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DR/CLIP (Class II-Associated Invariant Chain Peptides) and DR/Peptide Complexes Colocalize in Prelysosomes in Human B Lymphoblastoid Cells

Espen Stang,* Carolyn B. Guerra,† Miguel Amaya,‡ Yvonne Paterson,‡ Oddmund Bakke,2,3,* and Elizabeth D. Mellins2§

In APCs, MHC class II molecules (MHC class II) bind antigenic peptides after HLA-DM mediated removal of CLIP. To characterize intracellular sites of peptide loading in human B lymphoblastoid cell lines, we conducted immunoelectron microscopy studies with Abs recognizing MHC class II associated with CLIP or bound peptide, respectively, together with Abs to HLA-DM and endocytic markers. The distribution of these molecules indicates that peptide binding occurs in compartments with characteristics of normal late endosomes, and in compartments that show characteristics of late endosomes, but are not detectably accessed by endocytosed BSA-gold. The latter compartments may represent or give rise to recycling vesicles that deliver peptide-loaded class II molecules to the cell surface. In addition, we have compared cells in which HLA-DM and HLA-DR interaction is defective with cells in which this interaction is intact, and find that DM/DR interaction is not required for the proper localization of either molecule to peptide-loading compartments. The Journal of Immunology, 1998, 160: 4696–4707.

Major histocompatibility complex class II molecules are heterodimeric (α/β) transmembrane glycoproteins that fold to form a peptide-binding groove at their membrane distal (NH2-terminal) end. In APCs, MHC class II molecules bind peptides derived from endocytosed Ags and display them at the cell surface for inspection by CD4+ T cells. To bind endosomally derived peptides, class II molecules must localize to the endocytic pathway in a form that is competent to bind peptides there. To accomplish this, nascent class II molecules associate with invariant chain (Ii)4 trimers in the endoplasmic reticulum (ER). Ii serves a dual function: its association with MHC class II prevents binding of ligands in the ER (1), and two dileucine-based sorting motifs within its cytoplasmic tail direct the MHC class II-β complex to endocytic compartments (2–4). In endocytic compartments, Ii is degraded to a nested set of peptides (class II-associated invariant chain peptides, CLIP) that occupy the peptide-binding groove (5, 6). For many class II alleles, efficient exchange of CLIP for antigenic peptide requires HLA-DM, an MHC-encoded heterodimer that is targeted to endolysosomal compartments by a signal in its own β-chain cytosolic tail (7, 8). In vitro, HLA-DM acts in a catalytic manner to accelerate release of CLIP (9–12) and other peptides lacking optimal anchor residues (13, 14). Thus, in vivo HLA-DM is likely to edit the repertoire of class II/peptide complexes in favor of those that will be long-lived at the cell surface.

The intracellular site(s) in which MHC class II molecules are loaded with peptide cargo has been investigated in various APC types using several approaches. Whether peptide loading of nascent class II molecules occurs within a particular subset of endocytic compartments and whether peptide-loading compartments are specialized for this function in APCs remain matters of some debate (15, 16). In human B-LCLs and Langerhans cells, and in murine dendritic cells, intracellular compartments termed MIIC (MHC class II compartments) contain the bulk of intracellular MHC class II molecules (17–20) as well as HLA-DM molecules (21) and have been suggested to represent specialized compartments for Ag presentation. MIIC are positioned late in the endocytic pathway and contain lysosomal markers, such as lysosomal glycoproteins (LAMP-1, CD63) and enzymes such as cathepsin D and β-hexosaminidase, but not the mannose 6-phosphate receptor (MPR), generally located in late endosomes. MIIC are large (200–400 nm diameter) and resemble classical multivesicular bodies (mvb), with abundant internal membranes arranged in small vesicles, or are multilaminar with membranes forming concentric rings. In murine B cells, compartments with morphologic similarities to MIIC have been observed (22, 23). However, these lack lysosomal markers, and express small amounts of MPR and transferrin receptors. Such compartments have been named CIIV, for class II vesicles, in A20 B cells (22) and lysozyme-loading compartments in 2A4 B lymphoma cells (23). By physical characteristics, lysozyme-loading compartments and CIIV are distinct from conventional endosomes and lysosomes, and it has been speculated that they represent postendosomal recycling vesicles involved in transport of Ag-loaded MHC class II to the plasma membrane. There is only limited information concerning the route and regulation of class II/peptide transport from endosomal loading compartments to the cell surface, but

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2 These authors made equal contributions to this work.

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4 Abbreviations used in this paper: Ii, invariant chain; B-LCL, B lymphoblastoid cell line; BCS, bovine calf serum; CI-MPR, cation-independent mannose 6-phosphate receptor; CIIV, class II-containing vesicles; CLIP, class II-associated invariant chain peptide; CPL, compartments for peptide loading; ER, endoplasmic reticulum; GAM, goat anti-mouse-coated gold; GAR, goat anti-rabbit-coated gold; MIIC, major histocompatibility complex class II compartment; MPR, mannose 6-phosphate receptor; mvb, multivesicular bodies; PAG, protein A-coated gold; PVDF, polyvinylidene difluoride.

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recent data suggest that direct fusion of MHC-II-containing vesicles with the plasma membrane may be one route (24, 25).

Compartment for peptide loading (CPL) might be expected to contain both MHC class II with CLIP and MHC class II with bound peptide, as well as HLA-DM, and endocytosed material (at least in degraded form). On the basis of these criteria, we have looked for possible CPL in human B-LCL. We utilized cells with intact Ag-presenting function, as well as mutant derivatives that accumulate CLIP-loaded DR molecules. Using subcellular fractionation, we confirmed that these cells, like other B-LCL, contain an intracellular pool of HLA-DR molecules in organelle(s) that localizes in dense fractions. Immunocytochemical labeling of the cells with Abs that preferentially recognize HLA-DR with bound CLIP or HLA-DR with bound peptide allowed us to identify probable CPL. Uptake of BSA-gold and detection of endosomal/lysosomal marker proteins were used to further characterize these CPL relative to the endocytic pathway. In addition, by examining cells that lack HLA-DM or express DR molecules that are unable to interact with HLA-DM, we have also gained insight into the role of HLA-DM in the generation of and access to CPL.

Materials and Methods

Cell lines and transfectants

8.1.6 is a DR/DQ/DMB hemizygous B-LCL that expresses HLA class II specificities DR3, DRw52a, DQ2, and two copies of DP4.1 (26). Mutants 9.2, 9.3, 9.5, and 10.24.6 were derived in independent experiments by mutagenesis of 8.1.6 cells with ethylmethane sulfonate and immunoselection with mAb Ab 16.23 (27, 28). Mutant 9.2 is homozgyously deleted for the DRA gene and lacks expression of DR molecules, but retains expression of DP and DQ molecules. Mutant 9.5 lacks expression of HLA-DM mRNA (29); mutant 10.24.6 has a proline to serine substitution at residue 96 of DRβ1 that results in aberrant glycosylation of residue Asn-98 (30). The clonal cell line, 9.5.3-DMB, was derived by limiting dilution cloning of a population containing low levels of HLA-DM. This clone expresses lower levels of HLA-DM than the parental 9.5.3-DMB, but retains the ability to bind peptide, as well as HLA-DM, and endocytosed material (31). To identify other proteins, aliquots of fractions (either unboiled or boiled for 10 min in 10% SDS sample buffer with 2.5% β-mercaptoethanol) were spun for 10 min at 10,000 × g to remove Percoll. Samples were analyzed by SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membrane (Millipore, Bedford, MA). Membranes were incubated overnight at 4°C in blocking solution (5% casein, 0.05% Tween-20, 0.05% SDS, 25 mM Tris-HCl, pH 7.7, and 100 μM Na3C03). Proteins were detected by specific Abs and enhanced chemiluminescence (ECL), using a peroxidase-conjugated goat anti-mouse IgG (Jackson Immunoresearch, West Grove, PA), and visualized using a Fuji Film LAS 4000 (Rayon, Tokyo, Japan).

Pulse/chase immunoprecipitation

For immunoprecipitations, cells at 106 cells/ml were starved of methionine for 45 min in labeling medium (methionine-free RPMI 1640 medium supplemented with 2 mM l-glutamine, 10 mM HEPES, 0.1% dialyzed FBS, and antibiotics). The cells were then pulse labeled with 100 μCi/ml 131I-methionine (DuPontNEN, Wilmington, DE) in the presence of 10 μM cycloheximide, and chased for 20 min with 10 mM Tris-HCl, 150 mM NaCl, and 1% Triton X-100, pH 8. Pulse labeling was followed by a chase of 20 min with either 10 mM Tris-HCl, 150 mM NaCl, or 1% Triton X-100, pH 8. The bound immune complexes were eluted by boiling for 5 min in Laemmli sample buffer. Samples were analyzed by SDS-PAGE on 10% gels and transferred to nitrocellulose filters. Membrane-bound HLA-DR complexes with DRβ1 were determined by using specific Abs and enhanced chemiluminescence (ECL), as previously described (32). Immunoprecipitation was performed per manufacturer's instructions (DuPont NEN, Wilmington, DE). Briefly, membranes were incubated in primary Ab diluted in blocking solution for 1 h and then washed four times in PBS with 0.1% Tween-20. Membranes were treated with unlabeled goat anti-mouse IgG horseradish peroxidase conjugate (Life Technologies) or donkey anti-rabbit IgG horseradish peroxidase conjugate (Amersham, Arlington Heights, IL), as appropriate. After six washes, membranes were sealed in plastic bags with 500 μl of ECL substrate (DuPont NEN) and film was exposed.
against a 4-log-unit axis of fluorescence intensity. Background fluorescence was evaluated using medium and FITC-GAM alone.

Electron microscopy

To identify endocytic compartments, the cells were incubated with 5 nm BSA-coated colloidal gold in medium (RPML supplemented with 10% FCS) for 3 h, followed by an overnight chase in goldfree medium before a final incubation with 10 nm BSA-coated colloidal gold for 1 h. Colloidal gold of different sizes was prepared according to Slot and Geuze (45) and coated with BSA. Cells were fixed with 0.1% glutaraldehyde and 4% paraformaldehyde in Soerenson phosphate buffer, infused with polyvinylpyrrolidone (PVP)-sucrose (46), frozen, and stored in liquid nitrogen. Immunocytochemical labeling was performed on thawed cryosections, as described (47, 48), using the different primary Abs followed by either 15 or 20 nm gold coated with goat anti-rabbit IgG (GAR 15) or goat anti-mouse IgG (GAM 15 and GAM 20) (BioCell, Cardiff, U.K.). The intracellular distribution of labeled proteins was quantitated in randomly chosen cells from at least three different experiments for each single- or double-labeling experiment by counting the total number of both labeled and unlabeled endocytic compartments directly in the electron microscope at a magnification of \( \times 15,000 \) to \( \times 20,000 \). For each experiment, a minimum of 100 endocytic compartments from at least 20 different cells was analyzed. Under the conditions used for labeling, background staining of mitochondria and/or nuclei was limited to none or very few gold particles.

Results

In normal APCs (8.1.6), HLA-DR/peptide complexes localize in late endosomal compartments

As model APCs, we studied 8.1.6 cells, a DR/DQ hemizygous B-LCL that effectively presents endocytosed protein Ags to human CD4+ T cells (49) (see Table I for a summary of cell lines in this study). Our initial approach for detecting CPL in 8.1.6 was subcellular fractionation of cells, using 17% Percoll gradients. These gradients allowed a clear separation of plasma membrane, ER/Golgi, early endosomes, and the majority of late endosomes, which are in the light fractions, from a subset of late endosomes (indicated by \( \text{rab 7} \) expression) and the lysosomal compartments, which are in the dense fractions (Fig. 1A). MHC class I molecules were detected exclusively in the light fractions of the gradient. In contrast, HLA-DR molecules were found both in the light fractions, where they colocalized with class I molecules, and in the dense fractions, where they colocalized with mature HLA-DM molecules (Fig. 1B). This result suggested that, like other B-LCL (50), 8.1.6 cells contained relatively dense intracellular compartments, which were candidate CPL, based on their content of HLA-DR and HLA-DM molecules.

To identify organelles that represented CPL in 8.1.6 cells, we next used immunocytochemical labeling to look for intracellular compartments containing DR/peptide complexes. We detected the complexes with the anti-HLA-DR3 Ab, 16.23 (38). This Ab binds well to mature, cell surface DR3 molecules in 8.1.6 cells, but at low levels to surface DR3 in 8.1.6-derived DM mutants (e.g., 9.5.3), whose DR3 molecules are associated primarily with CLIP peptides (Fig. 2A). The 16.23 determinant is expressed by nascent DR3 molecules several hours after synthesis, subsequent to invariant chain degradation and CLIP release (Fig. 2, B and C). In immunoblots of cell lysates, 16.23 detects DR3 dimers that are SDS stable (but not shown, but see below), a characteristic of peptide-associated molecules. Thus, by several assays, the epitope recognized by 16.23 correlates well with normal peptide loading. By immunoelectron microscopy, 16.23 labeling was detected in 8.1.6 cells, but not in 9.5.3 cells (Table II). In 8.1.6 cells, the labeling was observed at the plasma membrane, most prominently at cytoplasmic extensions located at each pole of the cell (not shown). Small cytoplasmic vesicles were also labeled by 16.23 (Fig. 3A). Most strikingly, we observed a substantial amount of 16.23 labeling in intracellular vesicular compartments with the internal membrane characteristics of multivesicular bodies (Fig. 3, A and B). To confirm that the intracellular DR molecules labeled by Ab 16.23 included peptide-associated DR, we immunoblotted selected subcellular fractions from 8.1.6 cells (and 9.5.3 cells as controls) with 16.23 (Fig. 2D). Plasma-membrane rich light fractions from 8.1.6 contained molecules that were 16.23+ and SDS stable, as expected for surface-expressed, peptide-loaded DR; in light fractions from 9.5.3 (whose DR3/CLIP complexes are not stable in SDS (27)), 16.23 detected only monomeric β-chains. In 8.1.6, intracellular DR dimers found in dense fractions were also 16.23+ and SDS stable, implying that peptide-loaded DR molecules were being detected by 16.23 staining inside the cells.

As the 16.23+ compartments thus appeared to include peptide-loading compartments, we were interested to determine their position in the endocytic pathway. To this end, we first classified these compartments in the progenitor cell, 8.1.6 by content of endocytosed, BSA-coated colloidal gold and by immunocytochemical labeling with Abs to the late endosomal/lysosomal marker proteins CI-MPR and CD63. The late compartments (late endosomes, lysosomes) were labeled by BSA-gold taken up during a 3-h incubation and chased overnight. Immunocytochemical labeling with Abs to CI-MPR revealed that the overnight BSA-gold particles were found both in CI-MPR positive and negative compartments, i.e., late endosomes/prelysosomes and lysosomes, respectively (Table II). This was observed in both 8.1.6 and its derivatives. Early compartments were labeled by continuous uptake for 1 h, the time required in these cells for a small degree (<3%) of colocalization with overnight BSA-gold. A fraction of these compartments appeared to represent late endosomes/prelysosomes based on their expression of both CI-MPR and CD63.

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Table I. Cell lines used in this study

<table>
<thead>
<tr>
<th>Name</th>
<th>Genotype</th>
<th>Phenotype</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>8.1.6 (progenitor)</td>
<td>DR/DQ/DMB hemizygous</td>
<td>Normal APC; expresses DR3, DRw52, DQ2, DP4</td>
<td>26</td>
</tr>
<tr>
<td>9.5.3</td>
<td>DMB point mutation (120W→stop)</td>
<td>↓ ↓ ↓ DMB mRNA; no functional DM dimer; accumulates class II/CLIP complexes</td>
<td>27–29</td>
</tr>
<tr>
<td>10.24.6</td>
<td>DRA point mutation (96F→S)</td>
<td>Aberrant glycosylation of DRs; accumulates DR/CLIP complexes</td>
<td>28</td>
</tr>
<tr>
<td>9.5.3-DMB</td>
<td>DMB (wt-cDNA) transfected</td>
<td>Clone with ↓ DMB mRNA, less DM dimer than 8.1.6; partial restoration of Ag presentation defect</td>
<td>29</td>
</tr>
<tr>
<td>10.24.6-DRA</td>
<td>DRA (wt-cDNA) transfected</td>
<td>Expression of both wt and mutant DRs; partial restoration of DR-restricted Ag presentation</td>
<td>28</td>
</tr>
<tr>
<td>9.22.3</td>
<td>DRA homozygous deletion mutant</td>
<td>DR-null cell; normal Ag presentation to DQ-, DP-restricted T cells</td>
<td>27</td>
</tr>
</tbody>
</table>

*Cell lines 9.5.3, 10.24.6, and 9.22.3 were each derived from progenitor 8.1.6; see Materials and Methods for details.
II). Most compartments containing endocytosed gold had the morphology of mvb; however, some were filled with membranes in sheets, narrow tubules, or concentric rings, forming the multilaminar ultrastructure described for MIIC (17, 51). The intracellular DR/peptide (16.23) labeling in 8.1.6 cells mainly localized to endosomes containing overnight BSA-gold (Tables II and III). A semiquantitative analysis showed that 19% of the overnight gold-containing compartments labeled with mAb 16.23, whereas only 7% of the early compartments were 16.23 positive in these cells (Table II). The labeling of only a subset of overnight gold compartments by the 16.23 Ab may reflect the fact that 16.23 does not label all DR/peptide complexes, or that DR/peptide complexes are formed in a subset of compartments. In either case, the intracellular compartments in which DR/peptide complexes are detected are for the most part positioned late in the endocytic pathway.

Unexpectedly, the immunocytochemical studies of 8.1.6 also revealed that ~33% of 16.23<sup>+</sup> endosomal-like compartments contain no visible endocytosed BSA-gold (hereafter referred to as empty late endosomes) (Table III and Fig. 3). Morphologically, these compartments resemble endosomes containing overnight gold, having the same average diameter and internal vesicles or membrane structures arranged in sheets or narrow tubules. To insure that these empty late endosomes were not simply missed in the original labeling scheme, we conducted a prolonged incubation with BSA-gold. Despite continuous endocytosis of BSA-gold for 24 h, the empty compartments still represented 22% of 16.23<sup>+</sup> compartments (data not shown). The presence of the empty compartments may be a sampling/sectioning artifact: some vesicles are almost totally filled with gold, while others show a very spotty distribution. However, it is also possible that these compartments represent a functionally distinguishable site, defined by its accessibility to degraded, but not undegraded, endocytosed material.

**HLA-DR/peptide complexes and HLA-DM colocalize**

The efficient formation of DR/peptide complexes in 8.1.6 cells depends on expression of HLA-DM (29), which should thus be...
FIGURE 2. A. Flow-cytometric analysis of surface expression of total HLA-DR, DR/peptide complexes and DR/CLIP complexes on B lymphoblastoid cells. Progenitor cells (8.1.6), DM-null mutant (9.5.3.), DMB transfectant (9.5.3-DMB), DRA mutant (10.24.6), DRA transfectant (10.24.6-DRA), and DR-null mutant (9.22.3) were stained by indirect immunofluorescence using mAbs to total DR (L243), DR3-peptide (16.23), or DR/CLIP (I-5) and...
found in CPL. Therefore, we next localized HLA-DM in 8.1.6 cells by labeling with an anti-DMA antiserum (21). HLA-DM was not detected in significant amounts at the plasma membrane. The labeling was distributed in intracellular compartments similar to HLA-DR/peptide complexes (Tables II and III), although DM was found more often than DR/peptide (16.23) in compartments containing BSA-gold (Table II). This difference in DR/peptide vs DM labeling may reflect a difference in sensitivity of the Abs, more limited distribution of DR compared with DM molecules, or restricted expression of the 16.23 epitope on only a subset of peptide-loaded DR dimers.

To verify that intracellular HLA-DR/peptide complexes and HLA-DM molecules colocalize, we conducted double-labeling experiments (Fig. 3A). The results were quantified and are presented in Tables IV and V. As both the endosomal distribution of the double-positive compartments and the amount of colocalization between the different proteins were of interest, each was calculated. Table IV shows the distribution of the double-positive compartments among the different endosomes. Only double-positive compartments were counted, and the counted compartments were classified on basis of their content of endocytosed BSA-gold. The majority of the DR/peptide-HLA-DM double-positive structures are compartments late in the endocytic pathway; only 11% are endosomes with 1-h BSA-gold. Table V shows the amount of colocalization between the two proteins in these late compartments. The total number of each type of compartment positive for 16.23, as well as the number of double-positive compartments were counted, and the counted compartments were classified on basis of their content of endocytosed BSA-gold. The total number of each type of compartment positive for 16.23, as well as the number of double-positive compartments were counted, and the counted compartments were classified on basis of their content of endocytosed BSA-gold. Table V shows the amount of colocalization between the two proteins in these late compartments. The total number of each type of compartment positive for 16.23, as well as the number of double-positive compartments were counted, and the counted compartments were classified on basis of their content of endocytosed BSA-gold. The percentage of each type of endosome labeled with each Ab was then calculated.

Table II. Percentage of BSA-gold containing endosomes showing labeling with various Abs

<table>
<thead>
<tr>
<th>Ab</th>
<th>Progenitor 8.1.6 1 h</th>
<th>Progenitor 8.1.6 24 h</th>
<th>DM-Null 9.5.3 1 h</th>
<th>DM-Null 9.5.3 24 h</th>
<th>DMB Transfectant 9.5.3-DMB 1 h</th>
<th>DMB Transfectant 9.5.3-DMB 24 h</th>
<th>DRA Mutant 10.24.6 1 h</th>
<th>DRA Mutant 10.24.6-DRA 1 h</th>
<th>DRA Mutant 10.24.6-DRA 24 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>16.23 (DR/peptide)</td>
<td>7</td>
<td>19</td>
<td>0</td>
<td>0</td>
<td>3</td>
<td>21</td>
<td>0</td>
<td>0</td>
<td>11</td>
</tr>
<tr>
<td>I-5 (DR/CLIP)</td>
<td>0</td>
<td>0</td>
<td>11</td>
<td>27</td>
<td>9</td>
<td>14</td>
<td>9</td>
<td>22</td>
<td>7</td>
</tr>
<tr>
<td>α-DMA</td>
<td>25</td>
<td>48</td>
<td>0</td>
<td>0</td>
<td>30</td>
<td>60</td>
<td>14</td>
<td>43</td>
<td>32</td>
</tr>
<tr>
<td>α-MPR</td>
<td>36</td>
<td>63</td>
<td>40</td>
<td>73</td>
<td>19</td>
<td>56</td>
<td>39</td>
<td>72</td>
<td>22</td>
</tr>
<tr>
<td>α-CD63</td>
<td>35</td>
<td>67</td>
<td>25</td>
<td>88</td>
<td>8</td>
<td>63</td>
<td>21</td>
<td>70</td>
<td>10</td>
</tr>
</tbody>
</table>

* Cells were incubated with 5 nm BSA-gold for 3 h followed by overnight chase (“24 h”) before a final incubation with 10 nm BSA-gold for 1 h (“1 h”). Treated cells were sectioned and labeled with the different Abs shown. The total number of endosomes containing either of the two BSA-gold probes (1 h and 24 h) as well as the number of each type of endosome showing labeling with each Ab were counted. The percentage of each type of endosome labeled with each Ab was then calculated.

To further characterize the compartments containing HLA-DR/peptide complexes and HLA-DM, we conducted double-labeling experiments using anti-HLA-DM or 16.23 Abs in combination with Abs to the late endosomal/lysosomal marker proteins, CI-MPR and CD63. These experiments showed that peptide-loaded HLA-DR predominantly localizes to endosomes at a prelysosomal stage; more than 70% of the 16.23 + empty compartments labeled for CI-MPR (Fig. 3B, Table V). The 16.23 + BSA-gold overnight compartments also labeled with Abs to the lysosomal marker, CD63 (Tables IV and V), and similar results were seen for HLA-DM-containing compartments (Tables IV and V). Although some of the 16.23 and HLA-DM labeling was found in compartments that may represent lysosomes (BSA-gold overnight, CI-MPR negative), the majority of peptide-loading compartments in this B-LCL have characteristics of normal compartments at the prelysosomal stage of endocytosis.

In DM-null 9.5.3 cells, HLA-DR/CLIP complexes localize to compartments with the same characteristics as HLA-DR/peptide, HLA-DM+ compartments in progenitor 8.1.6 cells

Another predicted characteristic of CPLs is that they will contain DR/CLIP complexes. To localize HLA-DR/CLIP complexes intracellularly, we analyzed the 8.1.6-derived cell line, 9.5.3, which lacks DM expression and consequently expresses long-lived HLA-DR/CLIP complexes that are exported to the plasma membrane (Fig. 2, A and C). The steady state distribution of DR molecules in these DM-null cells resembles that of their DM-expressing progenitor, 8.1.6 (Fig. 1B). To look specifically at DR/CLIP complexes, we utilized an Ab, I-5, raised by immunizing DR3-transgenic mice with purified DR molecules from 9.5.3, which are 70% DR/CLIP complexes (29). Surface labeling with I-5 shows high levels of binding to 9.5.3 cells, weak

GAM-FITC as secondary Ab. DR-null cell 9.22.3 served as negative control. Control staining with secondary Ab alone (2') is also shown for each cell line. B, Kinetics of expression of mAb 16.23 epitope. The indicated cells (progenitor 8.1.6, DM-null cell 9.5.3, and DMB-transfectant 9.5.3-DMB) were pulse labeled with [35S]methionine and chased in excess methionine for the indicated periods of time (h). HLA-DR3 molecules were immunoprecipitated with mAb 16.23 at each chase point and analyzed by 12% SDS-PAGE and fluorography. The bands representing DR3 dimers and analyzed by 12% SDS-PAGE and fluorography. The bands representing DR3 dimers were SDS stable in 8.1.6 cells. The indicated cells (progenitor 8.1.6, DM-null 9.5.3) were subjected to subcellular fractionation using 17% Percoll gradients, as described in Materials and Methods. Aliquots of indicated fractions were combined (2+3, 4+5, light fractions; 12+13, 14+15, dense fractions), and one-half of the sample was boiled in SDS before electrophoresis on SDS 12% polyacrylamide gels and transfer to PVDF membranes. Immunoblotting with mAb 16.23 was conducted, as described in Materials and Methods. In 8.1.6 cells, 16.23 binds better to DR3 dimer than to DR3β monomeric chains, and thus there appears to be less material in the boiled samples; in 9.5.3 cells, there are some SDS-stable dimers that are not detected by 16.23, but give rise to the modestly increased amount of monomer detected in the boiled samples.
binding to 8.1.6 cells, and no binding to a DR-null cell, 9.22.3 (Fig. 2A), consistent with Ab recognition of CLIP/DR complexes. In addition, using complexes generated from recombinant DR and synthetic peptides, I-5 was found to bind preferentially to DR/CLIP compared with DR/antigenic peptide complexes (M. Amaya and E. Mellins, unpublished data). Labeling sections from 9.5.3 cells with the I-5 Ab showed specific labeling of the plasma membrane and also revealed DR/CLIP complexes in endosomal compartments (Fig. 4), whereas significant I-5 labeling of progenitor 8.1.6 was not observed.

The intracellular distribution of anti-DR/CLIP (I-5) labeling in DM-null 9.5.3 cells was very similar to that of anti-DR/peptide (16.23) in DM-expressing 8.1.6 cells: almost 60% of the labeled intracellular compartments were endosomes containing BSA-gold-chased overnight and 38% were empty late endosomes (Table III). The distribution of HLA-DR/CLIP complexes in 9.5.3 cells in compartments that are indistinguishable from HLA-DR/peptide complexes in 8.1.6 cells suggests that exchange of class II-associated CLIP for peptide occurs in these

FIGURE 3. Immunocytochemical characterization of CPL in 8.1.6 cells. 8.1.6 cells were incubated with BSA-coated 5-nm colloidal gold for 3 h, followed by an overnight chase in goldfree medium (overnight gold) before a final incubation with 10-nm BSA-coated colloidal gold for 1 h. (some 1-h gold pointed at by short arrows). To characterize possible CPL, ultrathin cryo sections were double labeled. A, Double labeling with Abs to HLA-DR/peptide (16.23) (GAM 20) and HLA-DM (GAR 15) revealed four different compartments (numbered 1–3 and ★) showing colocalization of the two markers. All compartments show an endosomal-like morphology, but vary in regard to the content of endocytosed gold. Within the visualized area, double-positive compartments containing overnight gold only (1); 1-h gold only (2); both 1-h and overnight gold (3) as well as empty late endosomes (★) can be seen. Overnight gold (5 nm) in compartment 3 is shown by the large arrow. A compartment negative for both markers but containing overnight gold is also present. Note the existence of small cytoplasmic vesicles labeling for DR/peptide complexes (16.23) only (arrowheads and inset). B, Double labeling for HLA-DR/peptide (GAM 20) and CI-MPR (GAR 15) shows the colocalization of these in similar compartments, as shown for 16.23 and HLA-DM. C, Double labeling for HLA-DM (PAG 15) and CI-MPR (PAG 20) confirms the presence of MPR in the putative CPL. The labeling shows colocalization of HLA-DM and MPR in both an empty endosomal-like compartment (★) as well as an endocytic compartment with overnight gold. Bars, 200 nm.
prelysosomal compartments. Moreover, double labeling of 9.5.3 cells with Ab to DR/CLIP and Abs to CI-MPR or CD63 shows substantial colocalization of DR/CLIP molecules with these markers (Tables IV and V), indicating that HLA-DM is unnecessary for the localization of HLA-DR/CLIP complexes in MPR- or CD63-positive compartments. Finally, if the empty endosomal compartments are unique to APCs, the data from 9.5.3 cells indicate that HLA-DM expression is not needed for their formation.

In 9.5.3-DMB cells and 10.24.6-DRA cells, HLA-DR/CLIP and HLA-DR/peptide complexes colocalize in late endocytic compartments

To further examine the hypothesis that maturation from DR/CLIP to DR/peptide complexes occurs in a compartment at the prelysosomal stage of endocytosis, we looked for colocalization of DR/CLIP and DR/peptide complexes. To do so, we examined a clone of 9.5.3 cells transfected with suboptimal amounts of DMB cDNA; these cells express detectable levels of both DR/peptide (16.23) and DR/CLIP (I-5) molecules by immunoprecipitation of metabolically labeled cells at late points of chase and by staining at the cell surface (Fig. 2, A, B, and C). Double labeling with 16.23 and I-5 Abs revealed clear colocalization in intracellular compartments positive for either of the HLA-DR epitopes (Fig. 4B; Tables IV and V). The majority of the double-labeled compartments contained gold chased overnight or were empty late endosomes, the bulk of which express MPR and CD63. About 75% of the DR/peptide (16.23) and DR/CLIP (I-5) compartments contained DR/peptide, and thus double labeling showed these compartments positive for HLA-DM labeling (Fig. 4C; Table V). Thus, the two maturational states of DR molecules, DR/CLIP and DR/peptide complexes, along with HLA-DM, the molecule that catalyzes the interconversion, colocalize in prelysosomal compartments.

Cell line 10.24.6, another mutant derived from the 8.1.6 progenitor cell, is also deficient in Ag presentation and expresses abundant HLA-DR/CLIP complexes. Similar to the DM mutant 9.5.3, FACS analysis of 10.24.6 cells shows reduced surface binding of the 16.23 Ab and increased surface binding of the I-5 Ab compared with 8.1.6 (Fig. 2A). However, the mutation in 10.24.6 cells is not in HLA-DM, but in the luminal domain of HLA-DRA, and causes the altered phenotype of DR molecules by introducing an aberrant glycosylation site (28). Immunochemical labeling of the 10.24.6 cells with the DR/CLIP Ab and DR/peptide Ab revealed clear colocalization in intracellular compartments (Tables II and III).

Double-labeling experiments showed DR/CLIP compartments of 10.24.6 cells to have a similar intracellular distribution to those of the DM-null 9.5.3 cells, and we observed a high degree of colocalization of the mutated HLA-DR and HLA-DM (results not shown). These findings again confirm the localization of DR/CLIP complexes to DM-containing compartments with characteristics of endosomes at a prelysosomal stage.

Transfection of 10.24.6 cells with wild-type HLA-DRA cDNA only partially corrects the phenotype, probably because there is competition between the mutant and wild-type DR molecules for binding to DRβ chains (Fig. 2A) (28). This transfectant offered an independent opportunity to look for colocalization of DR/CLIP complexes, DR/peptide complexes, and HLA-DM. In 10.24.6-DRA cells, we found the same intracellular distribution of molecules as observed in 9.5.3-DMB cells (Tables II and III). Thus, results with the 10.24.6-DRA cells support the model that the transition from a CLIP-loaded to a peptide-loaded class II molecule occurs in prelysosomal compartments in B-LCL.

Discussion

Using a unique set of Abs that recognize HLA-DR with either CLIP or bound peptide, we found that HLA-DR with CLIP and HLA-DR with peptide colocalize to multimembranous compartments, positioned late in the endocytic pathway. These intracellular compartments are most likely where CLIP is exchanged for peptide. Additionally, double labeling showed these compartments label for HLA-DM, which catalyzes this exchange, as well as for markers of late endocytic compartments, CI-MPR and CD63. From these analyses, we conclude that Ag loading can occur in normal compartments at the prelysosomal stage of endocytosis. It should be noted that the endocytic pathway in these human B-LCL is somewhat unusual overall. First, more than 1 h of uptake is needed for a second pulse of BSA-gold to colocalize with BSA-gold chased overnight, indicating that endocytosis is accomplished with relatively slow kinetics. Moreover, degradative processes in these cells also appear to have slow kinetics, allowing accumulation of proteolytic intermediates; for example, we observe invariant chain-processing intermediates (p21 and p10) without addition of the protease inhibitors that are generally required (Fig. 2C). These characteristics may influence the morphology and marker profile of CPL in these cells. Nonetheless, the results of this study are consistent with findings from two other studies: 1) in murine B cell lymphoma cells, Ag-loaded class II molecules (capable of stimulating T cells) were observed in high density-gradient fractions that also contained rab 7-positive compartments (52) and in melanoma cell line, Mel Juso, MHC class II, and HLA-DM colocalized in compartments expressing rab 7 (25). In addition, in a

Table III. Distribution (%) of HLA-DR with CLIP or peptide, HLA-DM, MPR, and CD63 in different endocytic compartmentsa

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Progenitor 8.1.6</th>
<th>DMB-Null 9.5.3</th>
<th>DMB Transfectant 9.5.3-DMB</th>
<th>DRA Mutant 10.24.6</th>
<th>DRA Transfectant 10.24.6-DRA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 h</td>
<td>24 h</td>
<td>Empty</td>
<td>1 h</td>
<td>24 h</td>
</tr>
<tr>
<td>16.23 (DR/peptide)</td>
<td>6</td>
<td>60</td>
<td>34</td>
<td>No labeling</td>
<td>4</td>
</tr>
<tr>
<td>I-5 (DR/CLIP)</td>
<td>No labeling</td>
<td>5</td>
<td>57</td>
<td>38</td>
<td>10</td>
</tr>
<tr>
<td>α-DMA</td>
<td>10</td>
<td>65</td>
<td>25</td>
<td>No labeling</td>
<td>9</td>
</tr>
<tr>
<td>α-MPR</td>
<td>7</td>
<td>65</td>
<td>24</td>
<td>8</td>
<td>69</td>
</tr>
<tr>
<td>α-CD63</td>
<td>11</td>
<td>73</td>
<td>16</td>
<td>4</td>
<td>63</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

a To show the relative distribution of labeling, the number of labeling-positive compartments of each type (compartments with BSA-gold endocytosed for 1 h, BSA-gold chased overnight (24 h), and endosomes with no detectable BSA-gold (empty)) were counted, and the percent distribution of the label in the different compartments was calculated. The total percent for each Ab on each cell line will therefore be 100%.

recent study of MHC class II/peptide complex assembly in a human B cell line, Morkowski et al. (53) found that peptide loading may start in multivesicular MIIC. These early MIIC most likely correspond to normal prelysosomes, as subcellular fractionation showed that they contain the membrane proteins (LAMP-1) and CI-MPR.

Our results differ from several reported studies that have found MIIC compartments to be MPR negative (17, 19, 20). The use of different Abs for MPR may be the cause of this discrepancy. Abs raised against bovine and chicken CI-MPR give strong labeling of mvb-like late endosomes (41, 54, 55). In contrast, Abs to the human CI-MPR and CD-MPR tend to give strong labeling of the trans-Golgi network and other tubular compartments, but reduced labeling of endosomal compartments (57–59). The reason for these varying results is unclear, but a likely explanation is that different antigenic epitopes are exposed to varying degrees in the different compartments. In the study by Morkowski et al. (53), the majority of peptide-loaded MHC class II molecules cofractionate with the majority of CI-MPR and, in Mel JuSo melanoma cells, anti-bovine CI-MPR Abs label MHC class II compartments (59). Thus, the finding of MPR+ class II compartments is not unique to our cells.

Our studies did not unambiguously distinguish lighter and denser lysosomal fractions, the latter of which have been reported in Mel JuSo melanoma cells to contain mature cathepsin D, minor amounts of HLA-DM, and no class II molecules (60). Higher resolution among vesicles that comigrate in dense fractions in our gradients will be necessary to determine whether 8.1.6 cells contain a class II-negative, dense lysosomal organelle.

The pathway(s) for transport of peptide-loaded HLA-DR to the plasma membrane remains enigmatic. Given that the ultimate destination of peptide-loaded class II molecules is the cell surface, the CPL we have described or transport vesicles derived from them must transport the peptide-loaded MHC II to the plasma membrane. One possibility is that the empty vesicles function in transport. In murine B cells, loading of degraded lysozyme onto MHC class II occurs in specialized lysozyme-loading compartments that morphologically resemble the empty late endosomes seen in this study (23). Lysozyme-loading compartments and CIIV compartments found in murine A20 B cells (22) contain small amounts of MPR and transferrin receptor. We were unable to detect transferrin receptor in class II-containing high density-gradient fractions of 8.1.6 cells, but did detect it in intermediate density fractions (Fig. 1A). Another route to the cell surface is described in a recent study of B-LCL by Raposo et al. (24), in which multivesicular MHC class II-positive compartments were observed to fuse with the plasma membrane and release Ag-presenting vesicles. These multivesicular compartments also contained endocytosed BSA-gold that was released together with the MHC II-positive vesicles. However, the process was reported to be sufficiently slow so as to be unlikely to represent the major pathway by which Ag-loaded MHC II reaches the plasma membrane. We found no evidence of exosome generation or release in the cells reported in this study. We did, however, observe small intracellular vesicles that contained DR/peptide complexes. The small vesicles were found both close to late endocytic compartments containing DR/peptide complexes and free in the cytoplasm in locations closer to the plasma membrane (see Fig. 3A and inset). The vesicles were typically without endocytosed gold and could in principle mediate the transport from the peptide-loading compartment to the plasma membrane.

The studies reported in this work provide some insights into the role of HLA-DM in the formation of peptide-loading compartments and in targeting conventional class II molecules to them. We find the same type of compartments (both BSA accessible and empty) in the HLA-DMB-negative cell line 9.5.3 and its DM-expressing progenitor, 8.1.6, indicating that HLA-DM is not.

### Table IV. Distribution (%) of double-positive compartments distinguished by their content of BSA-golda

<table>
<thead>
<tr>
<th>Ab</th>
<th>Progenitor 8.1.6</th>
<th>DMB-Null 9.5.3</th>
<th>DMB Transfectant 9.5.3-DMB</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>II</td>
<td>1 h</td>
<td>24 h</td>
</tr>
<tr>
<td>1-5</td>
<td>16.23</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>1.5</td>
<td>16.23</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>16.23</td>
<td>α-DMA</td>
<td>66</td>
<td>89</td>
</tr>
<tr>
<td>1-5</td>
<td>α-DMA</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>16.23</td>
<td>α-MPR</td>
<td>74</td>
<td>52</td>
</tr>
<tr>
<td>1-5</td>
<td>α-MPR</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>16.23</td>
<td>α-C6D3</td>
<td>90</td>
<td>96</td>
</tr>
<tr>
<td>1-5</td>
<td>α-C6D3</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>α-DMA</td>
<td>α-MPR</td>
<td>73</td>
<td>85</td>
</tr>
<tr>
<td>α-DMA</td>
<td>α-C6D3</td>
<td>84</td>
<td>87</td>
</tr>
</tbody>
</table>

aTo estimate the distribution of double-positive compartments, the number of each type of compartment (content of BSA-gold) was counted, and the percent distribution of double positive compartments was calculated. For example, in 8.1.6 cells, 11% of the DR/peptide (16.23)-DMA (α-DMA) double-positive compartments contained 1 h BSA-gold, 69% contained 24 h gold, and 20% were empty endosomes.

### Table V. Percentage of Ab I positive compartments also positive for Ab IIa

<table>
<thead>
<tr>
<th>Ab</th>
<th>Progenitor 8.1.6</th>
<th>DMB-Null 9.5.3</th>
<th>DMB Transfectant 9.5.3-DMB</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>II</td>
<td>24 h</td>
<td>Empty</td>
</tr>
<tr>
<td>16.23</td>
<td>1-5</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>1-5</td>
<td>16.23</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>16.23</td>
<td>α-DMA</td>
<td>66</td>
<td>89</td>
</tr>
<tr>
<td>1-5</td>
<td>α-DMA</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>16.23</td>
<td>α-MPR</td>
<td>74</td>
<td>52</td>
</tr>
<tr>
<td>1-5</td>
<td>α-MPR</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>16.23</td>
<td>α-C6D3</td>
<td>90</td>
<td>96</td>
</tr>
<tr>
<td>1-5</td>
<td>α-C6D3</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>α-DMA</td>
<td>α-MPR</td>
<td>73</td>
<td>85</td>
</tr>
<tr>
<td>α-DMA</td>
<td>α-C6D3</td>
<td>84</td>
<td>87</td>
</tr>
</tbody>
</table>

aThe relative level of colocalization between different proteins was quantitated by counting the total number of either DR/peptide (16.23), DR/CLIP (I-5), or DMA (α-DMA) positive compartments that were also positive for a second marker. The percent of double-positive compartments compared to the total number of compartments positive for the first protein was calculated. The table shows the amount of colocalization between the first protein (Ab I) and the second protein (Ab II); for example, in 8.1.6 cells, 38% of the DR/peptide (16.23) positive 1-h endosomes, 66% of the DR/peptide-positive 24-h endosomes, and 89% of the DR/peptide-positive empty endosomes were also positive for DMA.
needed for the formation of either type of compartment. Moreover, the distribution of DR in the DM-negative cells implies that HLA-DM is not necessary for transporting HLA-DR to this compartment, consistent with results from other studies (10, 61) and in striking contrast to its apparent role as lysosomal targeting molecule for HLA-DO (62). Our findings with 10.24.6 cells suggest that the aberrantly glycosylated DR molecules are not altered in their intracellular routing. This conclusion is supported by recent studies that follow trafficking of metabolically labeled DR molecules by subcellular fractionation.5 Thus, the expression of CLIP/DR complexes in these cells appears to result from inhibition of HLA-DM function by the glycosylated HLA-DR molecules, a finding in line with previous data showing that HLA-DM fails to release CLIP from the mutant HLA-DR molecules (9). The normal distribution of HLA-DM in this mutant also argues that transport of HLA-DM to peptide-loading compartments is not reduced in the absence of DM/DR interaction.

Electronmicroscopic studies, like this one, are static and thus may only implicate observed compartments in a given function, such as peptide loading. We have used standard markers of the endocytic pathway and by conventional classification, the CPL in these EBV-transformed lines are compartments at the prelysosomal stage of endocytosis. These compartments contain markers in common with lysosomes (CD63, overnight gold); thus, they are most likely proteolytic compartments that are able to degrade Ag. The environment in prelysosomes might be favorable for the formation of antigenic peptides, compared with lysosomes in which proteins are degraded to amino acids (63). The isolation of CPL will enable us to study their other molecular constituents, their proteolytic machinery, and, possibly, the signals and mechanisms involved in the final transport pathway of MHC class II back to the plasma membrane. Only then will we be able to discern whether these compartments are truly unique to the APC or just a part of the normal endocytic machinery of any cell.

FIGURE 4. Localization of DR/CLIP complexes in 9.5.3 and 9.5.3-DMB cells. A, Double labeling of 9.5.3 cells shows that HLA-DR/CLIP (I-5) (GAM 20) colocalizes with CI-MPR (GAR 15) in endocytic compartments. Within the area visualized, several different endocytic compartments displaying varying labeling patterns can be seen. Compartments containing overnight (5 nm) gold only (1), 1-h (10 nm) gold only (2), both 1-h and overnight gold (3) as well as empty late endosomes (★) show colocalization of CLIP and MPR. Morphologically similar compartments labeling for MPR only are also present. B, Colocalization of HLA-DR/peptide (16.23) (PAG 15) and HLA-DR/CLIP (I-5) (PAG 20) in 9.5.3 DMB cells. 16.23 and I-5 colocalize in endocytic compartments containing overnight gold (1) as well as in a compartment containing both 1-h and overnight gold (large arrow) (3) and in empty late endosomes (★). A 1-h gold endosome positive for DR/CLIP (I-5) but negative for DR/peptide (16.23) is also shown. C, Double labeling of 9.5.3 DMB cells for HLA-DR/CLIP (I-5) (GAM 20) and HLA-DM (GAR 15). Colocalization of the two markers in the same types of compartments in which DR/peptide (16.23) and DR/CLIP (I-5) colocalize. Bars, 200 nm.
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