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Involvement of MIIC-Like Late Endosomes in B Cell Receptor-Mediated Antigen Processing in Murine B Cells

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Currently, the involvement of classical vs novel endocytic compartments in the phenomenon of B cell receptor (BCR)-mediated Ag processing is a matter of considerable debate. In murine B cells, class II vesicles (CIIV) represent a novel endocytic compartment involved in BCR-mediated Ag processing and class II peptide loading. Alternatively, in human B cells, the MIIC class II-enriched compartment (MIIC) represents a lysosome (L)-like endocytic compartment that appears to be involved in this process. Presently, the relationship between CIIV, MIIC, and classical endosomes and L remains to be determined. Using density gradient centrifugation, a subcellular compartment morphologically and immunologically similar to human MIIC has been identified, isolated, and characterized in murine B cells. These MIIC-like vesicles represent a population of class II-positive late endosomes (LE) and are distinct from CIIV. MIIC-like LE are uniquely marked by the thiol protease cathepsin B, and along with mature L, appear to be the major repository of DM molecules in these cells. Importantly, both MIIC-like LE and CIIV isolated from Ag-pulsed B cells contain BCR-internalized Ag as well as antigenic peptide-class II complexes.

B cell receptor (BCR)-mediated Ag processing and presentation is the most physiologically relevant mechanism for Ag processing and presentation by B cells. This phenomenon involves the BCR-mediated internalization of specific Ag into class II containing endocytic compartments in which the Ag is processed and antigenic peptide-class II complexes are formed. These peptide class II complexes must then be transported to the surface of the B cell for recognition by peptide-specific class II-restricted T cells (1). Presently, the specific role of classical endosomes and lysosomes (L) vs that of novel endocytic compartments (i.e., class II vesicles (CIIV)) in BCR-mediated Ag processing remains a matter of intense investigation and debate.

Previous analysis of the A20 murine B cell line has demonstrated that a major fraction of the intracellular class II molecules in these cells is localized to a distinct population of class II vesicles (i.e., CIIV) (2). CIIV are a novel, class II-containing endocytic compartment that can be physically separated from endosomes, L, and other subcellular compartments by the technique of free flow electrophoresis (FFE) (2, 3). Moreover, the novel nature of CIIV was also illustrated by the identification of a putative CIIV-marker protein (i.e., p50Hex) that is immunologically related to the Igα subunit of the BCR (3). Within CIIV, newly synthesized class II molecules in the process of being loaded with Ag peptides are found to be extensively colocalized with BCR-internalized Ag. This observation strongly suggests a crucial role for CIIV in BCR-mediated Ag processing and class II peptide loading (3).

In contrast, immunoelectron-microscopic (immunoEM) analysis of the distribution of class II molecules in human B lymphoblastoid cells lead to the identification of a class II-positive, late endocytic compartment termed the MIIC class II-enriched compartment (MIIC) (4). MIIC have a multilaminar or multivesicular morphology (4) and characteristic immunological profile (e.g., CD63+, Lamp-1+, β-hexosaminidaseα, M6PR+, cathepsin D+, and HLA-DM+)(4, 5). Although BCR-internalized Ag can gain access to MIIC (6), it has not been determined if within morphologically defined MIIC this Ag is processed to peptides and loaded onto class II molecules and whether if formed, these complexes can gain access to the cell surface. Moreover, although the presence of DM molecules in MIIC has been cited as evidence of a role for this compartment in BCR-mediated Ag processing and class II peptide loading (5), the identification and characterization of an inhibitor of DM function (i.e., HLA-DO/H-2O) (7) that is expressed in B cells (8), suggests that the simple presence of DM within an intracellular compartment may not be an indication that peptide loading of class II molecules occurs at this site (9). Hence, the exact role of MIIC in BCR-mediated Ag processing and class II peptide loading remains to be determined.

More recently, immunoEM analysis of the distribution of transfected I-Aβ class II molecules in the A20 murine B cell was purported to show that no novel endocytic compartments exist within these cells and that both CIIV and MIIC represent classical endosomes or L (10). Although MIIC may represent a population of class II-positive late endosomes (LE) or L, the technique of immunoEM analysis is unable to establish the common nature of CIIV because the sole defining characteristic of this compartment is its electrophoretic mobility upon analysis by FFE. Although we have identified a putative marker...
protein for CIIV (i.e., p50^ag^ (3)), there are presently no monospecific anti-p50^ag^ Abs that could be used for the positive identification of CIIV by immunoEM (see Discussion). Therefore, the relationship between endosomes L, MIIC, and CIIV and the precise role of each of these compartments in BCR-mediated Ag processing remain to be determined. Accordingly, we have fractionated the A20 murine B cell line by the technique of Nycodenz (Nycomed Pharma, Oslo, Norway) density gradient centrifugation (DGC), a technique that allows for the separation of LE, L, and CIIV. Morphological and immunological analysis of LE isolated from A20 cells demonstrates that they are morphologically and immunologically similar to MIIC found in human B cells. These MIIC-like LE are physically and immunologically distinct from L and CIIV and are the major subcellular repository of the thiol protease cathepsin B. Moreover, these MIIC-like vesicles, like CIIV, contain class II molecules, BCR-internalized Ag, and complexes of class II and antigenic peptide (i.e., peptides derived from BCR-internalized Ags), suggesting a role for MIIC-like LE along with CIIV in BCR-mediated Ag processing.

Materials and Methods

Cell culture and homogenization

A20, A20huDM, and A20μWT cells were cultured, homogenized, and low density membranes (LDM) were isolated as previously reported (2, 3, 11, 12).

Nycodenz density gradient centrifugation

A20 LDM were fractionated on top-down or bottom-up continuous Nycodenz gradients as described below with the same distribution of subcellular compartments obtained by each method. Different percentage Nycodenz solutions were prepared by blending TEA_S250 (2) (i.e., 0% Nycodenz) with 30% Nycodenz (w/v) in TEA buffer (e.g., 18% Nycodenz = 6 ml of 30% Nycodenz and 4 ml TEA_S250). Top-down gradients. A total of 3 ml of LDM in TEA_S250 was added to a 12 ml ultracentrifuge tube. Then this was sequentially underlain with 3 ml each of 6, 12, and 18% Nycodenz stock solutions. Then the tube was tightly sealed and incubated on its side for 1 h at 4°C to allow a continuous gradient to form. The gradient was then brought to an upright position, loaded into an SW-41T rotor, and centrifuged for 2 h at 110,000 × g at 4°C. The 0.5 ml fractions were collected from the top to the bottom of the gradient and the refractive index of the odd number fractions were determined.

Bottom-up gradients. A total of 3 ml of TEA_S250 was added to a 12 ml ultracentrifuge tube. This was then sequentially underlain with 3 ml each of 6 and 12% Nycodenz. Finally, 3 ml of LDM in 18% Nycodenz (i.e., 1.2 ml of LDM in TEA_S250 and 1.8 ml of 30% Nycodenz) was added under the other layers. A continuous gradient was then formed and centrifuged as described for the top-down configuration.

Percoll density gradient centrifugation

A20 LDM or Nycodenz DGC-isolated LE were fractionated by Percoll DGC as previously reported (11).

Western blot analysis

Gradient fractions were diluted by the addition of 2 volumes of TEA_S250, and vesicles collected by centrifugation as previously reported (2). The samples were analyzed by SDS-PAGE, Western blot analysis, and enhanced chemiluminescence as previously described (2). The primary Abs used were GL2A7 (anti-IgG1, 1:10 of tissue culture supernatant; Ref. 14) and rabbit anti-rat cathepsin B (1:100) along with appropriate secondary Abs (i.e., donkey anti-rabbit Ig-Alexa 488; 1:100, 711-076-152) and donkey anti-rat Ig-DTAF (1:100, 712-015-153; Jackson Immunologicals, West Grove, PA). The cells were examined with a Zeiss Axioshot 2 microscope (Carl Zeiss, Thornwood, NY) using epi-illumination.

Immunoelectron microscopy

The three to five LE-containing gradient fractions were pooled, fixed, concentrated by centrifugation, and analyzed by immunoEM as previously reported (2). Subcellular fractions were probed on thawed, thin cryosections with rabbit anti-LAMP1 (Developmental Studies Hybridoma Bank, Iowa City, IO), rabbit anti-rat cathepsin B (1:100), rabbit anti-HLA-DMB (specific for the cytoplasmic tail of the human DM β-chain, 1:100; Ref. 12), rabbit anti-I-J^d^ (1:100; Ref. 2), and rabbit anti-OVA (1:100; RatOVALBUMI; East Acres Biologicals, Southbridge, MA).

T cell stimulation assay of gradient fractions

Gradient fractions from Ag-pulsed A20μWT cells (i.e., A20μWT pulsed with 5 μM phosphorylcholine-modified OVA (PC-OVA) for 15 min at 37°C) were assayed for the presence of antigenic peptide-class II complexes as previously described (11) including class II negative “feeder” cells. The specificity of peptide class II complex detection (i.e., IL-2 production) was demonstrated by the fact that no IL-2 was detected if gradient fractions from cells pulsed with irrelevant Ags (i.e., PC-rabbit γ-globulin) were used in the assay or if blocking anti-class II mAbs were first added to gradient fractions from PC-OVA-pulsed cells (11).

Results

Fractionation of LE, L, and CIIV from murine A20 B cells

In murine A20 B cells, BCR-mediated Ag processing and class II peptide loading has previously been demonstrated to occur exclusively in low buoyant density compartments including CIIV (2, 11). Although these results appear to rule out a role for high buoyant density, class II negative terminal L in BCR-mediated Ag processing in murine B cells, the role of LE in this phenomenon remain to be determined (see Table I for a summary of the immunological profiles of the endocytic compartments in murine B cells.). Accordingly, we have fractionated LE from murine A20 B cells and initiated a characterization of the role of this compartment in BCR-mediated Ag processing and class II peptide loading.

A20 cells were homogenized and total cellular membranes (i.e., LDM) were prepared as previously reported (2). LDM were fractionated on continuous Nycodenz gradients (Fig. 1). This approach allowed for the fractionation of LE from L (see Table I) and other subcellular compartments (e.g. plasma membrane (PM)). Importantly, LE were well separated from both the CIIV that were marked by the position of p50^ag^ (3) and early endosomes (EE) that were marked by the major peak of Rab4 (13). Interestingly, low buoyant density LE appear to be the major repository of the thiol protease cathepsin B that has been implicated in both the proteolytic maturation of the class II-associated invariant chain (II) and the proteolytic processing of exogenous Ag (15, 16). These results suggest that in murine A20 B cells, as reported in other cell types (17), lgp110 is a marker of both LE and terminal L and that lgp110-positive LE are uniquely marked by the thiol protease cathepsin B.

To further strengthen this interpretation, A20 cells were examined by double-labeled immunofluorescence microscopy. As shown in Fig. 2, A20 cells possess two distinct lgp110-positive intracellular compartments. The first type (most likely LE) contains cathepsin B and the second (most likely terminal L) does not contain detectable cathepsin B. Therefore, the fractionation of murine A20 B cells by Nycodenz DGC allows for the fractionation of LE and L and the resolution of these two endocytic compartments from CIIV.
Characterization of MIIC-like LE in murine B cells

To determine whether the LE isolated from A20 murine B cells were similar to the MIIC of human cells (4), we further refined their immunological profile and examined their morphology. As shown in Fig. 3, LE isolated from murine A20 B cells contain significant levels of class II molecules. Because previous analysis of A20 cells by Percoll DGC demonstrated that high buoyant density terminal L in these cells are class II negative (2), the low level of class II detected in the LE-enriched region of the gradient is most likely due to the presence of class II within “contaminating” PM or Golgi derived vesicles. Indeed, Percoll DGC analysis of Nycodenz DGC-isolated LE and L support this interpretation (see below). Importantly, the absence of a discernable peak of class II in the CIIV region of the Nycodenz gradient is most likely due to the extremely high level of class II molecules found in the closely migrating PM-derived vesicles that would overwhelm the CIIV signal.

As expected from previous reports of the intracellular trafficking of HLA-DM molecules (18), DM molecules (both transfected HLA-DM as well as endogenous H-2 M) were detected in both LE and L-containing gradient fractions. Although there were some differences in the distribution of the transfected human DM and murine DM molecules in the experiment shown, this was not a reproducible finding. Additionally, given our previous analysis of the distribution of these two molecules in A20 cells (12) that demonstrated a similar subcellular distribution for these two proteins, any minor differences in the steady state distribution of these proteins as detected by Nycodenz DGC is likely to be due to the effects of overexpression of the transfected human protein on trafficking within the cell. Importantly, in all experiments both the transfected human DM and endogenous murine DM molecules were found to be restricted to the LE- and L-containing regions of the gradient. Additionally, the LE region of the gradient also contained intact Ii molecules (the Ii molecules detected in the L region of the gradient may be due to the presence of “contaminating” Golgi-derived vesicles that are highly enriched in Ii molecules (2)).

To determine the morphology of the LE isolated from A20 B cells and to confirm that the immunological profile obtained by Western blot analysis is representative of a single type of vesicle (as opposed to the combined profile of two or more comigrating vesicle populations), LE-containing gradient fractions were collected by centrifugation and analyzed by immunoEM as previously reported (2). As shown in Fig. 4, LE isolated from A20 B cells have a MIIC-like multilaminar membrane structure (4) that is characteristic of LE (19). Interestingly, the presence of specialized physical and functional domains within these intravesicular membranes has been suggested (19). Within MIIC-like LE, class II molecules were found to be colocalized with both cathepsin B and the LE/L marker LAMP-1 (Fig. 4, A and B, respectively). Additionally, the MIIC-like LE also contained a significant level of HLA-DM molecules (Fig. 4, C). Thus, class II-positive LE in murine A20 B cells are morphologically and immunologically similar to the MIIC compartment as identified in human B cells (4). This finding is consistent with the idea that MIIC in murine B cells and possibly in human B cells may not represent a novel subcellular compartment but rather class II-containing LE (10).

**Table I. Summary of the immunological profiles of the endocytic compartments identified in A20 murine B cells**

<table>
<thead>
<tr>
<th>Marker</th>
<th>MIIC-like LE</th>
<th>L</th>
<th>EE</th>
<th>CIIV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lamp-1/Lamp-2 (lgp110)</td>
<td>+ (17)</td>
<td>+ (17)</td>
<td>− (17)</td>
<td>− (2)</td>
</tr>
<tr>
<td>β-Hex</td>
<td>−</td>
<td>+</td>
<td>−</td>
<td>− (2)</td>
</tr>
<tr>
<td>Cathepsin B</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>p50Igα</td>
<td>−</td>
<td>− (3)</td>
<td>− (3)</td>
<td>+ (3)</td>
</tr>
<tr>
<td>Rab4</td>
<td>+</td>
<td>− (13)</td>
<td>+ (13)</td>
<td>− (2)</td>
</tr>
<tr>
<td>Class II</td>
<td>+</td>
<td>− (2, 11)</td>
<td>?</td>
<td>+ (2)</td>
</tr>
<tr>
<td>HLA-DM/H-2M</td>
<td>++ (12)</td>
<td>++ (12)</td>
<td>− (12)</td>
<td>+ (12)</td>
</tr>
</tbody>
</table>

* The results summarized in this table are from this paper (no citation indicated) or from previously published reports (citation as indicated). For the purposes of this report, we have defined LE as a subcellular compartment that is rich in the LE and L markers Lamp-1 and Lamp-2 (lgp110), but that contain relatively low levels of the enzymatically active form of the lysosomal enzyme β-Hex. Contrarily, L are defined as a subcellular compartment rich in Lamp-1 and Lamp-2 (lgp110) and that contain relatively high levels of the enzymatically active form of β-Hex. EE are marked by the bulk of Rab4. CIIV are uniquely marked by the protein p50Igα.

**MIIC-like LE and BCR-mediated Ag processing**

Because Percoll DGC analysis of A20 B cells has previously been used to demonstrate that BCR-internalized Ags can be efficiently processed in low buoyant density endocytic compartments without the involvement of high buoyant density organelles (i.e., L) (11), it was of interest to determine the buoyant density of MIIC-like LE by Percoll DGC. As shown in Fig. 5, class II positive LE, like all other class II positive vesicles in A20 cells, exhibit a low buoyant density on Percoll density gradients, consistent with a possible role for MIIC-like LE in BCR-mediated Ag processing and class II peptide loading.

If MIIC-like LE are involved in BCR-mediated Ag processing and class II peptide loading, BCR-internalized Ags would be expected to be able to gain access to this intracellular compartment within the time frame in which this process occurs (3). As shown in Fig. 4D, BCR-internalized Ag can gain access to class II-containing MIIC-like LE by as little as 20 min after internalization. To further extend these findings, gradient fractions from Ag-pulsed A20μWT cells were analyzed for the presence of antigenic peptide (i.e., peptide derived from BCR-internalized Ag) class II complexes as detected by their ability to stimulate a class II-restricted OVA-specific T cell hybridoma. As shown in Fig. 6, MIIC-like LE-containing gradient fractions contained detectable antigenic peptide class II complexes, suggesting that MIIC-like LE may have a role in BCR-mediated Ag processing and class II peptide loading. Additionally, the CIIV-enriched region of the gradient also contained a variable lower level of complexes, again suggesting a role for CIIV in this phenomenon. Although the presence of a low level of Rab4 in the MIIC-like LE-containing gradient fractions (Fig. 1) suggests that the export of these peptide class II complexes from MIIC-like LE to the cell surface may occur, this process remains to be directly demonstrated.

**Discussion**

The results presented in this report demonstrate that murine A20 B cells contain a population of MHC class II positive LE that are...
morphologically and immunologically similar to the MIIC compartment that was originally identified and characterized in human B lymphoblastoid cells (4). Additionally, these class II-positive MIIC-like LE can be accessed by BCR-internalized Ag during the time frame in which the intracellular events of BCR-mediated Ag processing and class II peptide loading occur (11). Moreover, MIIC-like LE were demonstrated along with CIIV to contain antigenic peptides (i.e., peptides derived from BCR-internalized Ag) class II complexes, suggesting a role for both MIIC-like LE and CIIV in BCR-mediated Ag processing and class II peptide loading.

In light of our previous reports (12), the lack of detectable DM molecules in the CIIV-containing region of the gradient was a surprising result that is undergoing further analysis. Importantly, the formation of antigenic peptide class II complexes within a population of DM negative CIIV would be consistent with two previous observations concerning BCR-mediated Ag processing and presentation. The first report observes that BCR-mediated Ag processing and presentation, unlike the processing and presentation of Ag internalized via fluid-phase endocytosis, can occur in the absence of functional DM molecules (20). The second report finds that B cells express an inhibitor of DM function (i.e., HLA-DO (8)), suggesting that some or all of the DM molecules in B cells may be catalytically inactive (7).
Moreover, these results, along with our previous characterization of class II maturation within CIIV (21), suggest that CIIV are more than just transport vesicles for peptide-class II complexes formed at other intracellular sites and that peptide class II complexes are formed within CIIV.

Importantly, the results in this report are consistent with the previously published Percoll DGC analysis of BCR-mediated Ag processing in A20 cells (11) which demonstrated that BCR-internalized Ags can be processed in low density endocytic compartments without the involvement of high buoyant density organelles. Moreover, these results significantly extend the findings of Brachet et al. (22), who demonstrated that leupeptin treatment of A20 cells caused the accumulation of class II molecules in terminal L, by demonstrating that in the absence of leupeptin a low but significant number of class II molecules can be found in the MIIC-like LE of these cells.

Recent immunoEM analysis of the distribution of transfected I-A\(b\) class II molecules in the A20 murine B cell was purported to demonstrate that no novel endocytic compartments exist within these cells and that both CIIV and MIIC represent conventional endosomes or L (10). Although our results are consistent with the interpretation that MIIC represent a population of class II positive conventional LE, the physical separation of CIIV from conventional endosomes and L by two fractionation techniques (i.e., FFE (2, 3) and Nycodenz DGC) along with the identification of a putative CIIV marker protein (i.e., p50Ig\(\alpha\)) (3) strongly support the unique nature of CIIV. Indeed, immunoEM analysis of intact A20 cells presently precludes the use of immunoEM for the analysis of CIIV in whole cells.

In light of this information, one important question becomes: What are the roles of conventional vs novel endocytic compartments in BCR-mediated Ag processing and class II peptide loading? Presently, there are at least two pieces of evidence that suggest an obligate role for a novel endocytic compartment in BCR-mediated Ag processing in A20 cells (11) which demonstrated that BCR-internalized Ags can be processed in low density endocytic compartments without the involvement of high buoyant density organelles. Moreover, these results significantly extend the findings of Brachet et al. (22), who demonstrated that leupeptin treatment of A20 cells caused the accumulation of class II molecules in terminal L, by demonstrating that in the absence of leupeptin a low but significant number of class II molecules can be found in the MIIC-like LE of these cells.

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In light of this information, one important question becomes: What are the roles of conventional vs novel endocytic compartments in BCR-mediated Ag processing and class II peptide loading? Presently, there are at least two pieces of evidence that suggest an obligate role for a novel endocytic compartment in BCR-mediated Ag processing.
and class II peptide loading. First, and possibly most convincing, is the observation that a point mutation in the transmembrane region of the heavy chain of the BCR can completely abolish BCR-mediated processing and presentation while leaving completely unaffected the transport of major histocompatibility complex class II molecules to and from lysosomes.

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