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.

APC, 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 (mIg),[1] 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 ($\gamma 2a^+$, κ^+, H-2^d) 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

[1] *Abbreviations used in this paper:* APDE I, alkaline phosphodiesterase; BBS, CBS, borate- and citrate-buffered saline, respectively; CIIV, class II–containing vesicles; CIM6PR, cation-independent mannose 6-phosphate receptor; GAHμ, goat anti–human μ; HRP, horseradish peroxidase; LAMP-1, lysosome-associated membrane protein-1; mIg, membrane-bound Ig; MIIC, MHC class II–positive compartment; PC, phosphorylcholine; RGG, rabbit gamma globulin; TfR, transferrin receptor; t-OVA, tryptic fragments of OVA.

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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 $OVA_{323-339}$ 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 (R17 217.1.3), 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-$A^{b,d,q}$/I-$E^{d,k}$ (M5/114), I-$A^{b,f,k,p,q,r,s,u,v}$ (Y-3P), and PC (TEPC 15) were from hybridoma supernatants of the respective clones. Polyclonal rabbit antisera against bovine and rat cation-independent mannose 6-phosphate receptor (CIM6PR; 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). Diazo-PC was synthesized by dissolving 1.1 equivalents of $NaNO_2$ 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 $\geq 2 \times 10^8$) 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, δ = 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 1-ml fractions.

The distribution of intracellular organelles was defined using enzymatic assays for plasma membrane (alkaline phosphodiesterase I [APDE I] [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 mIgM, PC, TfR, CIM6PR, LAMP-1, and class II were determined by ELISA. TfR is distributed between plasma membrane and early endosome (18). CIM6PR 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. TfR, LAMP-1, class II, and CIM6PR 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 mIgM 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% H_2O_2 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 EIA 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 mIg by the ELISA technique was sensitive to ~0.01 ng/ml. For control cells not previously treated with anti-Ig, detection of mIg 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 mIg remained relatively constant within a given experiment and without variable losses caused by proteolytic degradation or competition from the internalizing antibody.

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 mIg-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 10^6/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, 10^5 of these cells were incubated with 10^5 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 [^{3}H]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 × 10^8 transfected A20 cells were washed and incubated for 15–30 min on ice at 10^8/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 sterilely homogenized and fractionated as described above. Fractions were sterilely 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 10^6 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, 10^5 of these APC were incubated with 10^5 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. Supernatant 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 Na_2HPO_4, 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 10^6 cells/ml in the presence of 100 μl of 4 mg/ml OVA for 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 triplicate wells with culture medium, OVA, and t-OVA were prepared similarly. These were allowed to dry at 37°C; 10^5 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-A^d) 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 mIg. Functional assays showed that processed antigen capable of stimulating OVA-specific T cells was detectable on the surface of mIgM-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 mIg 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 mIg versus fluid-phase pinocytosis, as well as in intracellular targeting. The fact that antigen bound and internalized in association with mIg 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 mIg would be present within a relevant processing compartment.

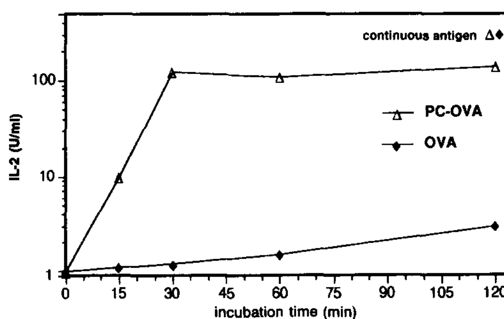
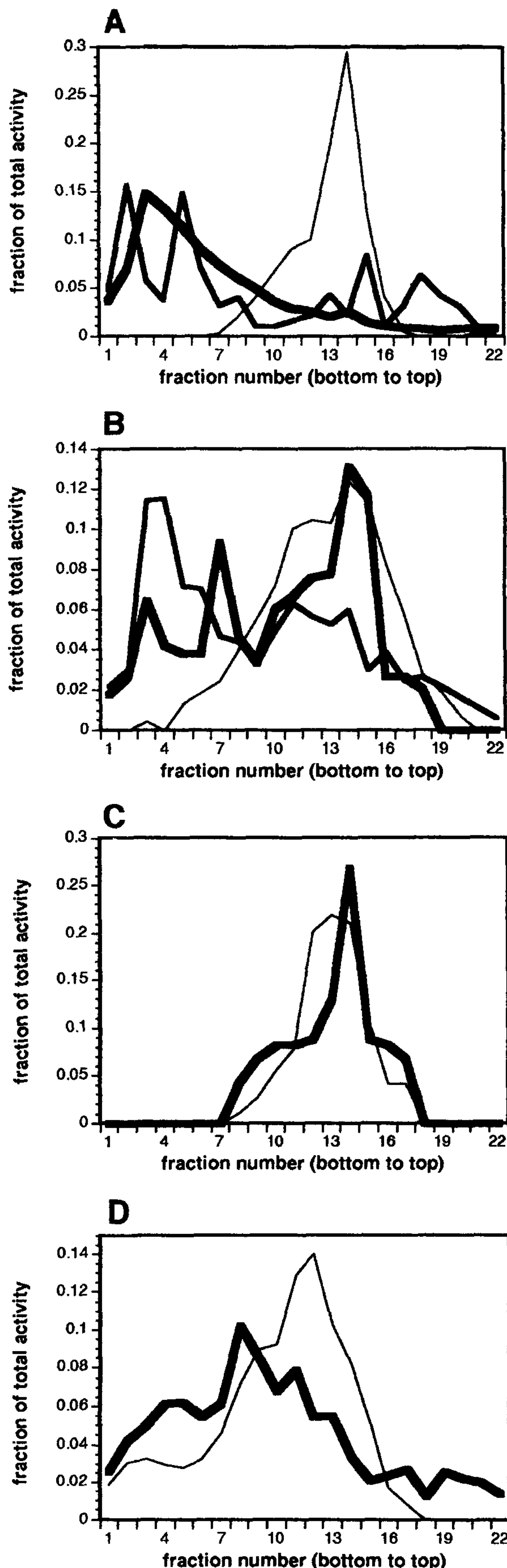


Figure 1. Kinetics of antigen processing and presentation. PC-specific human IgM-transfected A20 cells were incubated at 10^6/ml with 30 μg/ml PC-OVA (Δ) or 300 μg/ml OVA (◆) for various lengths of time at 37°C, followed by washing and fixation in 1% paraformaldehyde. 10^5 cells were incubated in triplicate culture wells with 10^5 DO.11 cells; after 24 h, IL-2 production in the culture supernatants was measured by HT2 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 mIg 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 TfR 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 mIgM and ligand after binding PC-OVA on ice (Fig. 2 *C*) were largely consistent with plasma membrane, although the mIgM peak frequently straddled the APDE I and TfR distributions. After internalizing PC-OVA for 15 min at 37°C, the bulk of mIgM was present at a density consistent with migration into a low density endosomal compartment (Fig. 2 *D*); mIgM remained at this density up to 30 min (not shown). Although mIg 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

Figure 2. Subcellular fractionation of transfected A20 cells; distribution of organellar markers, mIgM, and internalized antigen. Cells were incubated with 50 μg/ml PC-OVA at 4°C, followed by washing, internalization for 15 min at 37°C, and stopping with a large excess of ice-cold medium. Cells were ruptured by nitrogen cavitation and fractionated on self-forming Percoll gradients as described in Materials and Methods. Fraction 1 represents the highest density; fraction 22 is the lowest density. Characteristic enzymatic marker activities are shown in *A* (—, plasma membrane [APDI]; ▬, lysosomes [β-hexosaminidase]; ▬, Golgi complex [UDP-galactose galactosyl transferase]); characteristic antigenic profiles, as determined by ELISA, are shown in *B* (—, transferrin receptor; ▬, CIM6PR; ▬, lysosome-associated membrane protein-1). The antigenic distribution of PC and transfected human μ are shown in *C* at time 0, before internalization, and in *D* after 15 min at 37°C (—, transfected human μ [ELISA]; ▬, phosphorylcholine [ELISA]). Distributions are expressed as a fraction of total activity recovered from the entire gradient; data points are from single determinations from each fraction.

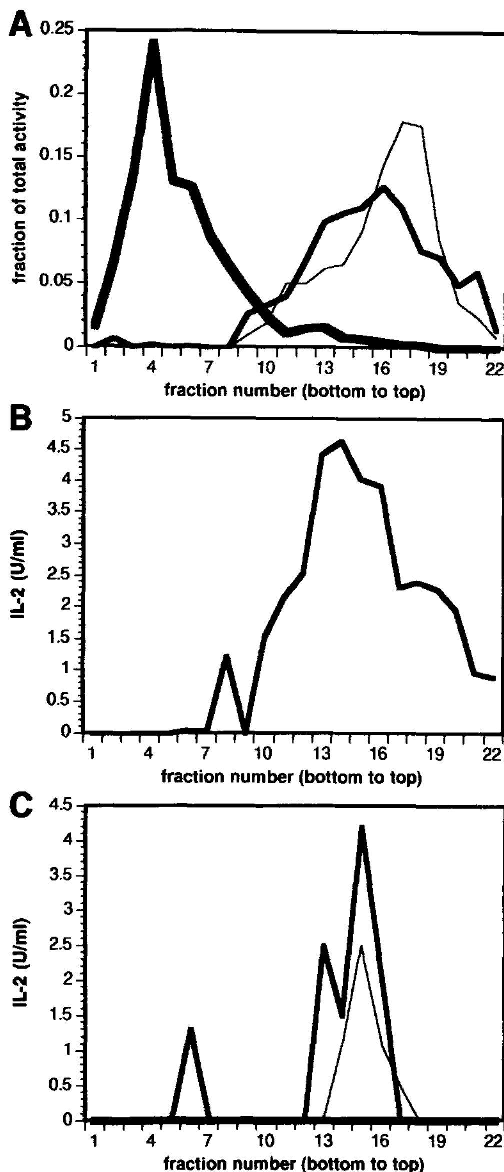


Figure 3. Distribution of processed antigen in subcellular fractions. Transfected A20 cells were incubated with 100 μg/ml PC-OVA or PC-F(ab')₂-RGG at 4°C followed by washing, internalization for 15 min at 37°C, and stopping with an excess of ice-cold medium. The cells were ruptured and fractionated as described in Materials and Methods. (*A*) Distributions of APDE I (—), β-hexosaminidase (▬), and TfR (▬). (*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 10^5 were incubated with 10^5 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 triplicate 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 10^5 were incubated with 10^5 DO.11 or 2R.50 as indicated in triplicate wells for 24 h, followed by HT2 assay for IL-2 production. 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. —, PC-OVA-treated cells/DO.11; ▬, PC-F(ab')₂-RGG-treated cells/DO.11; ▬, PC-F(ab')₂-RGG-treated cells/2R.50.

mIg at times up to 4 h. In contrast to the mIgM, the internalized hapten PC was found predominantly in denser fractions 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 endosomal lumen and was then passively carried along the normal endocytic 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 internalized on mIg 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 stimulation 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 measured 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 density endosomal compartments. The shoulder of antigenic activity 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 compartment (4, Fig. 4 *A*). This is in agreement with previously published work using immunogold-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 subcellular fractions were incubated either with fixed class II⁺ or class II⁻ APC. The APC were then washed and cultured

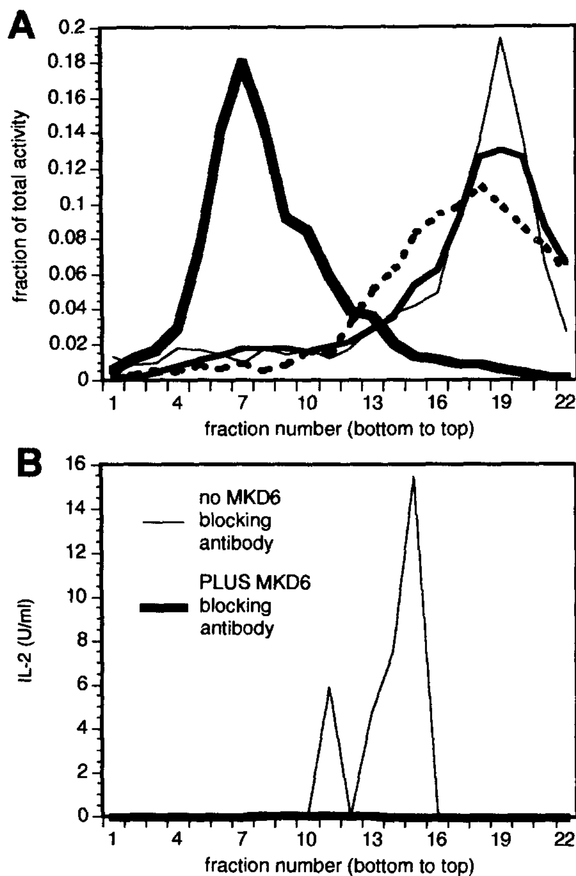


Figure 4. Recognition of intracellularly generated processed antigen is class II restricted. Transfected A20 cells were incubated with 100 μg/ml PC-OVA and fractionated as described in Fig. 3. (*A*) Distributions of APDE I, β-hexosaminidase, TfR, and class II. The distribution of class II antigenic activity was determined by ELISA using M5/114 antibody. —, plasma membrane; ▬, lysosomes; •••, transferrin receptor; ━, MHC class II. (*B*) Processed antigenic activity was determined as described in Fig. 3; blocking anti–I-A^d 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.

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 stimulatory activity results from free processed peptides cannot be excluded. Indeed, in A20 cells expressing tyrosine transmembrane mutants of the mIgM, 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

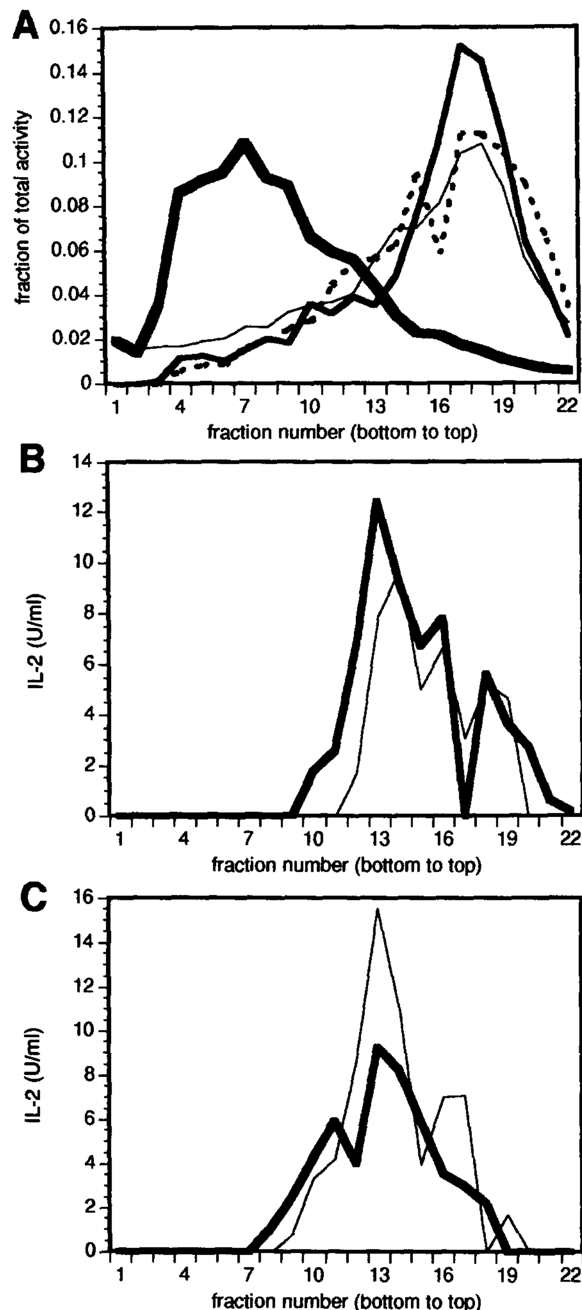


Figure 5. Characterization of the stimulatory activity from subcellular fractions. Transfected A20 cells were incubated with PC-OVA and fractionated as described in Fig. 3. (*A*) Distributions of APDE I, β-hexosaminidase, TfR, and class II. —, plasma membrane; ▬, lysosomes; •••, transferrin receptor; —, 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 wells (—) in triplicate and washed before adding 10^5 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 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, 0.4 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 reseparated 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.

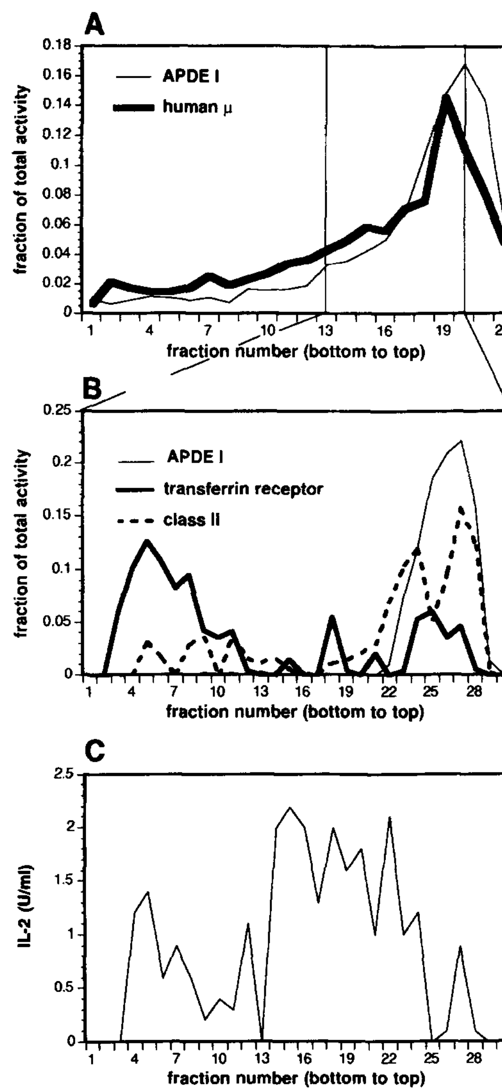


Figure 6. Separation of processed antigenic activity from plasma membrane and early endosomes. Transfected A20 cells were incubated with PC-OVA and initially fractionated as described in Fig. 3. (*A*) Characteristic activities in the 25.4% gradient. Fractions corresponding to the plasma membrane peak and indicated low density endosomal fractions were combined and refractionated over an 11% Percoll gradient as described in Materials and Methods. (*B*) Characteristic activities in the 11% gradient. (*C*) Processed antigenic activity in the 11% gradient was determined as described in Fig. 3.

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-

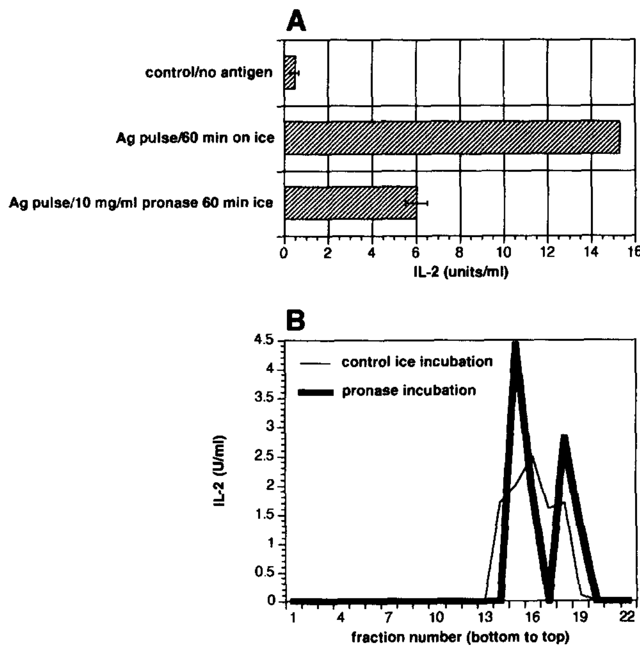


Figure 7. Processed antigenic activity is intracellular because it is not released from intact cells by proteolysis. (*A*) Transfected A20 cells were either kept on ice without antigen (*control/no antigen*) or pulsed with 100 μg/ml PC-OVA and incubated 60 min at 37°C. Antigen-pulsed cells were then washed and incubated on ice for 1 h with or without 10 mg/ml pronase; the control cells received 10 mg/ml pronase for 1 h on ice. All cells were then washed, fixed, and used as APC in culture with DO.11 as described in Fig. 1; 24-h supernatants were assayed for IL-2. (*B*) Transfected A20 cells were pulsed with 100 μg/ml PC-OVA and incubated 30 min at 37°C; the cells were then washed and treated with or without 10 mg/ml pronase as described in *A*. After washing, cells were fractionated and assayed for processed antigenic activity as described in Fig. 3.

dosomes accounted for 37.5% of the total activity across the gradient, while lysosomes accounted for 62.5% (n = 15). Although smaller shoulders of activity in intermediate densities were occasionally seen, no distinct antigen-processing activity attributable to higher density endosomes was consistently identified. Control experiments with medium containing 10% calf serum or with RGG in place of OVA showed no activity. Pepstatin A, a cathespin D inhibitor previously shown to abrogate OVA processing (42), inhibited both early endosome and lysosome proteolytic activities to the same extent (65% inhibition ± 30%; n = 4). Thus, processed peptide–class II complexes are generated only in low density endosomes in intact cells, although both endosomes and lysosomes contain the enzymes necessary for antigen processing.

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-GAHμ since the PE fluorescence signal is readily detected and quantifiable.

A20 cells were incubated with PE-GAHμ 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 μM) 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 bioassays 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.

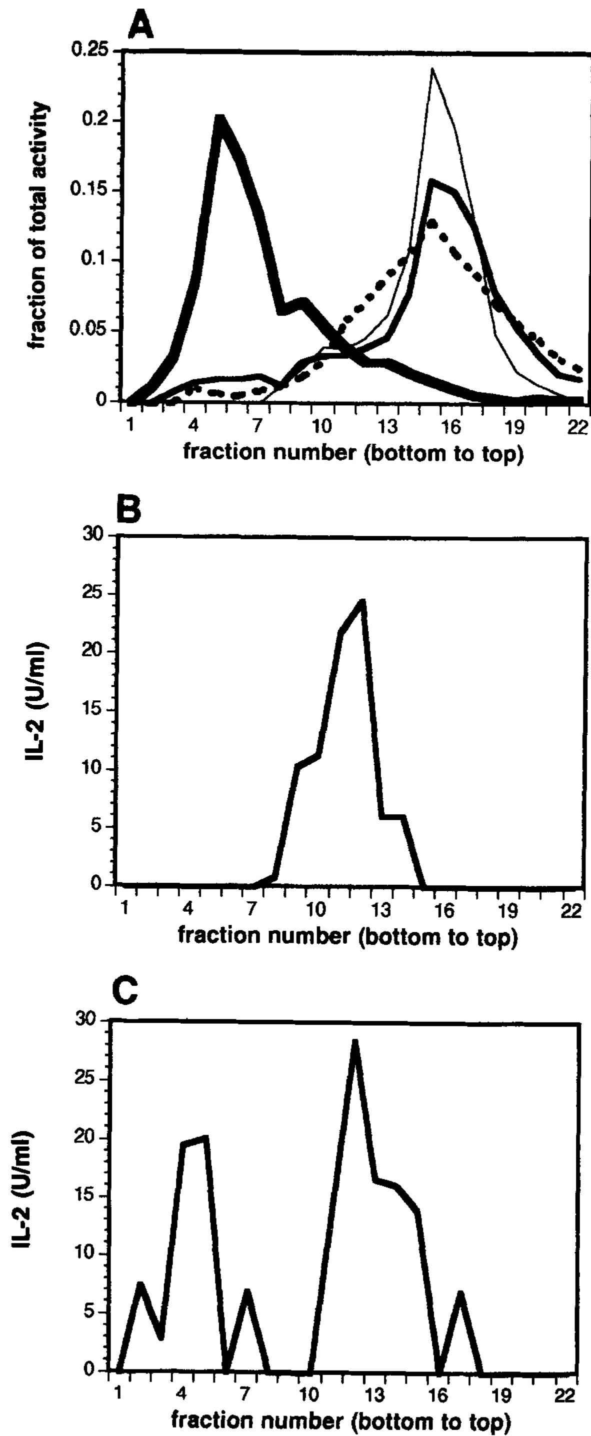


Figure 8. Subcellular distribution of proteolytic activity capable of generating stimulatory antigenic fragments. Transfected A20 cells were either preincubated with PC-OVA, fractionated, and incubated with fixed M12.4.1 as described in Fig. 3 (*B*), or they were fractionated without preincubation with 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, 17 U/ml. IL-2 values for control incubations in *C* 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.

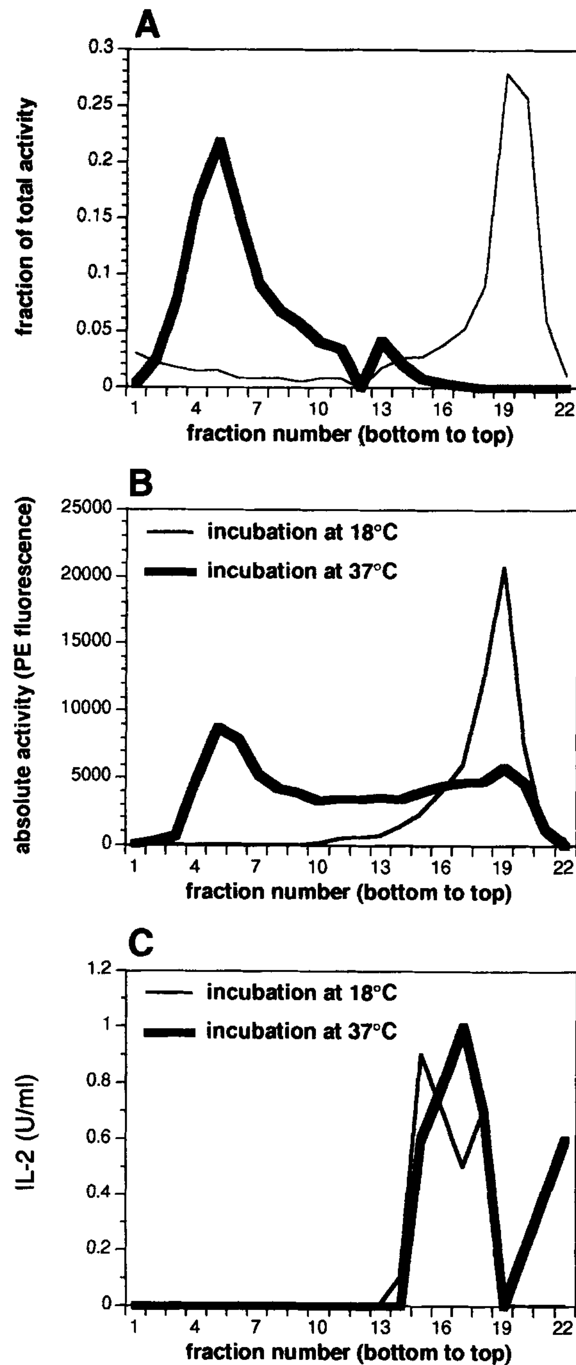


Figure 9. Effect of low temperature incubation on intracellular trafficking and antigen processing. Transfected A20 cells were incubated at 4°C with PE-GAHμ (*B*) or PC-OVA (*C*), washed, and internalized for 30 min at either 18°C (—) or 37°C (▬). Characteristic markers, shown in *A*, were not altered by the incubation at 18°C. —, plasma membrane; (APDE 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 × 10^8 cells, each with 1–2 × 10^5 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 (or fragments) 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 ($OVA_{323-339}$) 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.

Germane 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 mIg 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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