Detection of Functional Class II–associated Antigen: Role of a Low Density Endosomal Compartment in Antigen Processing

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Summary

We have developed a functional assay to identify processed antigen in subcellular fractions from antigen-presenting cells; stimulatory activity in this assay may be caused by either free peptide fragments or by complexes of peptide fragments and class II molecules present on organellar membrane sheets and vesicles. In addition, we have developed a functional assay to identify proteolytic activity in subcellular fractions capable of generating antigenic peptides from intact proteins. These techniques permit the direct identification of intracellular sites of antigen processing and class II association. Using a murine B cell line stably transfected with a phosphorylcholine (PC)-specific membrane-bound immunoglobulin (Ig), we show that PC-conjugated antigens are rapidly internalized and efficiently degraded to generate processed antigen within an early low density compartment. Proteolytic activity capable of generating antigenic peptide fragments from intact proteins is found within low density endosomes and a dense compartment consistent with lysosomes. However, neither processed peptide nor peptide–class II complexes are detected in lysosomes from antigen-pulsed cells. Furthermore, blocking the intracellular transport of internalized antigen from the low density endosome to lysosomes does not inhibit the generation of processed antigen. Therefore, antigens internalized in association with membrane Ig on B cells can be efficiently processed in low density endosomal compartments without the contribution of proteases present within denser organelles.

A PC, including B cells, macrophages, and dendritic cells, internalize exogenous antigens, process them within an intracellular compartment(s), and then express on the plasma membrane newly generated peptide fragments complexed to class II MHC molecules (1-3). Proteases within an acidic environment capable of generating antigenic peptide fragments are present within both low and high density endosomes, as well as within lysosomes; class II molecules are found predominantly in the plasma membrane and low density endosomal compartments (4). In B cells, where antigen is primarily internalized in association with membrane-bound Ig (mlg), a class II–containing endocytic compartment (called MIIC or CIIV) with characteristics distinct from endosomes and lysosomes has been identified; this may be the major site of peptide–class II association in these cells (4-6). Conversely, macrophages, which efficiently internalize molecules via a receptor-independent pinocytic pathway or by phagocytosis, appear to use lysosomes or lysosome-like organelles for processing before class II association (7, 8). Moreover, reduction of disulfide-linked markers, presumably important in the subsequent proteolytic degradation of some antigens, also occurs predominantly within lysosomes (9).

A significant limitation in many of the studies done to identify the intracellular site(s) of antigen processing and class II association is the reliance on indirect approaches for studying processing. In this article, we describe a series of assays that directly identify, within subcellular fractions of B cells, either functional peptide–class II complexes capable of stimulating T cells, free antigenic peptide, or proteolytic activity capable of generating antigenic peptides. We also present evidence that although proteolytic enzymes are present in low density endosomes and in denser lysosomal compartments, the generation of functional peptide–class II complexes does not require a contribution of the denser organelles. Therefore, antigen internalized bound to mIg may be exclusively processed within low density endosomes.

Materials and Methods

Cell Lines. A20 murine B lymphoma cells (γ2a+, λ+, H-2d) were transfected with phosphorylcholine (PC)-specific human μ heavy chain constructs, cloned, and maintained as described previously; surface expression of the transfected mIgM averaged 1-2 × 10^5 molecules per cell (10). The I-A^d-expressing M12.4.1 cell line...
antigens were distributed between plasma membrane and early endosomes (18). CIMP6PR is used as a marker for late endosomes/prelysosomes (19, 20), although it is also found in most organelles of the biosynthetic and endocytic pathways, with distributions that vary among cell types (21). LAMP-1 is a heavily glycosylated protein originally described in lysosomal membranes (22), but it is also present in the late endosome/prelysosome and early endosomal compartments (23).

ELISA was performed on 25–50 μl of Percoll fractions, which had been dried onto flexible microtiter plates (Micro Test III; Falcon Plastics, Cockeysville, MD). After drying, the wells were blocked with 200 μl of 2% BSA in borate-buffered saline (BBS) for 1–2 h at 37°C, washed with PBS/0.01% Triton-X 100, and dried. TIR, LAMP-1, class II, and CIMP6PR antigenic distributions were evaluated by incubating plates with 100 μl of 1:20–1:100 dilutions of the relevant hybridoma supernatants or polyclonal antisera in 2% BSA/BBS for 1–2 h at 37°C, washing with PBS/Triton-X 100, and probing with 100 μl of appropriate HRP-conjugated antisera at 1:1,000 dilutions in 2% BSA/BBS for 1–2 h at 37°C. Transfected human IgM was probed with 100 μl of 1:1,000 dilutions of isotype- and species-specific HRP-conjugated antisera in 2% BSA/BBS. PC was localized by using antibody from the PC-specific murine IgA myeloma, TEPC 15, followed by HRP-goat anti-mouse IgA. After 1–2 h at 37°C, the ELISA plates were washed in PBS/Triton-X 100, and captured HRP activity was assayed by adding 100 μl of citrate buffer (0.1 M sodium citrate, 0.1% Triton-X 100, pH 4.2) plus 50 μl of 1 mM substrate/0.03% H2O2 in citrate buffer (substrate = 2,2'-azino-di[3-ethylbenzthiazoline-6-sulfonic acid] diammonium salt [Sigma Chemical Co.]) for 15–45 min at room temperature. Color was quantitated at 414 nm on an ELISA reader (model 2550; Bio-Rad Laboratories, Richmond, CA) using standard dilution curves of HRP-conjugated antibodies or human IgM myeloma protein (Cappel Laboratories) dried onto the microtiter plates. Compared with starting material applied to the Percoll gradients, recoveries of enzymatic or antigenic activities were within 15% for all time points in a given experiment, and varied from 60 to 130% of total applied activities.

In control experiments, detection of mlgM by the ELISA technique was sensitive to ~0.01 μg/ml. For control cells not previously treated with anti-Ig, detection of mlgM was virtually quantitative (10). After binding of monoclonal or polyclonal anti-IgM at 4°C, up to 50% of the surface μ remained detectable using the ELISA technique; for up to 30 min after internalization, the total recovery of antigenic activity from the subcellular fractions was comparable. Thus, the absolute levels of detectable mlgM remained relatively constant within a given experiment and without variable losses caused by proteolytic degradation or competition from the internalizing antibody.

and its class II + variant, M12.C.3 (11), were generous gifts of Dr. Laurie Glimcher (Harvard School of Public Health, Boston, MA). The T cell hybridomas used were DO.11, specific for OVA peptide fragment OVA233-249 in the context of I-A^d (12), and 2R.50, specific for processed F(ab')2 fragments of rabbit Ig (RGG; specific peptide unknown), in the context of I-A^d (13).

Antibodies. F(ab')2 fragments of RGG (F(ab')2-RGG) were from Cappel (Organon Teknika, Rockville, MD), and intact RGG and goat gamma globulin were from Sigma Chemical Co. (St. Louis, MO). FITC-, PE-, biotin-, and horseradish peroxidase (HRP)- conjugated antibodies were from Southern Biotechnology Associates, Inc. (Birmingham, AL); reagents containing azide were dialyzed against PBS before use. TIB 219 (R172.1.3), a rat monoclonal antibody against mouse transferrin receptor (TfR), was from the hybridoma supernatant (American Type Culture Collection, Rockville, MD). Antibody against lysosome-associated membrane glycoprotein-1 (LAMP-1) was from 1D4B hybridoma supernatants (generated by T. August, distributed through the Developmental Studies Hybridoma Bank, Iowa, City, IA, and maintained on a contract from the National Institute of Child Health and Human Development [NO1-HD-6-2915]). mAbs to I-A^d (MKD6), I-A^d/M5(114), I-A^d/M5-I-A^k (Y-3P), and PC (TEPC 15) were from hybridoma supernatants of the respective clones. Polyclonal rabbit antisera against bovine and rat calmodulin-dependent mannose 6-phosphate receptor (CIMP6PR; insulinlike growth factor-II receptor; [14]) were generous gifts from Drs. G. Gary Sahagian (Tufts University, Boston, MA) and Michael Czech (University of Massachusetts Medical Center, Worcester, MA), respectively.

Preparation of PC-conjugated Proteins. OVA (Sigma Chemical Co.) and F(ab')2-RGG were PC-haptenated as described previously (10). Diazot-PC was synthesized by dissolving 1.1 equivalents of NaNO2 into a solution of p-aminophenylphosphorylcholine (Sigma Chemical Co.) in 0.1 N HCl maintained at pH 1–2 and 15–18°C for ~15 min.

Internalization of Bound Ligand. Cells (typically ≥ 10^6) were incubated in 1–2 ml of culture medium containing 50–100 μg/ml of specific antibody (PE-conjugated goat anti-human μ [GAHμ]) or antigen (PC-OVA) for 30 min on ice, followed by a wash in culture medium. Treated cells were split into equal volumes and either maintained on ice (“time 0” control) or incubated at 37°C for varying periods of time. Incubations were terminated with a 15-fold excess of ice-cold medium followed by centrifugation (10 min, 300 g) at 4°C.

Experiments to disrupt intracellular trafficking were performed by pretreating cells with antigen or antibody at 4°C, followed by washing and incubation for 30 min at 18°C.

Subcellular Fractionation. Cells were suspended in 7 ml homogenization buffer (0.3 M sucrose, 0.01 M Hepes, 10 μg/ml leupeptin, 40 μg/ml pepstatin A, and 200 μM PMSF [Sigma Chemical Co.], pH 7.2) and ruptured by nitrogen cavitation after equilibration at 450 psi for 5 min at 4°C, yielding 85–90% rupture. After centrifugation at 850 g for 10 min to remove intact cells and nuclei, 1 ml of supernatant material was removed to quantify total activity applied; the remaining 6 ml was centrifuged over 24 ml of 25.4 Percoll (Sigma Chemical Co.) with 0.3 M sucrose, 0.01 M Hepes, and the same protease inhibitors as above (pH 7.2, 5 × 1.129 g/ml) for 1 h 45 min at 20,000 rpm in a centrifuge (model J2-21 M/E, rotor model JA-20; Beckman Instruments, Inc., Fullerton, CA; average force ~30,000 g). Fractions (0.8 ml) were recovered from each gradient by gravity siphon; the approximate density of fractions was assessed by adding density marker beads (Pharmacia Inc., Piscataway, NJ) to parallel gradients. To evaluate proteolytic activity in subcellular fractions, homogenization buffer and Percoll were prepared without protease inhibitors. In some experiments, fractions comprising the plasma membrane and low-density endosomes were pooled and centrifuged for 45 min at 20,000 rpm over 11% Percoll gradients; the entire gradient was then collected in 30-ml fractions.

The distribution of intracellular organelles was defined using enzymatic assays for plasma membrane (alkaline phosphodiesterase 1 [APDE 1] [15]), Golgi apparatus (UDP-galactose galactosyl transferase [16]), and lysosomes (β-hexosaminidase [17]), all adapted to microtiter plates. The distribution of PE was determined by solubilizing 100–200 μl of fractions in 2 ml of PBS/0.01% Triton-X 100 and reading fluorescence at 488 nm excitation/580 nm emission; background fluorescence due to Percoll was determined by measuring parallel “blank” Percoll gradients.

Measurement of Antigenic Activity in Subcellular Fractions by ELISA. The distributions of mlgM, PC, TfR, CIMP6PR, LAMP-1, and class II were determined by ELISA. TIR is distributed between plasma membrane and early endosomes (18). CIMP6PR is used as a marker for late endosomes/prelysosomes (19, 20), although it is also found in most organelles of the biosynthetic and endocytic pathways, with distributions that vary among cell types (21). LAMP-1 is a heavily glycosylated protein originally described in lysosomal membranes (22), but it is also present in the late endosome/prelysosome and early endosomal compartments (23).
Distributions for APDE I, β-hexosaminidase, and TfR were routinely performed in all experiments and for every time point to standardize for slight gradient-to-gradient variations. The distributions of the other markers were not routinely assayed; however, when examined, their distributions did not vary significantly as a function of mlg-mediated antigen internalization or time of incubation.

Antigen Processing and Presentation. To determine the kinetics of the generation of processed antigen, A20 cells were washed and incubated at 10⁶/ml with 30 µg/ml PC-OVA or 300 µg/ml OVA in culture medium for various lengths of time before washing and fixation in 1% paraformaldehyde/PBS for 30 min at room temperature. After washing, 10⁶ of these cells were incubated with 10⁵ DO.11 cells in 0.2 ml of culture medium in triplicate for 24 h. Generation of IL-2 in the cultures was assessed by an HT-2 assay as described previously (10) using [³H]dT incorporation (New England Nuclear, Boston, MA) and/or XTT (Sigma Chemical Co.) colorimetric assay (24) to quantitate IL-2 levels. IL-2 standard curves were generated using IL-2 generously provided by Dr. Andrew Lichtman (Harvard Medical School, Boston, MA) from the IL-2-transfected X63 cell line (25). Results are expressed as the average of triplicate cultures; standard deviations were consistently <15%. To remove processed peptide-class II complexes from the cell surface, cells were incubated 1 h on ice with 10 mg/ml pronase (Calbiochem Corp., La Jolla, CA), and washed in 5% BSA/RPMI before further use.

To assay processed antigen in subcellular fractions, 2–4 x 10⁶ transfected A20 cells were washed and incubated for 15–30 min on ice at 10⁶/ml in RPMI with 100 µg/ml PC-OVA. After washing, the cells were incubated for 15 min at 37°C in 1–2 ml culture medium, washed in ice-cold culture medium, and then steriley homogenized and fractionated as described above. Fractions were steriley sonicated (model 250; Branson Ultrasonics Corp., Danbury, CT) with a microtip at power level 1.5 for 10 s; 0.25 ml of each fraction was incubated for 4 h at 37°C with 0.5 ml of paraformaldehyde-fixed (1%, 30 min at room temperature) and washed APC at 10⁶ cells/ml in PBS at pH 7.2. In most experiments, the APC used were M12.4.1 (class II⁺); in some experiments, the class II⁻ cell line M12.C.3 was used. After washing with culture medium, 10⁶ of these APC were incubated with 10⁶ DO.11 in triplicate in a final volume of 0.2 ml culture medium; in experiments involving the presentation of RGG, 2R.50 T cells were substituted for the DO.11. Supernatants IL-2 activity was assessed after 24 h as described above. Control incubations included 0.5 ml of fixed APC with 0.25 ml of homogenization buffer or Percoll medium plus 100–200 µl of (a) culture medium; (b) OVA or PC-OVA at 4 mg/ml; and (c) tryptic fragments of OVA (t-OVA; [26]) at 4 mg/ml.

To assay the distribution of proteolytic antigen-processing activity in untreated cells, nitrogen cavitation and Percoll fractionation were performed in the absence of protease inhibitors. Fixed APC were incubated with subcellular fractions at pH 5.0 in citrate-buffered saline (CBS; 0.015 M citrate, 0.01 M Na₂HPO₄, pH 5.0). This pH value was used because endosomal and lysosomal proteases have acidic pH optima, pH 5 approximates the normal physiologic pH of these organelles (27), and the formation of antigen–class II complexes is accelerated at lower pH (28). However, Percoll precipitated at pH 5; therefore, subcellular fractions were first sonicated, precipitated with an equal volume of CBS, and centrifuged for 10 min at 850 g. One-half milliliter of the supernatants was then incubated with 0.5 ml of fixed APC at 10⁶ cells/ml in the presence of 100 µl of 4 mg/ml OVA for 1 h at 37°C. Control incubations with homogenization buffer or Percoll in place of subcellular fractions were performed as described above. APC were washed and incubated with DO.11 cells as described above. In some experiments, pepstatin A was added to the subcellular fraction–APC-OVA mixture at a final concentration of 36 µg/ml.

To examine whether subcellular fractions could be directly stimulatory for DO.11, 25 µl of sonicated fractions was plated in triplicate and 75 µl of water was added; control trilplate wells with culture medium, OVA, and t-OVA were prepared similarly. These were allowed to dry at 37°C; 10⁵ DO.11 per well in 0.2 ml were then added and IL-2 production after 24 h was assessed as described above. The ability of MKD6 (anti-I-Aβ) to block antigen presentation was assessed by adding 100 µl of hybridoma supernatants to the final APC + DO.11 cultures (final vol = 0.2 ml). Parallel control experiments used isotype-matched antibodies against an irrelevant class II molecule (Y-3P).

Results

Subcellular Distribution of Internalized Antigen and mlg. Functional assays showed that processed antigen capable of stimulating OVA-specific T cells was detectable on the surface of mlgM-transfected A20 cells within 15 min of incubation with PC-OVA (10, 29, Fig. 1). This is consistent with the rapid rates of internalization of mlg described by ourselves and others (29–32). In comparison, nonhaptenated OVA was much more slowly processed to stimulatory antigenic fragments, with 50–100-fold less stimulatory activity, even after a 2-h antigen pulse (Fig. 1). The difference in the efficiency of processing of PC-OVA versus OVA is presumably caused by different efficacy of antigen uptake when bound to mlg versus fluid-phase pinocytosis, as well as in intracellular targeting. The fact that antigen bound and internalized in association with mlg is rapidly processed to antigenic fragments suggests that processing occurs in an early endocytic compartment.

Based on this functional assay, we predicted that by 15 min of internalization, a significant proportion of antigen and mlg would be present within a relevant processing compartment.

![Figure 1](https://example.com/figure1.png)

**Figure 1.** Kinetics of antigen processing and presentation. PC-specific human lgM-transfected A20 cells were incubated at 10⁶/ml with 30 µg/ml PC-OVA (Δ) or 300 µg/ml OVA (●) for various lengths of time at 37°C, followed by washing and fixation in 1% formaldehyde. 10⁵ cells were incubated in triplicate culture wells with 10⁵ DO.11 cells; after 24 h, IL-2 production in the culture supernatants was measured by HT-2 assay. The results are expressed as the average of triplicate cultures; standard deviations are <15%. The data points indicated as "continuous antigen" represent cultures where antigen, transfected A20 cells, and DO.11 were present together continuously in culture for 24 h.
In the next set of experiments, we used subcellular fractionation to determine the intracellular location of mlg and antigen. Transfected A20 cells were incubated with PC-OVA on ice, washed, and either ruptured immediately (time 0) or incubated for 15 min at 37°C before rupture. Subcellular fractions were then assayed for organellar marker profiles and for transfected human μ and PC.

Fig. 2 A shows a characteristic distribution of enzymatic activity from fractionated A20 cells. Plasma membranes were identified by the low density peak of APDE I activity (δ = 1.04 g/ml) and lysosomes by the high density peak of β-hexosaminidase activity (δ = 1.08 g/ml). The distribution of antigenic activities from the same fractions is shown in Fig. 2 B; the lower density peak of TIR activity was interpreted as plasma membrane associated, whereas the slightly denser second peak was consistent with early endosomes. The distribution of CIM6PR showed a major peak of activity corresponding to plasma membrane, with a smaller shoulder of activity interpreted as early endosomal; the densest peak of activity (fraction 3) was attributable to CIM6PR in association with elements of the Golgi complex. The peak of activity at fractions 6–8 was attributed to late endosomes/prelysosomes based on its intermediate density (δ = 1.07 g/ml) and increased CIM6PR activity (33). The distribution of LAMP-1 showed a predominant dense peak of antigenic activity corresponding to lysosomes, with a lesser peak coincident with early endosomes.

The initial distributions of both mlgM and ligand after binding PC-OVA on ice (Fig. 2 C) were largely consistent with plasma membrane, although the mlgM peak frequently straddled the APDE I and TIR distributions. After internalizing PC-OVA for 15 min at 37°C, the bulk of mlgM was present at a density consistent with migration into a low density endosomal compartment (Fig. 2 D); mlgM remained at this density up to 30 min (not shown). Although mlg that has been endocytosed without bound antigen, or after binding monovalent antigen or monoclonal anti-μ antibodies, can recycle back to the plasma membrane (30, 34, 35), we have been unable to document such recycling with cross-linked...
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4 mg/ml ILGG: 0 U/ml; IL-2 values for control incubations of fixed APC cate cultures (SD g20%). Ib2 values for control incubations of fixed APC DO.11 in triplicate wells for 24 h. II.-2 levels in the supernatant were de-
37~ The APC were then washed and 10 s were incubated with 10 s

cubated with paraformaldehyde-fixed M12.4.1 (class II*) APC for 4 h at
37~ and stopping with an excess of ice-cold medium. The cells were
Fractions from cells treated with PC-OVA (---) were sonicated and in-
37~ and adding an anti-I-A a antibody (MKD6) to the culture of

Figure 3. Distribution of processed antigen in subcellular fractions. Transfected A20 cells were incubated with 100 μg/ml PC-OVA or PC-F(ab')2-RGG at 4°C followed by washing, internalization for 15 min at
37°C, and stopping with an excess of ice-cold medium. The cells were
ruptured and fractionated as described in Materials and Methods. (A) Distributions of APDE I (---), β-hexosaminidase (-----), and TIR (---). (B) Fractions from cells treated with PC-OVA (-----) were sonicated and incubated with paraformaldehyde-fixed M12.4.1 (class II+) APC for 4 h at
37°C. The APC were then washed and 10 s were incubated with 105
DO.11 in triplicate wells for 24 h. IL-2 levels in the supernatant were
determined by HT2 assay, and they are expressed as the average of the tripli-
cate cultures (SD <20%). IL-2 values for control incubations of fixed APC are as follows: control (medium only), 0 U/ml; 4 mg/ml OVA, 0.4 U/ml; 4 mg/ml t-OVA, 12 U/ml. (C) Fractions from cells treated in another experiment with PC-OVA or PC-F(ab')2-RGG were sonicated and incubated for 4 h at 37°C with fixed and washed M12.4.1. The APC were
then washed and 105 were incubated with 105 DO.11 or 2R.50 as indi-
cated in triplicate wells for 24 h, followed by HT2 assay for IL-2 produc-
tion. IL-2 values for control incubations of fixed APC with DO.11 or 2R.50 are as follows: control (medium only), 0 U/ml; 4 mg/ml OVA, 0 U/ml; 4 mg/ml RGG: 0 U/ml; IL-2 values for control incubations of fixed APC with 4 mg/ml t-OVA and DO.11, 29.7 U/ml, or 2R.50, 0 U/ml. ---,

mlg at times up to 4 h. In contrast to the mlgM, the internal-
ized hapten PC was found predominantly in denser frac-
tions at 15 min, including a peak of activity corresponding to
lysosomes. It is likely that internalized PC-OVA was released
by a shift in pH and/or digested by proteases into the en-
dosomal lumen and was then passively carried along the normal
dendocytic pathway (36, 37), accumulating in the lysosome.
These results demonstrate that by the time class II peptide
complexes can be detected on the cell surface, ligand inter-
nalized on mlg is found within both low and high-density
intracellular compartments, including lysosomes.

Functional Processed Antigen in Subcellular Fractions. In the
next series of experiments, we used a functional T cell stimu-
lation assay to identify the intracellular compartment where
antigen is processed. Transfected A20 cells were pulsed with
PC-OVA or PC-F(ab')2 RGG, ruptured, and fractionated.
Fractions were sonicated and incubated with a fixed class II B

cell line (M12.4.1); after washing unbound material, the
fixed B cells were incubated with specific T cell hybridomas
(DO.11 for OVA or 2R.50 for RGG), and IL-2 was mea-
sured in 24-h supernatants.

As shown in two representative experiments illustrated in
Fig. 3, B and C, processed OVA and RGG capable of
stimulating specific T cells were detected mainly in low den-
sity endosomal compartments. The shoulder of antigenic ac-
tivity in lower density fractions (Fig. 3 B, fractions 17–19)
was not consistently seen, and may reflect material that has
already returned to the plasma membrane during the 15-min
incubation. The response in this assay was antigen specific,
since transfected A20 cells incubated with PC-F(ab')2 RGG
did not stimulate the OVA-specific T cell hybridoma, DO.11
(Fig. 3 C). Moreover, the recognition of processed antigen
was class II restricted, since it was completely blocked by
adding an anti-I-A d antibody (MKD6) to the culture of
fixed APC and T cells (Fig. 4 B); an irrelevant isotype-matched
antibody, Y-3P, had no effect (not shown). In these assays,
maximal responses of DO.11 cells were typically 1–15 U/ml,
with responses to fixed APC plus t-OVA fragments being
10–125 U/ml. Intact OVA was not presented by the fixed
APC, indicating that the assay detects processed antigenic
fragments. It is noteworthy that in A20 cells (and other B
cell lines), class II is present largely within plasma membrane
fractions, with only a small fraction corresponding to the
low density endosomal compartments (4, Fig. 4 A). This is
in agreement with previously published work using im-
munogold–electron microscopy (5, 38–40).

To determine if the processed antigen detected by this assay
consisted of free or class II–associated peptides, transfected
A20 cells were pulsed with PC-OVA, ruptured, and subcel-
ular fractions were incubated either with fixed class II + or
class II APC. The APC were then washed and cultured

PC-OVA–treated cells/DO.11; , PC-F(ab')2-RGG–treated cells/DO.11;
PC-F(ab')2–RGG–treated cells/2R.50.
with DO.11 cells. T cell stimulatory activity was seen even when the subcellular fractions from antigen-pulsed cells were incubated with fixed class II- APC (Fig. 5 B) or when dried onto culture wells (Fig. 5 C). In three experiments, incubation of fractions with fixed class II- cells resulted in peak stimulations that were 75 ± 30% of those obtained by incubation with class II- APC. Therefore, the assay detects predominantly processed peptide-class II complexes. However, the possibility that a smaller fraction of the stimulator activity results from free processed peptides cannot be excluded. Indeed, in A20 cells expressing tyrosine transmembrane mutants of the mlgM, antigen is bound and internalized to an intracellular compartment(s) where processing occurs, but no class II association takes place (29); subcellular fractions from these antigen-pulsed mutant A20 cells can stimulate

Figure 4. Recognition of intracellularly generated processed antigen is class II restricted. Transfected A20 cells were incubated with 100 μg/ml PC-OVA and fractionated as described in Fig. 3. (A) Distributions of APDE I, β-hexosaminidase, TRR, and class II. The distribution of class II antigenic activity was determined by ELISA using M3/114 antibody. —, plasma membrane; —, lysosomes; —, transferrin receptor; —, MHC class II. (B) Processed antigenic activity was determined as described in Fig. 3; blocking anti-I-Aa antibody MKD6 was added as 100 μl of hybridoma supernatant to the final APC/DO.11 culture (total volume = 0.2 ml). IL-2 values for control incubations of fixed APC are as follows: control (medium only) with and without MKD6, 0 U/ml; 4 mg/ml OVA with and without MKD6, 0 U/ml; 4 mg/ml t-OVA without MKD6, 110 U/ml; 4 mg/ml t-OVA with MKD6, 43.3 U/ml.

Figure 5. Characterization of the stimulatory activity from subcellular fractions. Transfected A20 cells were incubated with PC-OVA and fractionated as described in Fig. 3. (A) Distributions of APDE I, β-hexosaminidase, TRR, and class II. —, plasma membrane; —, lysosomes; —, transferrin receptor; —, MHC class II. (B) Processed antigenic activity was determined as described in Fig. 3, and by incubating subcellular fractions with either fixed MHC II+ (M12.4.1) or fixed MHC II- (M12.C.3) cells, and culturing with DO.11. IL-2 values for control incubations are as follows: control (medium only) with either APC, 0 U/ml; 4 mg/ml OVA with either APC, 0 U/ml; 4 mg/ml t-OVA with M12.4.1 (MHC II+ APC), 8 U/ml; 4 mg/ml t-OVA with M12.C.3 (MHC II- APC), 0.01 U/ml. (C) Fractions were either incubated with fixed M12.4.1 APC as described in Fig. 3 or they were sterilely dried onto microtiter plates (●) in triplicate and washed before adding 10⁵ DO.11 in a final volume of 0.2 ml. IL-2 values for control incubations of fixed APC are as follows: control (medium only), 0 U/ml; 4 mg/ml OVA, 0.4 U/ml; 4 mg/ml t-OVA, 67.3 U/ml. IL-2 values for controls dried onto microtiter plates are as follows: control (medium only), 0 U/ml; 4 mg/ml OVA, 0 U/ml; 4 mg/ml t-OVA, 0 U/ml.
T cells when incubated with class II⁺ APC, but not when incubated with class II⁻ APC. Thus, by using class II⁺ or class II⁻ APC, free processed peptide and peptide-class II complexes in subcellular fractions can be distinguished (29). That the results with wild-type transfected mIgM are similar whether subcellular fractions are incubated in the presence of either class II⁺ or class II⁻ fixed APC indicates that processed peptide fragments are rapidly and efficiently complexed to class II molecules in the low density compartment. It is also worth emphasizing that this assay detected peptide-class II complexes that are generated after mIg-mediated antigen internalization, but it fails to detect such complexes after unhaptenated OVA is internalized by fluid-phase pinocytosis (not shown). This is presumably related to the inefficient concentration of processed peptides in any one intracellular site after uptake by pinocytosis.

Percoll fractionation does not yield a large separation of plasma membrane and low density endosomes; therefore, it was important to demonstrate that the processed peptide-class II complexes we detected were in fact intracellular, and not simply complexes that had already recycled to the plasma membrane. We verified this in two ways. First, fractions containing plasma membrane and low density endosomes from a standard 25.4% gradient of antigen-pulsed A20 cells were pooled and reserparated over a second 11% Percoll gradient (Fig. 6). In this manner, early endosomes, as marked by the denser TfR peak, are cleanly separated from the low density plasma membrane marked by APDE I (Fig. 6 B). Fractions from this same gradient, assayed for processed peptide, showed a broad prominent peak at an intermediate density (δ = 1.044 g/ml) between the plasma membrane and early endosomes, and clearly distinct from both (Fig. 6 C). Interestingly, a smaller amount of activity was detected coincident with the early endosomes, suggesting that processed peptides may be present within that organelle as well. Finally, the intermediate compartment containing processed peptide has relatively little class II (Fig. 6 B); this may result from the relative insensitivity of the solid-phase ELISA assay used to detect class II molecules, but is in general agreement with others who showed that the majority of class II is plasma membrane associated (4, 41).

The second approach we used to demonstrate that the stimulatory peptide-class II complexes were not on the plasma membrane involved removing surface complexes by proteolytic digestion. As shown in Fig. 7 A, a 1-h incubation with pronase reduced the ability of antigen-pulsed and subsequently fixed A20 cells to stimulate DO.11 cells by >60%. Fig. 7 B shows that subcellular fractions from antigen-pulsed A20 cells yielded comparable amounts of processed peptide whether or not the cells were treated with pronase before fractionation. Therefore, the complexes detected are within intracellular organelles.

Proteolytic Activity in Subcellular Fractions. A possible reason that we did not detect processed antigen in denser organelles may be that these compartments lack the enzymes required to generate stimulatory peptide fragments. To examine the subcellular distribution of proteolytic activity, sonicated fractions from A20 cells not exposed to antigen or protease inhibitors were incubated with OVA at pH 5 in the presence of fixed M12.4.1 cells. Extracellular (ex vivo) processing and class II association were allowed to occur, followed by washing and incubation of the fixed APC with DO.11 cells; 24-h supernatants were then assayed for IL-2. As shown in Fig. 8 C, both low density endosomes and denser lysosomes were capable of generating antigenic peptide fragments that could associate with class II molecules. A parallel gradient from antigen-pulsed A20 cells again showed that processed peptide-class II complexes were only seen in the low density compartment (Fig. 8 B). On average, proteolytic activity in en-
Internalized Antigen Does Not Require Processing in Lysosomes.

Internalized antigen does not require processing in lysosomes. From Fig. 2 D, it is apparent that after 15 min of incubation, internalized antigen is found in both endosomal and lysosomal compartments. Moreover, both compartments have enzymes capable of generating stimulatory fragments from intact OVA (Fig. 8 C). Thus, it is conceivable that processed antigen-class II complexes detected within the low density endosome derive from antigen initially catabolized in lysosomes and then recycled to a lower density compartment, where it associates with class II molecules.

Since the trafficking of material from low density endosomes to late endosomes and lysosomes is dependent on microtubules and therefore may be inhibited by low temperature incubation (36, 43, 44), we asked if incubation at 18°C would inhibit the intracellular generation of processed peptide. To demonstrate that this treatment blocks trafficking of an internalized ligand, we used PE-GAH\(\mu\) since the PE fluorescence signal is readily detected and quantifiable.

A20 cells were incubated with PE-GAH\(\mu\) on ice and then shifted to either 18 or 37°C for a subsequent 30-min incubation, followed by subcellular fractionation and assay of PE fluorescence. In parallel, A20 cells were incubated with PC-OVA and allowed to internalize at 18 or 37°C for 30 min before fractionation and assay of fractions for processed peptide. As shown in Fig. 9 B, incubation at 18°C blocked the accumulation of PE in denser organelles; however, the generation of processed peptide was not affected (Fig. 9 C). This result is discrepant with data demonstrating that incubation at 18°C interferes with the presentation of hen egg lysozyme taken up by nonspecific pinocytosis in macrophages and a B cell clone TA3 (45). However, this may be due to a blockade in surface expression of newly generated peptide–class II complexes, which possible complication is avoided by use of the subcellular fractionation assay. Notably, Roederer et al. (46) have found that catabolism of a cathepsin B substrate internalized by fluid-phase pinocytosis was not blocked by incubation at 13–21°C, although they did not examine the ability of this internalized antigen to be processed. Additional experiments using microtubule inhibitors to interfere with accumulation of endocytosed ligand in denser organelles showed that concentrations of colchicine or nocodazole (up to 100 \(\mu\)M) sufficient to disrupt microtubules in nontransfected A20 cells (as evaluated by fluorescence microscopy [47]) did not affect the accumulation of PE in dense organelles (results not shown). Nevertheless, the incubation at 18°C demonstrates that, at least for the case of OVA, proteolytic degradation within the low density endosome is sufficient to generate antigenic fragments without any contribution from proteolytic activity in denser organelles.

Discussion

We have developed a set of sensitive and interrelated biosays that can examine the various steps in the sequence of antigen processing and class II–associated presentation. By using subcellular fractions from antigen-pulsed APC incubated with class II\(^{+}\) cells, processed peptide in various intracellular sites can be assayed; by incubating fractions with class II\(^{+}\) cells or in plastic wells, processed antigen–class II complexes may be identified. In addition, by using subcellular fractions from untreated APC in the presence of intact antigen and fixed class II\(^{+}\) cells, the proteolytic processing capacity of various compartments can be directly assessed. These assays have an advantage over others that rely exclusively on the conformational change of class II molecules to assess intracellular formation of peptide–class II complexes (4, 6, 48) since class II conformational change only indirectly infers the intracellular site of antigen processing.
Figure 8. Subcellular distribution of proteolytic activity capable of generating stimulatory antigenic fragments. Transfected A20 cells were either preincubated with PC-OVA, fractionated, and incubated with fixed M12.4.1 as described in Fig. 3 (B), or they were fractionated without preincubation with antigen (and in the absence of protease inhibitors) and incubated at pH 5 with fixed M12.4.1 in the presence of 0.36 mg/ml OVA as described in Materials and Methods (C). (A) Distribution of markers from cells preincubated with PC-OVA and fractionated in the presence of protease inhibitors; similar distributions were seen with untreated cells fractionated in the absence of protease inhibitors. ---, plasma membrane; - - lysosomes; ---, transferrin receptor; - - MHC class II. IL-2 values for control incubations in B are as follows: control (medium only), 0 U/ml; 4 mg/ml OVA, 0 U/ml; 4 mg/ml t-OVA, 21.7 U/ml.

Figure 9. Effect of low temperature incubation on intracellular trafficking and antigen processing. Transfected A20 cells were incubated at 4°C with PE-GAHα (B) or PC-OVA (C), washed, and internalized for 30 min at either 18°C (---) or 37°C (---). Characteristic markers, shown in A, were not altered by the incubation at 18°C. --- plasma membrane; (APPE I); - - lysosomes (β-hexosaminidase). The distribution of PE fluorescence is shown in B, and antigenic activity (as determined in Fig. 3) is shown in C.
The ability to detect functional in vivo processed antigen probably results from several features of our experimental system. The A20 cell line transfected with a PC-specific human mIgM construct is an extremely efficient APC, and the cells can be routinely cultured to yield 2-4 × 10⁶ cells, each with 1-2 × 10⁴ mIgM molecules per cell. The antigenic specificity allows antigen binding in the cold, followed by a synchronous wave of internalization upon warming. Thus, large amounts of processed antigen can be generated in a relatively narrow temporal window, permitting accurate intracellular localization. By comparison, analysis of the intracellular sites of processing for pinocytosed antigen gave equivocal results (not shown), in large part due to the long incubation times required to load these cells by pinocytosis (Fig. 1). With increasing incubation times, a greater proportion of pinocytosed antigen may enter organelles other than where processing is occurring. Moreover, at early time points, up to 2 h, the amount of antigen internalized by pinocytosis was not sufficient to generate a detectable stimulatory signal from subcellular fractions. The use of the DO.11 T cell hybridoma with specificity for processed OVA peptide in the context of I-A^d (12) was also advantageous in that DO.11 can respond to peptide-MHC class II complexes on planar membranes without a requirement for accessory molecules (49). Consequently, small amounts of processed antigen-MHC complexes could be detected by a very sensitive biologic assay. In addition, the pertinent antigenic fragment of OVA (OVA323-339) is in a relatively accessible portion of the molecule near a major open α-helical loop (50). Thus, degradation of PC-OVA by the transfected A20 cells may proceed rapidly and efficiently because of specific binding and intracellular targeting via the surface mIg (29, 51, 52), and because of physical and biochemical characteristics of the molecule. Although the antigenic epitope from F(ab')₂-RGG that is recognized by 2R.50 is not known, the results with PC-F(ab')₂-RGG (Fig. 2 C) suggest that the technique may prove useful in determining the intracellular processing site(s) for a variety of antigens.

The data in this article demonstrate that antigens internalized in association with mIg on B cells can be efficiently processed to class II-associated antigenic peptide fragments in low density endosomal compartments without the contribution of lysosomal proteolysis. Processed antigen is detected at 15 min in a compartment denoted as low density endosome based on the early entrance of internalized ligand (PC-OVA or PE-GAHμ); it is distinct from early endosomes (marked by TfR) based on its density in an 11% gradient (Fig. 6 C). Moreover, the low density compartment where processed antigen-class II complexes are detected after 15 min of internalization is clearly distinct from lysosomes identified by β-hexosaminidase activity and the predominant peak of LAMP-1 antigenicity. Similar prelysosomal degradation (and subsequent lysosomal accumulation) of antigen in B cells has been observed by others (34, 35). The results are also in agreement with immunogold-electron microscopy data demonstrating early (2–30 min) colocalization of cathepsins, class II, and mIg within an acidic, rapidly accessed endocytic compartment (38).

Although T cell stimulatory processed antigen is detected only within this low density endosomal compartment, enzymatic activity capable of degrading OVA to relevant antigenic fragments is present in both low density endosomal and lysosomal compartments (Fig. 8 C). The demonstration of processing activity in low density endosomes is in agreement with other work (34, 35, 53–55); in addition, cathepsin D, an aspartyl protease inhibitable by pepstatin A and present within low density endosomes (56), has been shown to degrade OVA to fragments that will complex with class II molecules and stimulate DO.11 T cells (42). As expected, antigen-processing activity is also present within dense organelles consistent with lysosomes (7, 9, 34, 35, 57). However, in contrast to previous work with macrophages (8, 39, 40) or B cell lymphoblastoid cell lines (5, 41), little, if any, class II could be detected by an ELISA in the same compartment (Figs. 4, 5, and 8). In fact, class II appears to be largely confined to plasma membrane and low density organelles in A20 cells (4). Therefore, even if antigen is proteolytically degraded in lysosomes in these cells, it may not have access to class II molecules and may not be protected from complete proteolysis (58). Incubation at 18°C blocks the transit of internalized antigen to lysosomes, but it does not affect internalization or the processing of PC-OVA to stimulatory peptide fragments (Fig. 9). This is further evidence that antigen processing can occur within low-density compartments, accessed within minutes of endocytosis. Moreover, the results with fixed class II+ and class II− cells (Fig. 5 B) suggest that processed peptide rapidly and efficiently associates with class II molecules in the low density endosomal compartment.

Our results are somewhat dissimilar to those described by Qiu et al. (41), who used a slightly different technique to assay for processed peptide in subcellular fractions. By directly incubating Percoll subcellular fractions from antigen-pulsed APC with responding T cells, they found that the majority of processed antigen taken up by mIg in a CH27 B cell lymphoma line was present in dense compartments colocalizing with lysosomes (41). However, their assay does not exclude the possibility that the subcellular fractions from antigen-pulsed cells were generating processed peptides de novo during the 24-h incubation with T cells (41). Alternatively, the differences between the two sets of results may be related to the use of different antigens (cytochrome C in the case of Qiu et al. and OVA and RGG in this study) with different processing requirements for the generation of antigenic peptide fragments. Finally, the dense organelle with processed antigen in the CH27 murine B cell lymphoma line (41) may be analogous to the relatively dense MIIC compartment described by Peters et al. (5) in a human B lymphoblastoid cell line and a similar higher density organelle (δ = 1.06) described by West et al. (6), also in human B lymphoblastoid cells. The low density endosomal organelle described in our work with transfected murine A20 B cell lymphoma cells (δ ~1.044) is most likely the low density CIIV described by Amigorena et al. (4), also in A20 cells. It is possible that all of these organelles represent a similar specialized processing compartment in B cells, differing primarily in their physical characteristics between various cell lines.
to this point are the results of Harding and Geuze (8), who used a technique similar to Qiu et al. (41) to assay processed antigen–class II complexes in subcellular fractions from macrophages. They found that in macrophages where antigen was internalized by pinocytosis, processing and class II association occurred largely in a class II-enriched, high density tubulovesicular lysosomal compartment. Since the lysosomal compartment(s) is likely to be the end repository for the bulk of pinocytosed protein fragments in macrophages, the tubulovesicular lysosome may be the specialized organelle in these cells analogous to the CIIV or MIIC in B cells.

In summary, antigen bound to mlg can be rapidly and efficiently processed to peptide–class II complexes within low density endosomes without the contribution of lysosomal degradation. Both proteolytic activity and the availability of class II molecules determine the intracellular site where functional processed antigen is generated.

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