keene, NH). A mAb 4G10 anti-phosphotyrosine (Upstate Biotechnology Inc., Lake Placid, NY) Western blot was then performed.

In other experiments, lysates of intestinal epithelial cells were immunoprecipitated with murine anti-gp180 mAb B9, IgG1 (isotype control), or the anti-class I mAb W6/32 (negative control) to determine whether an association between gp180 and class I MHC existed through the TCR. A Sepharose beads were coated with 10 μg/ml rabbit IgG anti-mouse immunoglobulin followed by primary antibody (isotype control, anti-gp180, or anti-class I MHC). Each of these steps was performed for 1 h at room temperature while rotating. The beads were then washed thoroughly with wash buffer (2 mM Tris, pH 7.4, 0.5% v/v EDTA, 4 mM NaCl), and the epithelial cell lysate was rotated with the coated beads overnight at 4 °C. The beads were washed five times with RIP buffer (10 mM
Association of CD1d with gp180 on IECs

Tris, pH 8.0, 1.0 mM EDTA, 0.5% Nonidet P-40, 0.1 mM NaCl, 1 mg/ml ovalbumin, and 0.02% sodium azide) and resolved on SDS-PAGE. After transferring to nitrocellulose, an anti-CD1d (D5) mAb followed by an anti-gp180 (B9) mAb Western blot was performed.

When kinase assays were performed, bands were resuspended in 30 μl of kinase buffer (10 mM MgCl₂, 50 mM Tris, pH 7.4) and mixed with 10 μCi of [γ-³²P]ATP (Amersham Pharmacia Biotech) for 30 min at room temperature. The enzyme reaction was stopped by adding 15 μl of 4× reducing buffer (as described previously) and boiling for 5 min.

Western Blot—A 10% SDS-PAGE gel was prepared, and one million cell equivalents of protein was loaded per lane. The protein was transferred from the gel to nitrocellulose paper and blocked with 5% milk for 1 h at room temperature. The membrane was then incubated with 1 μg/ml of primary antibody (4G10, B9, D5, or isotype control) overnight at 4 °C. The membrane was washed with PBS several times and incubated in 1 μg/ml of HRP-conjugated goat anti-mouse Ig (Cappel, Durham, NC) antibody for 1 h at room temperature. The membrane was washed thoroughly with wash buffer (PBS, 0.05% Tween-20) prior to development using enzyme-linked chemiluminescence (NEP Life Science Products) reagent.

Enzyme-linked Immunosorbant Assays—gp180 was purified using a B9 affinity column and diluted in coating buffer (dH₂O, 0.015 M Na₂CO₃, 0.03 M NaHCO₃, 3 mM Na₂SO₄). An optimal dilution of gp180 (4 × 10⁵ cell equivalents) was used to coat 96-well Nunc ELISA plates at 4 °C overnight. The plates were washed five times with 100 μl of coating buffer (PBS, 0.05% Tween-20) and blocked with 1% bovine serum albumin-PBS for 1 h at room temperature. After washing, 1 μg/ml CD1d-GST fusion protein or GST alone was added to the plates and incubated at room temperature for 1 h. The plates were then washed five times. Binding was detected by incubating the plates with 5 μg/ml mouse anti-GST antibody for 1 h followed by 1 h of incubation with 10 μg/ml HRP-conjugated goat anti-mouse Ig secondary antibody. After 100 μl of HRP substrate (dH₂O, 0.2 M Na₃PO₄, 0.2% NaN₃, 0.001% H₂O₂, 2 mM phenol, 1.2 mM 4-aminantipyrine) was added, the plate was read by a Genetic Systems microplate ELISA reader at 490 nm.

Flow Cytometry Cell Staining—Intestinal epithelial cells were isolated as described, and 1 × 10⁵ cells per condition were stained in a V-bottom 96-well plate. The cells were washed three times with 0.1% bovine serum albumin in PBS. The intestinal epithelial cells were incubated with 10 μg/ml isotype control mAb (murine IgG1 or murine IgG2b), 10 μg/ml mouse IgG1 anti-gp180 (B9), or 10 μg/ml mouse IgG2b anti-CD1d (D5) monoclonal antibody for 30 min at 4 °C. Cells were washed several times and incubated with 10 μg/ml fluorescein isothiocyanate-conjugated goat anti-mouse Ig (BIOSOURCE, Camarillo, CA) for an additional 30 min at 4 °C. Cells were washed and resuspended in 400 μl of PBS. The percentage of positive cells and mean fluorescence were analyzed by an Epics Profile III flow cytometer.

RESULTS

CD1d Associates with gp180 on the Surface of Intestinal Epithelial Cells—Our previous studies have shown that co-culturing IECs with PBTs results in the activation of the TCR-associated p56lck and CD1d-associated p56lck tyrosine kinases. Purified gp180 was found to be responsible for the activation of the protein tyrosine kinase p56lck but not p56yn (29). It was, therefore, hypothesized that another molecule might associate with gp180 and interact with the TCR. Because anti-CD1d antibodies have been shown to inhibit IEC-induced proliferation of CD8⁺ T cells, CD1d was an eligible candidate to aid gp180 in the activation of CD8⁺ T cells. Thus, the hypothesis that gp180 associates with CD1d was studied.

Initial studies were performed to confirm that normal IECs express both gp180 and CD1d on their surface. 1 × 10⁵ cells per condition were stained with B9, D5, and appropriate isotype controls to confirm cell surface expression of gp180 and CD1d, respectively. The cells were analyzed by an Epics Profile III flow cytometer. Fig. 1A illustrates that freshly isolated intestinal epithelial cells do express both gp180 and CD1d. A co-immunoprecipitation study was then performed using these same freshly isolated intestinal epithelial cells (data not shown) or HT29 cells. An intestinal epithelial cell lysate was prepared by lysing 1 × 10⁷ intestinal epithelial cells in lysis buffer containing 1% digitonin. The lysate was immunoprecipitated with a murine IgG2b mAb isotype control or mAb D5. An anti-gp180 (B9) followed by an anti-CD1d (D5) Western blot was then performed. Fig. 1B documents the association of gp180 (180 kDa) and CD1d (37 kDa). The D5 mAb immunoprecipitated a 180-kDa band consistent with gp180 (Fig. 1B, left panel, right lane), as well as a doublet at 37-kDa (right panel, right lane), consistent with the form of CD1d isolated from intestinal epithelial cells (24, 30). Similar results were observed when 3C11 and 1H1 monoclonal antibodies were used (data not shown). Although this figure provides data with intestinal epithelial cells lysed in a digitonin based lysis buffer, there appeared to be no difference in intestinal epithelial cells lysed with either Nonidet P-40 or Brij 97 (data not shown). Thus, the interaction between gp180 and CD1d appeared to be quite strong. In addition, it appears that neither gp180 nor CD1d co-precipitated with the conventional restriction element class I MHC (data not shown).

In order to confirm these findings more directly, an enzyme-linked immunosorbant assay was performed. An optimal concentration (4 × 10⁵ cell equivalents) of gp180 was used to coat ELISA plates, and 1 μg/ml CD1d-GST or control proteins were added to the wells. A mouse anti-GST antibody (5 μg/ml) was then added followed by a HRP-conjugated goat anti-mouse IgG antibody to detect binding. As seen in Fig. 2, the CD1d-GST fusion protein bound gp180 to a significantly greater extent in comparison to the control GST protein. Thus, by two experimental approaches, CD1d appeared to be capable of associating with gp180.

CD1d Alone Does Not Activate the CD8-associated Protein Tyrosine Kinase p56lck but Does Appear to Activate the TCR-associated Kinase p56yn—Initial experiments conducted in our laboratory suggested that CD1d was not involved in the activation of CD8-associated p56lck in IEC-T cell co-cultures. This was determined by mAb inhibition studies in which neither the 3C11 mAb nor 1H1 mAb inhibited IEC activation of p56lck. However, in order to confirm that CD1d was not involved in the activation of p56lck more directly, FO-1 cells stably transfected with human CD1d cDNA (FO-1 D5) were co-cultured with peripheral blood T cells for varying incubation periods (0, 2, and 5 min). Reaction products were then added to cold lysis buffer. The T cell lysates were immunoprecipitated with mouse IgG anti-human p56lck antibody, the proteins were resolved by SDS-PAGE, and an anti-phosphotyrosine Western blot was performed. In this experiment (Fig. 3A), no phosphorylation of the CD8-associated protein tyrosine kinase p56lck was seen. As a control, untransfected FO-1 cells were cultured with peripheral blood T cells and subjected to the same conditions as the FO-1 D5 cells. In order to document that the CD1d expressed by the transfectant was functional, we utilized the same cell lysates to determine whether CD1d could activate the TCR-associated kinase p56yn. FO-1 D5 cells were co-cultured with peripheral blood T cells for varying incubation periods (0, 2, and 5 min) in the presence or absence of the mouse IgG anti-human CD1d mAb, D5. As a control, untransfected FO-1 cells were cultured with peripheral blood T cells and subjected to the same conditions as the FO-1 D5 cells. All reactions were stopped with cold lysis buffer. The T cell lysates were immunoprecipitated with rabbit anti-human p56yn antibody, and an anti-phosphotyrosine Western blot was performed. As seen in Fig. 3B, phosphorylation of p56yn was readily seen with FO-1 D5-T cell co-cultures, which was blocked by the monoclonal antibody D5. FO-1 cells do not express either CD1d or gp180, thus explaining the inability of this cell line to activate p56lck or p56yn. In addition, the ability of FO-1 D5 cells to activate p56yn indicates that CD1d is functional (31). The identification of Lck in these lysates serves as a control for equal loading of protein, given the absence of bands in Fig. 3A.
Antibodies against CD1d Do Not Inhibit CD8-associated Kinase p56<sup>lck</sup> but Do Inhibit the TCR-associated Kinase p59<sup>fyn</sup> in Freshly Isolated Intestinal Epithelial Cell-T cell Co-cultures—We have previously shown that co-culturing purified gp180 with CD8<sup>+</sup>T cells results in the activation of CD8-associated p56<sup>lck</sup>. The studies represented in Fig. 3 appear to indicate that CD1d may be responsible for the activation of the TCR-associated kinase p59<sup>fyn</sup> but not of the CD8-associated kinase p56<sup>lck</sup>. Because the use of a CD1d transfectant may not actually reflect in vivo events, we set out to confirm this in our isolated IEC-T cell co-culture system.

An antibody to CD1d (D5) was incubated with IECs prior to being co-cultured with T cells. Excess antibody was removed by washing three times with RPMI 1640. After varying incubation periods at 37 °C, the T cells were lysed, immunoprecipitated with rabbit anti-human p56<sup>lck</sup>, resolved on SDS-PAGE, and subjected to an anti-phosphotyrosine Western blot. As seen in Fig. 4A, the D5 monoclonal antibody was incapable of inhibiting the activation of p56<sup>lck</sup>. To determine the impact that this same antibody has on the activation of the TCR-associated kinase p59<sup>fyn</sup>, the same T cell lysates from IEC-T cell co-cultures treated with D5 were immunoprecipitated with the rabbit anti-human p59<sup>fyn</sup> antibody covalently bound to Sepharose beads (Santa Cruz). As can be seen in Fig. 4B, the anti-CD1d monoclonal antibody D5 was capable of inhibiting the activation of the TCR-associated protein tyrosine kinase p59<sup>fyn</sup> in this co-culture system. These results suggested that CD1d was capable of interacting with the TCR and activating the TCR-associated protein tyrosine kinase p59<sup>fyn</sup>.
Fig. 3. A, CD1d does not activate CD8-associated protein tyrosine kinase p56<sup>fyn</sup>. Either the cell line transfected with CD1d cDNA, FO-1 D5, or the untransfected control FO-1 was cultured with peripheral blood T cells for 0, 2, or 5 min. The cells were then lysed and were immunoprecipitated with an anti-p56<sup>fyn</sup> antibody (Santa Cruz), and an anti-pan-T lymphocyte Western blot was performed. Lane 1, negative control of T cells alone. Lane 2, control of T cells stimulated with anti-CD3 (OKT3) mAb and cross-linked with RAM for 2 min. Lane 3, positive control of T cells stimulated with anti-CD8 (OKT8) mAb and cross-linked with RAM for 2 min. Lanes 4–6, FO-1 cells co-cultured with PBTs for 0, 2, and 5 min. Lanes 7–9, FO-1 cells treated with anti-CD1d mAb D5 for 30 min prior to being co-cultured with PBTs for 0, 2, and 5 min. Lanes 10–12, FO-1 D5 cells co-cultured with PBTs for 0, 2, and 5 min. Lanes 13–15, FO-1 D5 cells treated with anti-CD1d mAb D5 for 30 min prior to being co-cultured with PBTs for 0, 2, and 5 min. Inset, the identification of Lck protein in these lysates serves as a control for equal loading of protein given the absence of bands. Compared with the positive control, in which T cells were incubated with anti-CD8 antibodies, CD1d alone does not appear to activate the CD8-associated protein tyrosine kinase p56<sup>fyn</sup>. This experiment was repeated three times. B, CD1d appears to activate the TCR-associated protein tyrosine kinase p56<sup>lck</sup>. Lysates from FO-1 D5 or FO-1-PBT co-cultures (treated or untreated with the anti-CD1d mAb) were immunoprecipitated with an anti-p56<sup>lck</sup> antibody (Santa Cruz), and an anti-pan-T lymphocyte Western blot was performed. Lane 1, negative control of T cells alone. Lane 2, control of T cells stimulated with anti-CD8 (OKT8) mAb and cross-linked with RAM for 2 min. Lane 3, positive control of T cells stimulated with anti-CD8 mAb and cross-linked with RAM for 2 min. Lanes 4–7, FO-1 cells co-cultured with PBTs in the presence of an irrelevant isotype control mAb. Lane 4, control of T cells stimulated with anti-CD3 mAb and cross-linked with RAM for 2 min. Lanes 5–7, FO-1 cells stimulated with anti-CD1d mAb D5 for 30 min prior to being co-cultured with PBTs for 0, 2, and 5 min. Lanes 8–11, FO-1 cells preincubated with anti-CD1d antibody (D5) and then co-cultured with PBTs for 0, 1, 2, or 5 min. This figure is representative of two experiments. B, antibodies against CD1d appear to inhibit the activation of TCR-associated protein tyrosine kinase p56<sup>lck</sup>. Lysates from IEC-PBT co-cultures (treated or untreated with the anti-CD1d D5 mAb) were immunoprecipitated with anti-p56<sup>lck</sup> antibody, and an anti-pan-T lymphocyte Western blot was performed. Lane 1, negative control of T cells alone. Lane 2, negative control of T cells stimulated with anti-CD3 mAb and cross-linked with RAM for 2 min. Lane 3, positive control of T cells stimulated with anti-CD3 mAb and cross-linked with RAM for 2 min. Lanes 4–7, IECs co-cultured with PBTs in the presence of the isotype control for 0, 1, 2, and 5 min. Lanes 8–11, IECs preincubated with anti-CD1d antibody (D5) and then co-cultured with PBTs for 0, 1, 2, or 5 min. This figure is representative of four experiments.

T-Cell Receptor-Associated p56<sup>lck</sup> Kinase but was incapable, directly or indirectly, of activating p56<sup>fyn</sup>. It therefore appeared that CD1d was capable of interacting with the T cell receptor and activating p56<sup>lck</sup> in both in vitro (CD1d transfectants) and in vivo (freshly isolated intestinal epithelial cells) systems. Because the anti-CD1d mAb D5 appeared to inhibit the ability of intestinal epithelial cells to phosphorylate p56<sup>lck</sup> in T cells, we next asked whether this observation could be confirmed using another anti-CD1d monoclonal antibody, 51.1.3. Intestinal epithelial cells were incubated with either 51.1.3 or D5 mAb (10 μg/ml) for 30 min on ice and then washed thoroughly. The intestinal epithelial cells were co-cultured with PBTs for 0, 2, or 5 min at 37 °C. The T cells were lysed and immunoprecipitated with a rabbit anti-p56<sup>fyn</sup> antibody, and the protein was resolved by SDS-PAGE, transferred to nitrocellulose paper, and subjected to an anti-pan-T lymphocyte Western blot. As seen in Fig. 5, the relative intensities of the bands resolved at 59 kDa indicated that whereas the D5 mAb almost completely

Fig. 4. A, anti-CD1d antibodies do not appear to be capable of inhibiting IEC-induced activation of p56<sup>fyn</sup>. Lysates from IEC-PBT co-cultures (treated with the anti-CD1d D5 mAb or untreated) were immunoprecipitated with an anti-p56<sup>fyn</sup> antibody (Santa Cruz), and an anti-pan-T lymphocyte Western blot was performed. Lane 1, negative control of T cells alone. Lane 2, negative control of T cells stimulated with anti-CD3 mAb and cross-linked with RAM for 2 min. Lane 3, positive control of T cells stimulated with anti-CD8 mAb and cross-linked with RAM for 2 min. Lanes 4–7, IECs co-cultured with PBTs in the presence of the isotype control for 0, 1, 2, and 5 min. Lanes 8–11, IECs preincubated with anti-CD1d antibody (D5) and then co-cultured with PBTs for 0, 1, 2, or 5 min. This figure is representative of four experiments. B, antibodies against CD1d appear to inhibit the activation of TCR-associated protein tyrosine kinase p56<sup>lck</sup>. Lysates from IEC-PBT co-cultures (treated or untreated with the anti-CD1d D5 mAb) were immunoprecipitated with anti-p56<sup>lck</sup> antibody, and an anti-pan-T lymphocyte Western blot was performed. Lane 1, negative control of T cells alone. Lane 2, negative control of T cells stimulated with anti-CD3 mAb and cross-linked with RAM for 2 min. Lane 3, positive control of T cells stimulated with anti-CD3 mAb and cross-linked with RAM for 2 min. Lanes 4–7, IECs co-cultured with PBTs in the presence of the isotype control for 0, 1, 2, or 5 min. Lanes 8–11, IECs preincubated with anti-CD1d antibody (D5) and then co-cultured with PBTs for 0, 1, 2, or 5 min. This figure is representative of four experiments.
inhibited the ability of intestinal epithelial cells to activate the TCR-associated p59\textsuperscript{kinase} in CD8\textsuperscript{+} suppressor T cells. Lysates from intestinal epithelial cell-T cell co-cultures treated with isotype control, anti-CD1d D5 mAb, or anti-CD1d 51.1.3 mAb were immunoprecipitated with an anti-p59\textsuperscript{kinase} antibody, resolved by SDS-PAGE, and subjected to an anti-phosphotyrosine Western blot. Lane 1, negative control of T cells alone. Lane 2, positive control of T cells stimulated with anti-CD3 mAb and cross-linked with RAM for 2 min. Lane 3, negative control of T cells stimulated with anti-CD8 mAb and cross-linked with RAM for 2 min. Lanes 4–6, IECs preincubated with an irrelevant isotype control were co-cultured with PBTs for 0, 2, and 5 min. Lanes 7–9, IECs preincubated with anti-CD1d antibody (D5) and then co-cultured with PBTs for 0, 2, and 5 min. Lanes 10–12, IECs preincubated with anti-CD1d antibody (51.1.3) and co-cultured with PBTs for 0, 2, and 5 min. The relative intensity depicted represents the percent of density of phosphorylated p59\textsuperscript{kinase} under antibody-treated conditions compared with the isotype control lanes.

**FIG. 5.** Anti-CD1d antibody 51.1.3 partially inhibits the ability of intestinal epithelial cells to activate TCR-associated p59\textsuperscript{kinase} in CD8\textsuperscript{+} suppressor T cells. Lysates from peripheral blood T cells treated with FO-1 cells (control transfection cells), FO-1 D5 cells (FO-1 cells transfected with human CD1d cDNA), purified gp180, or gp180-pulsed FO-1 or FO-1 D5 cells were immunoprecipitated with an anti-p56\textsuperscript{kinase} antibody, resolved by SDS-PAGE, and subjected to an anti-phosphotyrosine Western blot. Lane 1, negative control of T cells alone. Lane 2, negative control of T cells stimulated with anti-CD3 monoclonal antibody and cross-linked with RAM for 2 min. Lane 3, positive control of T cells stimulated with anti-CD8 mAb and cross-linked with RAM for 2 min. Lanes 4 and 5, PB Ts cultured with untransfected FO-1 cell for 0 or 2 min. Lanes 6 and 7, PB Ts were co-cultured with human CD1d cDNA transfected cells (FO-1 D5) for 0 or 2 min. Lanes 8 and 9, PB Ts cultured with purified gp180 for 0 or 2 min. Lane 10, FO-1 D5 cells were preincubated with purified gp180 prior to being co-cultured with PB Ts for 2 min. Lane 11, FO-1 D5 cells were preincubated with purified gp180 and washed four times prior to being co-cultured with PB Ts for 2 min. Lane 12, FO-1 cells were preincubated with purified gp180 prior to being co-cultured with PB Ts for 2 min. Lane 13, FO-1 cells were preincubated with purified gp180 and washed four times prior to being co-cultured with PB Ts for 2 min. This figure is representative of two separate experiments.

**FIG. 6.** gp180 is able to bind extracellularly to CD1d. Lysates from peripheral blood T cells incubated with CD1d (FO-1 D5) for 0 or 2 min. Lane 1, negative control of T cells alone. Lane 2, positive control of T cells stimulated with anti-CD3 monoclonal antibody and cross-linked with RAM for 2 min. Lanes 3, 5, and 7, PB Ts cultured with untransfected FO-1 cell for 0 or 2 min. Lanes 4 and 6, PB Ts cultured with purified gp180 and washed four times prior to being co-cultured with PB Ts for 2 min. Lanes 8–13, PB Ts were co-cultured with human CD1d cDNA transfected cells (FO-1 D5) for 0 or 2 min. Lanes 8 and 9, PB Ts cultured with purified gp180 for 0 or 2 min. Lane 10, FO-1 D5 cells were preincubated with purified gp180 prior to being co-cultured with PB Ts for 2 min. Lane 11, FO-1 D5 cells were preincubated with purified gp180 and washed four times prior to being co-cultured with PB Ts for 2 min. Lane 12, FO-1 cells were preincubated with purified gp180 prior to being co-cultured with PB Ts for 2 min. Lane 13, FO-1 cells were preincubated with purified gp180 and washed four times prior to being co-cultured with PB Ts for 2 min. This figure is representative of two separate experiments.

**DISCUSSION**

Although the role of classical restriction elements has been relatively clearly defined, the function of the structurally related class I b proteins has not yet been thoroughly investigated. Recent studies from several groups suggest that class I b molecules may be involved in stress responses or in T cell responses against nonprotein antigens (34, 35). These molecules include the CD1 family of proteins. The CD1 family of nonclassical class I-like proteins are encoded outside the MHC, and most members have limited homology to classical MHC class I restriction elements (21, 22, 30, 34, 36). CD1d is a nonpolymorphic isoform of the CD1 family that is prominently expressed by intestinal epithelial cells. The mouse CD1 homologue has been shown to be capable of binding peptides generally larger that those bound by classical restriction elements (36); however, the recent X-ray crystallographic structure of CD1d (37) suggests that it more likely binds lipids or glycolipids, similar to CD1b and CD1c. In addition, CD1d may be endosomally localized, like CD1b (38), and hence peptide and/or lipid (37) association with CD1d could conceivably occur within classical class II loading compartments (exogenous antigen pathway) or on the cell surface. The immunoregulatory function of CD1d has not been clearly defined, but its presence on the surface of intestinal epithelial cells lends support to the notion that it may serve as a regulator of mucosal immune responses to antigens of undefined origin (39). Indeed, previous studies have shown that intraepithelial lymphocyte killing of intestinal epithelial cell lines can be CD1d-restricted, and PBT cell proliferation induced by intestinal epithelial cells is inhibited by anti-CD1d antibodies (26, 27).

Mucosal surfaces, such as those found in the gastrointestinal tract, are continuously exposed to antigen; however, immune responses are not mounted to most dietary and microbial antigens. This state of systemic nonresponsiveness, which can also be induced by feeding antigen to a host (oral tolerance), is a unique feature of the mucosal immune system. Although the mechanisms involved in the induction of mucosal tolerance may be multifaceted, several groups have reported that CD8\textsuperscript{+} T cells play a role in transferring tolerance to naive animals. Previous studies have demonstrated that antigen presentation by intestinal epithelial cells results in the activation of CD8\textsuperscript{+} CD28\textsuperscript{-} T cells, which appear to have suppressor activity (17). This suggests that the intestinal epithelial cell may play a role in the activation of a subset of T cells, which may be involved in the suppression of local immunity and, potentially, systemic immune responses as well.

The activation of these CD8\textsuperscript{+} T cells by intestinal epithelial
cells appears to involve the CD8 molecule. Monoclonal antibodies against CD8 but not CD4 inhibit IEC-induced proliferation of T cells in response to IECs. Interestingly, antibodies recognizing conventional restriction elements do not inhibit IEC-induced activation and proliferation of CD8+ T cells (20). Therefore, other molecular interactions were analyzed.

Two monoclonal antibodies, B9 and L12, identify a novel 180-kDa intestinal epithelial cell glycoprotein, gp180, which is capable of interacting with the CD8 molecule. We have shown that gp180 binds to CD8 molecules and activates the CD8α chain associated protein tyrosine kinase p56lck (29). When the anti-gp180 monoclonal antibody B9 is added to IEC-T cell cocultures, no intestinal epithelial cell induced activation or proliferation of CD8+ T cells is observed. This supports the fact that gp180 is critical to the activation of these CD8+ T cells.

Data generated in this study clearly indicate that gp180 does not act alone in the activation and proliferation of CD8+ T cells in response to IECs. Because it has been previously determined that anti-CD1d antibodies inhibit the proliferation of CD8+ T cells by intestinal epithelial cells (27), we examined the role that CD1d plays in the activation of these T cells. It was clearly demonstrated that CD1d associates with the glycoprotein gp180 in the absence of β2-microglobulin (31). More specifically, it was shown that CD1d is not responsible for the activation of the CD8-associated kinase p56lck but is responsible for the activation of the TCR-associated kinase p59fyn. This is consistent with previous studies that failed to show binding of CD1d to CD8 molecules.

The key question relates to the nature of the antigen presented by the nonpolymorphic molecule, CD1d, in our alloge neic co-culture system. Several possibilities exist. First, alloreactive T cells may recognize processed class I or class II fragments in the antigen binding cleft of CD1d. Alternatively, proteins found in serum (both human serum and fetal calf serum used in cell culture) may be taken up by the nonpolymorphic restriction element, CD1d, expressed on IECs and presented to T cells. Thus, CD1d on cell lines and freshly isolated intestinal epithelial cells could be constantly loaded with peptides (or lipids/glycolipids) derived from serum. Finally, it may be that CD1d has limited polymorphisms not previously appreciated. Zeng et al. (37) suggested that CD1 is incapable of binding peptide due to its deep hydrophobic pocket; however, Castano et al. (36), using a peptide display library, showed that CD1 was capable of associating with peptides larger than those bound by classical II Molecules.

The response to CD1d-presented peptide (or nonpeptide) may be limited, but it is present. The other issue relates to the cell population activated. Clearly, there are CD1d-restricted double negative T cells in the peripheral blood and presumably within the mucosa. The subpopulations of CD8+ T cells expanded in our co-culture system may exist as “presuppressor” cells. If such regulatory cells arise from a distinct subpopulation, it could well explain the difficulty in clearly identifying suppressor T cells in the past.

In our postulated model, gp180 associates with the nonclassical class I molecule CD1d on the surface of intestinal epithelial cells. More specifically, we postulate that each component of this complex has a unique function: gp180 binds to the CD8 molecule, resulting in the activation of the CD8-associated kinase p56lck, whereas CD1d interacts with the TCR, causing the phosphorylation of the TCR-associated kinase p59fyn. The gp180-CD1d complex thus appears more like classical class I, capable of interacting with the TCR-CD8 co-receptor complex. This postulated model may account for several features observed in the gastrointestinal tract. The existence of this complex could explain the presentation of exogenous antigen to CD8+ T cells by IECs (if CD1d exists in an endosomal compartment, as does CD1b), the presence of predominantly CD8+ T cells in the IEC compartment, and the poor activation of mucosal lymphocytes by conventional antigen-presenting cells. The necessity for the activation of suppressor T cells in the gut is clear. Any inability to do so is costly, resulting in inflammation and the loss of functional integrity in the gastrointestinal tract. The use of distinct molecules and restriction elements provides further evidence for the differences between systemic and mucosal immunity. This dichotomy may evolve from the differences in antigen load and the requirement for controlled rather than active immune responses in the gastrointestinal tract.

REFERENCES