# Separation of Subcellular Compartments Containing Distinct Functional Forms of MHC Class II

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*Abstract.* Antigen processing in B lymphocytes entails initial binding of antigen to the surface Ig and internalization of the antigen into acidic compartments where the antigen is degraded, releasing peptides for binding to major histocompatibility complex class II molecules. Using subcellular fractionation techniques we show that functional, processed antigen–class II complexes capable of activating antigen-specific T cells in vitro are first formed in dense vesicles cosedimenting with lysosomes which are distinct from early endosomes and the bulk of late endosomes. With time, processed antigen–class II complexes appear in vesicles sedimenting with early endosomes and finally cofractionate with plasma membrane. A separate compartment is identified which contains major histocompatibility complex class II receptive to peptide binding but which does not have access to processed antigen in the B cell. These class II molecules are in the so-called "floppy" form in contrast to the class II molecules in the very dense vesicles which are in the "compact" form. These results demonstrate a correlation between the floppy and compact forms of class II molecules and their association with processed antigen and show that floppy and compact forms of class II reside in distinct and physically separable subcellular compartments.

THE helper T lymphocyte response to antigen requires that the antigen be processed and presented by class II-expressing antigen-presenting cells (APC)[1] such as macrophages, B cells, and dendritic cells (7). For B lymphocytes processing is initiated by antigen binding to the surface Ig. Bound antigen is internalized into acidic compartments where degradation proceeds and the resulting peptides bind to class II molecules for subsequent cell surface display. The processing and presentation of antigen to helper T cells is an early crucial event in the initiation of immune responses. Thus, delineating the molecular events underlying the transport of antigen into B cells, the proteolysis of the antigen, and the assembly of processed antigen–class II complexes is central to an understanding of the activation of B and T cells. A key question to be answered is where in the cell these events occur. Indeed, an identification of the microenvironments in which processing occurs is likely to be important as the conditions within subcellular compartments may dramatically influence the assembly of processed antigen–class II complexes.

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1. *Abbreviations used in this paper*: APC, antigen-presenting cells; DMc, Drosophila melanogaster cytochrome *c*; HB, homogenization buffer; KLH, keyhole limpet hemocyanin; LAMP-1, lysosomal associated membrane glycoprotein; PM, plasma membrane; PNS, postnuclear supernatant; TfR, transferrin receptor; TGR, *trans*-Golgi reticulum; THMc 81-103, tobacco hornworm moth cytochrome *c* residues 81-103.

The $\alpha$ and $\beta$ chains of the class II are synthesized in the ER where they rapidly associate with invariant chain (Ii) (36, 40), and the resulting complexes are transported out of the ER, through the Golgi complex, to a post-Golgi compartment, and ultimately to the plasma membrane. At present, it is controversial whether only newly synthesized class II, or class II–peptide complexes recycled from the cell surface, participate in the presentation of newly processed antigen (44). Ii serves both to direct the class II to a post-Golgi compartment (2, 4, 38) and to prevent peptide binding to the $\alpha\beta$ chain dimers in the ER and Golgi (52, 61). Pulse–chase experiments show that class II resides in a post-Golgi compartment for 1–2 h before transport to the cell surface (16, 45, 46). During this time Ii dissociates from the $\alpha\beta$ dimer, which is accompanied by proteolysis of Ii (48). The $\alpha\beta$ chain dimers bind processed antigen which prevents aggregation of empty class II (22, 23) and results in the formation of SDS-stable dimers (22, 45, 54, 58, 65). Two forms of SDS-stable dimers have been described, termed floppy and compact (20). The floppy form may represent an intermediate in the formation of stable dimers (55).

The compartmental localization of individual antigen-processing events is the subject of intensive investigation, but as yet is not fully resolved. The endocytic pathway plays a central role in antigen processing and a number of studies implicate early and late endosomes in these events. Indeed, class II has been shown to reside in endosomes. Enzymes entering the cell by receptor-mediated endocytosis or by pinocytosis intersect class II (16, 46). By immunoelectron

microscopy, antigen bound to surface Ig is rapidly internalized into endosomes containing class II, Ii, the transferrin receptor (TfR), as well as proteases implicated in antigen processing, namely cathespins B and D (25). Using immunoelectron microscopy, class II was observed highly concentrated in vesicular structures distinct from both *trans*-Golgi reticulum (TGR) and lysosomes (46). Neefjes and Ploegh (45) later showed that inhibition of endosomal proteolysis activity by leupeptin blocks the conversion of $\alpha\beta$ dimers to the SDS-stable compact form, suggesting that endosomes are sites of assembly of processed antigen–class II complexes. This work is in agreement with the finding that antigen processing is defective in cells which bear mutations selectively blocking acidification of endosomes but not that of lysosomes (43).

However, other evidence in various cell types supports a key role for very late endocytic or lysosomal compartments in antigen processing. By immunoelectron microscopy, Peters, et al. (50) described a post-Golgi compartment in B cells, termed MIIC, which has many characteristics of lysosomes and contains class II but not Ii. Class II molecules expressed in stably transfected L cells were localized to late endosomes (56). Similarly, Pieters, et al. (51) identified both Ii and class II molecules in prelysosomes as well as in endosomes and multivesicular bodies in a melanoma cell line. Class II in macrophages which function in phagocytic processing of *Listeria monocytogenes* was localized to lysosomes and phagolysosomes (27). Recently, processed antigen–class II complexes were found in vesicles identified as prelysosomes in macrophages (28). Using a different approach, Harding, et al. showed that antigens encapsulated in liposomes which are released at the pH of lysosomes were more efficiently processed as compared to antigens encapsulated into liposomes which release their content in endosomes (26). Finally, when antigen is restricted to early endosomes, by binding to the TfR, antigen processing does not proceed (47). However, cross-linking the TfR to which antigen is bound, causing transport of the TfR to lysosomes (64), results in efficient antigen processing.

Thus, taken together, current data indicate that class II molecules and Ii are present throughout the endocytic pathway from early endosomes to lysosomes. However, only indirect data link any single subcellular compartment to antigen processing. The critical question which remains to be answered is: in which compartment are functional processed antigen–class II complexes, capable of activating T cells, formed? Here we present direct evidence using subcellular fractionation that in B cells which initiated processing by binding antigen to the surface Ig, functional, processed antigen–class II complexes are first detected in dense compartments which cosediment with lysosomes and subsequently, in fractions containing endosomes and the plasma membrane. We also describe a compartment which does not contain processed antigen–class II complexes but contains class II which is receptive to peptide binding and can present peptide provided in vitro. Significantly, this compartment contains the floppy form of class II while the lysosomal compartment contains the compact form. Thus, it is possible to correlate the compact form of class II with the formation of functional processed antigen–class II complexes and the floppy form with the ability to present peptide antigen. Furthermore, we show that the compact and floppy forms reside in physically separable compartments.

# Materials and Methods

## Antibodies and Cell Lines

The I-$E^k$–specific mAb producing hybridoma 17.3.3s (49) was obtained from the American Tissue Culture Collection, (ATCC, Rockville, MD) and maintained in this laboratory. Antibodies were purified from culture supernatants by protein A affinity chromatography. IN-1, mAb-producing hybridoma specific for the $NH_2$ terminus of Ii was kindly provided by Dr. N. Koch (Immunbiologie Zoologisches Institut Universitat, Bonn, Germany) (35). The mAb-producing hybridoma, 1D4B, specific for the lysosomal associated membrane glycoprotein (LAMP-1) was generated and characterized by Drs. J. W. Chen and J. T. August (The Developmental Studies Hybridoma Bank, Johns Hopkins University, Baltimore, MD) (13) and the supernatant generously provided by Dr. A. Sant (University of Chicago, Chicago, IL). A polyclonal antibody specific for rab 7 was generated by immunizing rabbits with a 32–amino acid peptide corresponding to the hypervariable COOH terminus of dog rab 7 (KQETEVELYNEFPEPIKLDKNDRAKTSAESCSC). For use as an immunogen the peptide was coupled to the carrier keyhole limpet hemocyanin (KLH) using benzoquinone (39). Rab 7–specific antibodies were affinity purified on a column containing rab 7 protein covalently coupled to affi-gel 10 resin (Bio Rad Labs., Hercules, CA). An mAb specific for rab 5 was generated by immunizing mice with a 32–amino acid peptide corresponding to the hypervariable COOH terminus of the rab 5 (PKNEPQNPGANSARGRGVDLTEPTQPTRSQCCSN). For immunization, the peptide was coupled to KLH as above. Hybridoma cell lines were selected by ELISA using a peptide immunoabsorbant and Western blot assays against the rab 5 protein. The mAb 4F11 was found to react preferentially with the full-length peptide and failed to react with a peptide consisting of the 16 $NH_2$ terminus amino acids (PKNEPQNPGANSARGR), therefore, the epitope resides within the COOH-terminal last 18 amino acids. The antibody was used in the form of ascites fluid without further purification and is isotype $IgG2a_\kappa$.

The B cell lymphoma CH27 (30) was characterized and kindly provided by Dr. G. Haughton (University of North Carolina, Chapel Hill, NC). The mouse T cell hybridoma TPc 9.1, generated in this laboratory, is specific for *Drosophila melanogaster* cytochrome *c* (DM*c*) presented by I-$E^\kappa$–expressing APC and secretes IL-2 upon activation (10). The CTLL-2 cell line (24) obtained from ATCC, is an IL-2–dependant cell line. The human fibroblast cell line, Niemann-Pick (5), was obtained from ATCC. Cell lines are routinely monitored for mycoplasma infection and are free of this contamination. All cell lines are maintained in 15% FCS containing DME supplemented as described (32).

## Antigens

DM*c* was purified as a recombinant protein from the yeast strain GM-3C-2 transformed with the plasmid YE p DM*c* 01, kindly provided by Dr. E. Margoliash (University of Illinois at Chicago, Chicago, IL). A peptide representing the COOH terminus of tobacco hornworm moth cytochrome *c* residues 81–103 (THM*c* 81–103) was synthesized as detailed previously (11). DM*c* was covalently coupled to affinity-purified rabbit antibodies specific for mouse Ig (DM*c*–anti-Ig) as previously described (42).

## Subcellular Fractionation

CH27 cells ($6 \times 10^8$) were washed with PBS (pH 7.4) and homogenization buffer (HB) (10 mM Tris, 1 mM EDTA, 0.25 M sucrose, pH 7.4) before homogenization. Cells were resuspended in 2 ml of HB and dounced gently in a Dounce Tissue Grinder (Wheaton 357542; Wheaton Inds., Millville, NJ) for 20 cycles, followed by gentle pipetting by a transfer pipette. The homogenate was centrifuged at 900 *g* to yield postnuclear supernatant (PNS). The pellet was gently resuspended in 1 ml HB, the centrifugation repeated, and the supernatant combined with the first PNS. The combined PNS was centrifuged at 10,000 *g* to remove mitochondria. 2 ml of the PNS was layered onto a 9-ml Percoll gradient (Pharmacia LKB Biotechnology Inc., Piscataway, NJ) (1.05 g/ml) prepared according to manufacturer's directions and centrifuged at 34,809 $g_{max}$. 0.5-ml samples were collected using a fractionator (Buchler Auto Densiflow II C; Buchler Instruments, Inc., Lenexa, KS). All procedures were performed at 4°C. The total protein present in each fraction was determined by measuring the radioactivity in subcellular fractions derived from $^{35}$S-Met–labeled CH27 cells.

## Enzyme Assays

Subcellular fractions were assayed for the following. The pinocytosis of HRP was used to mark endosomes as described (1). Briefly, CH27 cells

($10^8$) were incubated at 37°C with HRP (3 mg/ml) in DME containing 10% FCS. After 3 min the cells were rapidly cooled to stop uptake and washed extensively with PBS and HB before homogenization. To mark lysosomes, CH27 cells ($10^8$) were incubated with HRP for 3 min at 37°C, washed twice, and incubated at 37°C for 25 min. The cells were rapidly cooled and washed before homogenization. HRP was detected in the gradient fractions as follows: 100-μl samples were incubated for 5–15 min in the dark with 100 μl of a solution containing the substrate 2,2-azino-*bis* 3-ethylbenzthiazoline-6-sulfonic acid at 5 mg/ml in 0.1 M sodium citrate buffer to which a 1:2,000 dilution of a 30% $H_2O_2$ stock was added. The samples were centrifuged in a microfuge at the maximum speed for 20 min to remove Percoll before the absorbance at 405 nm was determined. β-hexosaminidase activity was measured in gradient fractions as a marker for endosomes/lysosomes. 20-μl samples of each fraction were assayed as described (60). Cytochrome *c* oxidase activity was measured as a marker for mitochondria as described (41). Briefly, horse heart cytochrome *c* (Sigma Chemical Co., St. Louis, MO), 12 mg in 2 ml of a 0.4 M phosphate buffer (pH 6.2), was reduced to ferrocytochrome *c* with sodium hydrosulfite. 50-μl gradient fractions were quickly mixed with 50 μl of ferrocytochrome *c* and 50 μl of acidified $H_2O$ (pH 6.2) at 37°C in a 96-well plate. The rate of decay in the absorbance (550 nm) was measured immediately as an indication of the oxidation of ferrocytochrome *c* using a kinetic–statistic program in the microplate autoreader. $Na^+/K^+$ ATPase activity was measured as a marker for the PM as described (34). 80-μl gradient fractions were incubated with 0.5 ml of a freshly prepared ATP-containing substrate solution (34) for 30 min at 37°C. The reaction was stopped by the addition of 0.1 ml of 50% TCA solution. Samples were incubated on ice for 15 min and centrifuged in microfuge for 8 min at the maximal speed. For phosphate determination (21), supernatants were mixed with 0.1 ml of 2.5% molybdate (in 5 N sulfuric acid) and 0.1 ml of a reducing solution prepared by dissolving 0.5 g aminonaphtholsufonic acid in 190 ml of a 15% sodium bisulfite solution and slowly adding ~10 ml of 20% sodium sulfite until solution is completely dissolved. Samples were incubated for 20 min and centrifuged to remove Percoll before the absorbance (660 nm) was determined. Control samples contained 5 mM ouabain. Cholinephosphoretransferase activity was measured as a marker for the ER as described (15). 40-μl gradient fractions were mixed with 0.2 ml of a solution containing diolin and $^{14}$CDP-cholin and reacted for 30 min at room temperature. Samples were extracted by centrifugation at 300 *g*. α-mannosidase II was measured as a marker for the Golgi apparatus as described (62). 20-μl samples of gradient fractions were assayed at both pH 5.8 and 7.4.

## APC Assay

CH27 cells (1–2 × $10^8$) were cultured in DMc-anti-Ig (2 nM DMc) for various lengths of time, washed, and fractionated as described above. 100-μl subcellular fractions were added to wells of 96-well plates, diluted with 50 μl $H_2O$ and rapidly frozen (−20°C), and thawed (37°C) to break the subcellular organelles. Samples were warmed to 37°C, and 50 μl of a 4× concentrated 5% DME (+/− peptides) was added to each well, followed by 1–5 × $10^5$ TPc 9.1 cells. The cells were incubated at 37°C for 24 h and the IL-2 content of the culture supernatant determined as described (24).

## Immunoprecipitation and Western Blotting

Immunoprecipitations were carried out as described (19). Briefly, CH27 cells (4 × $10^8$) were metabolically labeled with $^{35}$S-Met for 4 h at 37°C, washed, and fractionated as above. For I-$E^k$, 500-μl subcellular fractions were precleared with protein A and immunoprecipitated with 30 μg of affinity-purified anti-I-$E^k$ mAb (17-3-3s) and protein A. For Ii, 300-μl fractions were precleared with protein G and immunoprecipitated with 140 μl IN-1 culture supernatant and protein G. For LAMP-1, 500-μl fractions were precleared with protein G and immunoprecipitated with 500 μl of culture supernatant and protein G.

Subcellular fractions were detected by Western blot analysis for the presence of rab 5 and rab 7 (39). Briefly, subcellular fractions were subjected to SDS-PAGE using 15% mini polyacrylamide gels. The proteins were transferred to poly(vinylidine difluoride) membranes and stained with the affinity-purified polyclonal or monoclonal antibodies followed by the appropriate HRP-conjugated secondary antibody (Amersham Corp., Arlington Heights, IL). Antibody dilutions were made in 3% newborn calf serum, 0.05% (wt/vol) Tween 20, 50 mM Tris, pH 7.5, 200 mM NaCl and incubations were performed for 1–2 h at room temperature. Antibody binding was detected using a chemiluminescent substrate for HRP (Amersham Corp.). Typical exposure times were less than 2 min.

# Results

## Subcellular Fractionation of B Cells

Cells of the B cell lymphoma line, CH27, were homogenized and the homogenate clarified by centrifugation to remove nuclei and unbroken cells. The PNS was then subjected to centrifugation at 10,000 *g* to remove mitochondria and any large protein aggregates. The pellet and supernatant were assayed for the mitochondrial enzyme marker cytochrome *c* oxidase (41) and the pellet contained greater than 85% of the enzyme activity while the remainder of the activity appeared in the supernatant (Fig. 1). The mitochondria-depleted PNS was applied to a Percoll density gradient, after a 20-min centrifugation step, 0.5-ml fractions were collected. The top fraction was discarded to avoid sampling soluble material contained within the PNS and the very bottom of the gradient was not collected to avoid sampling aggregated material. Individual fractions were assayed for several enzyme activities (Fig. 1) including: β-hexosaminidase, marking en-

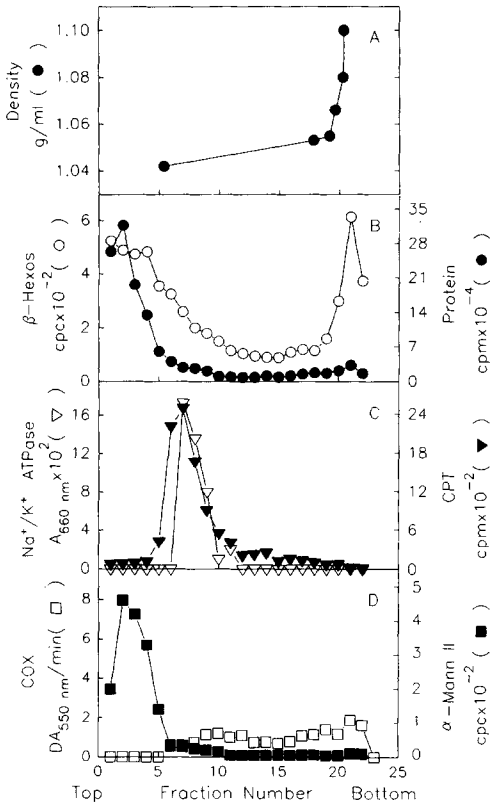


*Figure 1.* Separation of subcellular organelles by Percoll density gradient centrifugation. The mitochondria depleted PNS of homogenized CH27 cells was layered onto a 1.05 g/ml Percoll gradient and centrifuged at 34,809 $g_{max}$ for 20 min at 4°C. (*A*) Given is the density of the gradient fractions determined using density beads (●). Fractions (0.5 ml) were collected and assayed for the following enzymatic markers: (*B*) β-hexosaminidase (*β-Hexos*), marking endosomes and lysosomes (○); (*C*) $Na^+/K^+$ ATPase (▽), marking the PM and cholinephosphotransferase (*CPT*) marking the ER (▼); (*D*) cytochrome *c* oxidase (*COX*) marking mitochondria (□) and α-mannosidase II marking the Golgi (■). All enzyme activities were measured as described in Materials and Methods. The total radioactivity in Percoll density gradient fractions derived from $^{35}$S-Met–labeled CH27 cells is given as cpm in *B* (●).

dosomes and lysosomes (60); $Na^+/K^+$ ATPase, marking the plasma membrane (PM) (34); choline-phosphotransferase, marking the ER (15); cytochrome *c* oxidase, marking the mitochondria (41), and α-mannosidase II, marking the Golgi (62). In addition, CH27 cells were allowed to pinocytose HRP for 3 min to label early endosomes or allowed to pinocytose HRP for 3 min followed by a 25-min chase to identify late endosomal/lysosomal compartments (1). Fractions were also assayed for the presence of the GTP-binding proteins, rab 5, marking early endosomes and the PM and rab 7, marking late endosomes (12) and LAMP-1, a lysosomal marker (13). A summary of the subcellular distribution of these markers in CH27 cells is given in Table I. Hexosaminidase activity was detected in two regions of the Percoll gradient, in light fractions 1–6 and in dense fractions 19–22 (Fig. 1). The activity in the light fractions was highest in fractions 1–4 and trails off into fractions 5–7. The activity in the dense fractions shows a sharp peak of activity in fraction 21. The hexosaminidase activity in the light fractions 1–6 and in dense fractions 19–22 tentatively identified these as containing endosomal and lysosomal compartments. The hexosaminidase activity was contained within intact vesicles in both the light, 2–6, and dense fractions, 19–22, demonstrated by the ability to sediment the enzyme activity by centrifugation at 100,000 *g*. In a separate experiment the protein in each fraction was estimated by the radioactivity in fractions derived from $^{35}$S-Met–labeled CH27 cells (Fig. 1).

$Na^+/K^+$ ATPase activity was detected in fractions 7–9 indicating that these fractions contained the PM (Fig. 1). Fractions 7–9 also contained cholinephosphotransferase activity indicating that the ER was contained in these fractions (Fig. 1). Thus, the PM and ER do not separate on this gradient, although a clear separation of endosomes, lysosomes, and PM/ER was achieved.

The α-mannosidase II activity measured under conditions specific for the Golgi enzyme was detected only in fractions 2–4 (Fig. 1). Thus, Golgi elements sediment with endosomes. No significant cytochrome *c* oxidase activity, a mitochondrial marker, was detected in any of the gradient fractions (Fig. 1) in agreement with our observation that over 85% of the mitochondrial cytochrome *c* oxidase activity was removed from the PNS applied to the gradient.

To confirm that fractions 2–6 contained endosomes and fractions 19–22 lysosomes, the pinocytosis of HRP was followed. CH27 cells were incubated with HRP for 3 min, washed, homogenized, and fractionated as above. Following

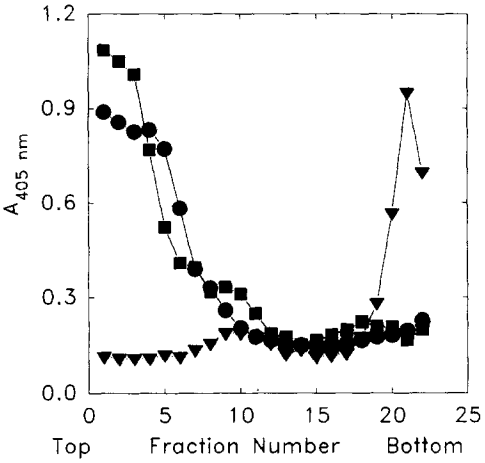


*Figure 2.* Identification of endosomes and lysosomes by HRP uptake. CH27 cells were pulsed with HRP (3 mg/ml) at 37°C for 3 min to label endosomes (●) or pulsed with HRP at 37°C for 3 min and chased in DME for 25 min to label lysosomes (▼). As a control, CH27 cells were incubated with HRP for 35 min at 18°C, a temperature at which transport to lysosomes is blocked (■). Cells were cooled rapidly and washed extensively prior to homogenization and fractionation. HRP activity was measured in gradient fractions as described in Materials and Methods.

a 3-min pulse, pinocytosed-HRP activity was found only in the light fractions 1–6 (Fig. 2). To label lysosomes, CH27 cells were incubated with HRP for 3 min, washed, and the cells incubated in the absence of HRP for 25 min to chase the HRP into lysosomes. After the chase, HRP was detected only in the dense fractions, 19–22 (Fig. 2). The appearance of HRP in the dense fractions was blocked when cells were incubated at 18°C (Fig. 2), a temperature which blocks transport of endosomal content to lysosomes (59). The HRP was contained within intact vesicles by the criteria that greater than 95% of the HRP activity was sedimented by centrifugation at 100,000 *g*. Thus, the presence of HRP only in the light fractions after a 3-min incubation and the temperature-dependent chase of the HRP from the light fractions to the dense fractions with time supports the conclusion that the light fractions contain early endosomes and the dense fractions contain late endosomes/lysosomes.

To further characterize the gradient fractions, Western blot analyses were carried out to detect rab 5 and rab 7. By immunoelectron microscopy, rab 5 has been found to be as-

*Table I. Summary of the Distribution of Organelle Markers in the Subcellular Fractions*

| Fraction numbers: | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | 21 | 22 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| HRP 3 min | + | + | + | + | + | + | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − |
| HRP 25 min | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | + | + | + | + | + |
| β-Hexos | + | + | + | + | + | − | − | − | − | − | − | − | − | − | − | − | − | − | + | + | + | + |
| COX | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − |
| $Na^+/K^+$ ATPase | − | − | − | − | − | − | + | + | + | − | − | − | − | − | − | − | − | − | − | − | − | − |
| CPT | − | − | − | − | − | + | + | + | + | − | − | − | − | − | − | − | − | − | − | − | − | − |
| α-Mann II | + | + | + | + | + | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − |
| rab 5 | * | + | + | + | + | + | + | + | − | − | − | − | − | − | − | − | − | − | − | − | − | * |
| rab 7 | * | + | + | + | + | + | + | + | − | − | − | − | − | − | − | − | − | − | + | + | + | + |
| LAMP-1 | * | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | + | + | + | * |

All assays are as described in Materials and Methods. A * indicates not tested. *HRP 3 min*, HRP pinocytosed for 3 min; *HRP 25 min*, HRP pinocytosed for 3 min followed by a 25-min chase; *β-Hexos*, β-hexosaminidase; *COX*, cytochrome *c* oxidase; *CPT*, cholinephosphotransferase; *α-Mann II*, α-mannosidase II.

sociated with early endosomes and the plasma membrane and rab 7 has been shown to be associated with late endosomes (12). A mAb specific for the COOH terminus of rab 5, mAb4F11, was recently generated and characterized. As shown here, the 4F11 mAb detected a 25-kD band in whole cell lysates (Fig. 3). The 4F11 mAb did not bind in the presence of a peptide corresponding to the COOH terminus of rab 5 but the presence of a peptide corresponding to the COOH terminus of rab 7 had no effect (Fig. 3 *A*). The 4F11 mAb detected a 25-kD band in pooled fractions 2–5 (Fig. 3 *B*, *Pool 1*) and 7–9 (*Pool 2*) but not in the pooled dense fraction, 13–15 (*Pool 3*) or 19–21 (*Pool 4*). The presence of rab 5 in the light fractions 2–5 (*Pool 1*) and its absence in the very dense fractions 19–21 (*Pool 4*) is consistent with the identification of these as early endosomes and lysosomes, respectively. Rab 5 is also a component of the cytosolic face of the PM consistent with its detection in fractions 7–9 (*Pool 2*) which contain PM (12). Rab 7 was detected using affinity-purified rabbit antibodies specific for the COOH terminus of rab 7 recently generated and characterized. As shown here, the purified antibodies detected a 24-kD band in whole cell lysates (Fig. 3 *A*). No binding was detected in the presence of peptide corresponding to the COOH terminus of rab 7 but the presence of a peptide corresponding to the COOH terminus of rab 5 had no effect. Rab 7 was most abundant in fractions 7–9 (*Pool 2*) which also contained the PM and ER (Fig. 3 *B*). The light fractions 2–5 (*Pool 1*) also contained rab 7 although less than the PM/ER fraction. Thus, some late endosomes cosediment with early endosomes. The denser fractions 13–15 (*Pool 3*) were negative for rab 7. However, some rab 7–containing vesicles were also detected in the densest fractions 19–21 (*Pool 4*). Subsequent Western blot analysis of the individual fractions 19 through 22 showed rab 7 to be present in each fraction in similar amounts. Thus, a portion of rab 7-positive endosomes are apparently extremely dense and cosediment with the lysosomal fraction. This would appear to be a discrete subpopulation of late endosomes as no rab 7 is found in intermediate fractions between the lightest 2–9 (*Pools 1* and *2*) and most dense 19–22 fractions (*Pool 4*).

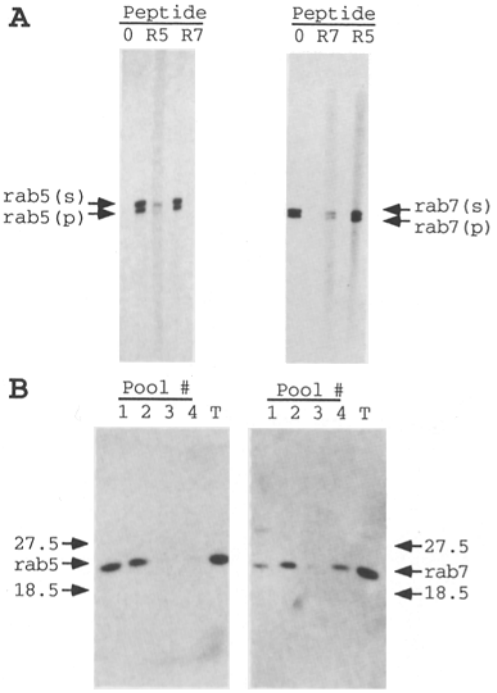


*Figure 3.* Distribution of rab 5 and rab 7 in pooled gradient fractions. (*A*) Cell lysates were prepared from human fibroblasts transfected with rab 5 or rab 7. These were subjected to 15% SDS-PAGE and analyzed by Western blot using mAb (4F11) to rab 5 (*left*) or affinity-purified rabbit antibodies to rab 7 (*right*). Membranes were probed with antibodies alone (lane *0*), or antibodies with the addition of a peptide representing the COOH-terminus of rab 5 (lane *R5*) or a peptide representing the COOH-terminus of rab 7 (lane *R7*). Peptides were added in a 70-fold molar excess over the antibody concentration ($\sim 3 \times 10^{-7}$ M). Two forms of the rab 5 and rab 7 proteins are detected representing the soluble form (*s*) and the prenylated form (*p*). (*B*) Gradient fractions were pooled: 2–5 (*Pool 1*); 7–9 (*Pool 2*); 13–15 (*Pool 3*) and 19–21 (*Pool 4*). A 10-$\mu$l aliquot of each pool and 3 $\mu$l of the total PNS (*T*) were subjected to 15% SDS-PAGE and analyzed by Western blot. Membranes were probed with the rab 5-specific mAb 4F11 (*left*) or with affinity-purified rabbit antibodies specific for rab 7 (*right*). The immunoblot staining was visualized using HRP-conjugated secondary antibodies and a chemiluminescent detection system.

The subcellular fractions were further analyzed by immunoprecipitation for the presence of LAMP-1 using the mAb 1D4B (Fig. 4). The 1D4B mAb detected a diffuse 110-kD band only in fractions 19–21 (*Pool 4*), consistent with the identification of fractions 19–22 as containing lysosomes. LAMP-1 was not detectable in fractions 13–15 (*Pool 3*), fractions 2–5 (*Pool 1*), or in fractions 7–9 (*Pool 3*).

### *Subcellular Localization of Functional Processed Antigen–Class II Complexes*

In order to identify the compartments in which functional processed antigen–class II complexes are formed, subcellular fractions of B cells which had processed antigen were assayed in vitro for their ability to activate an antigen-specific T cell which is dependent on the presence of processed antigen bound to the class II molecules. Thus, CH27 cells were incubated at 37°C with the antigen DM*c* covalently coupled to rabbit antibodies specific for mouse $F(ab')_2$ (DM*c*–anti-Ig). As detailed previously (42), processing of DM*c*–anti-Ig requires ~1/1,000 the concentration of conjugated DM*c* to maximally activate T cells as compared to DM*c* alone but is otherwise indistinguishable from the processing of DM*c*. At low concentrations of DM*c*–anti-Ig, as used here, only Ig-expressing B cells are able to process sufficient antigen so as to result in T cell activation. CH27 cells were incubated with DM*c*–anti-Ig for 15 min, 2 h, or 4 h, harvested, and fractionated on Percoll gradients as described above. Individual fractions were tested in dilution for their ability to

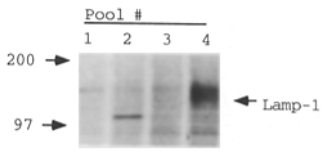


*Figure 4.* Identification of LAMP-1 in the subcellular fractions. Gradient fractions from $^{35}S$-labeled CH27 cells were pooled, immunoprecipitated with the LAMP-1 mAb, 1D4B, and the immunoprecipitates subjected to SDS-PAGE on a 10% gel. Gradient fractions shown are: 2–5 (*Pool 1*); 7–9 (*Pool 2*); 13–15 (*Pool 3*); and 19–21 (*Pool 4*). Immunoprecipitates of pooled fractions using an isotype-matched mAb showed no proteins in the molecular weight range of LAMP-1 in any fraction.

activate an I-E$^k$-restricted T cell hybrid, TPc 9.1 to secrete IL-2. The experiment was carried out three times with similar time points and similar results. Shown is the maximal T cell stimulatory activity detected in the fractions of a typical gradient (Fig. 5). At time zero no T cell stimulatory activity was detected. After a 15-min incubation with DMc-anti-Ig, the lysosome fractions 21-22 contained processed antigen-class II complexes capable of stimulating T cells (Fig. 5). Two hours after processing was initiated, the lysosome-like compartments still contained functional, processed antigen-class II complexes and the complexes began to appear in vesicles which cosediment with early endosomes. At this time, T cell stimulatory activity was barely detectable in the PM fractions. Four hours after processing began the T cell stimulatory activity decreased in the lysosomal compartments was no longer detected in the endosomal compartments, and was present in the PM compartments. The time course of appearance of the functional processed antigen-class II complexes first in vesicles cosedimenting with lysosomes followed by endosomes and finally PM, suggests that the complexes were assembled in dense vesicles and subsequently transported through endosomal compartments to the PM. In control experiments, T cell activation by fractions 21-22 was blocked by mAb 17.3.3s specific for I-E$^k$ (Fig. 6) indicating that the activation of the T cell did indeed require the class II molecule. T cell stimulation was also antigen specific requiring the presence of the antigen DMc-anti-Ig as identical fractions from CH27 cells which had not processed antigen showed no activity in the assay (Fig. 6).

The above experiment identified the B cell subcellular compartments in which processed antigen-class II complexes capable of activating T cells are first formed following the internalization of antigen bound to surface Ig. It is also of significant interest to localize class II molecules in B cells actively processing antigen which have not yet bound processed antigen but are competent to bind peptides and present peptides to T cells. To identify these class II molecules, the gradient fractions described above were analyzed for class II molecules capable of presenting an antigenic peptide provided in vitro. Aliquots of the subcellular fractions described in Fig. 5 were incubated with TPc 9.1 cells in vitro at pH 7 in the presence of the COOH terminal peptide of THMc 81-103. TPc 9.1 cells recognize THMc 81-103 presented on I-E$^k$ and the peptide does not require processing. Surprisingly, class II molecules capable of presenting the peptide antigen in vitro appeared in fractions 13-15 (Fig. 7). These fractions contained no measurable processed antigen-I-E$^k$ complexes at any time during 4 h of processing of

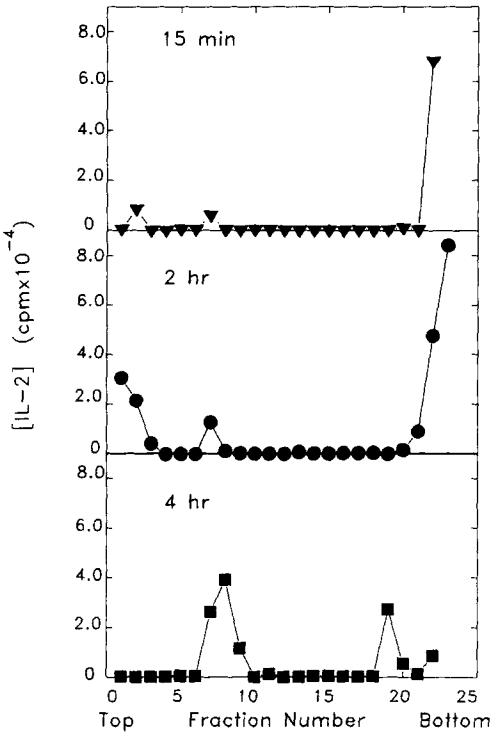


*Figure 5.* Subcellular distribution of functional, processed antigen-MHC class II complexes. CH27 cells were incubated with DMc-anti-Ig (2 nM DMc) at 37°C for 15 min (▼), 2 h (●), or 4 h (■). Cells were washed, homogenized, and applied to a Percoll gradient. Fractions were collected and added, in dilution, to wells of a 96-well microtiter plate. TPc 9.1 cells were added to the fractions and the culture supernatants assayed for their IL-2 content 24 h later as a measure of T cell activation.

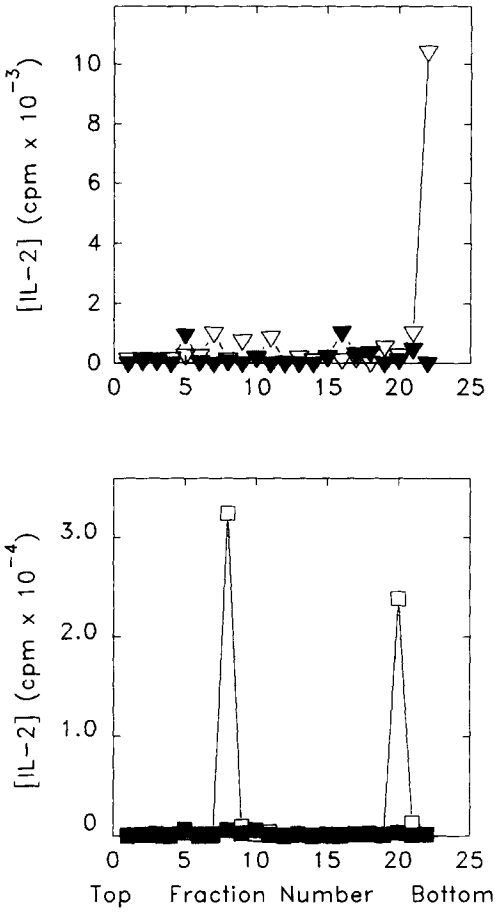


*Figure 6.* T cell activation by subcellular fractions is dependent on antigen and is blocked by I-E$^k$-specific antibodies. (*Top*) CH27 cells were incubated for 3 h with DMc-anti-Ig (▽) or in media alone (▼). Cells were homogenized, fractionated and fractions were tested for their ability to stimulate TPc 9.1 cells to IL-2 secretion. (*Bottom*) Fractions from CH27 cells incubated with DMc-anti-Ig for 4 h, were cultured in vitro with THMc 81-103 (1 μM) in the presence (■) or absence (□) of I-E$^k$-specific mAb 17.3.3s.

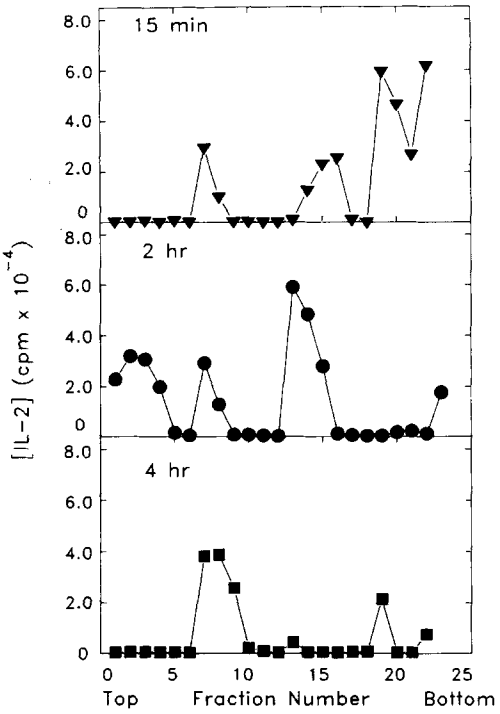


*Figure 7.* The subcellular distribution of class II molecules capable of presenting peptides in vitro. Aliquots of the identical gradient fractions described in Fig. 5 derived from CH27 cells incubated with DMc-anti-Ig for 15 min (▼), 2 h (●), or 4 h (■) were incubated in vitro with TPc 9.1 cells and graded concentrations of the antigenic peptide THMc 81–103 (1 μM). Shown are the results obtained for 1 μM THMc 81–103. Cells were cultured for 24 h and the IL-2 content of the culture supernatant determined as a measure of T cell activation.

DMc-anti-Ig (Fig. 5). The peptide-presenting activity was clearly measurable in fractions 13–15, 15 min after processing was initiated. Two hours after processing began fractions 13–15 showed significant T cell stimulatory activity which disappeared by 4 h. It is interesting to note that the class II molecules readily presented peptide at pH 7. Several studies have shown that the majority of mature class II molecules require reduced pH (4.5–5.0) for efficient peptide binding (33, 28, 54, 65, 66), further suggesting that the class II molecules in fractions 13–15 are indeed distinct from mature class II molecules. Thus, fractions 13–15 define a compartment which contains class II molecules capable of presenting peptide antigen provided in vitro but which do not have access to processed antigen in the B cell. Thus, in B cells which have bound antigen to their surface Ig, it is possible to separate compartments containing class II molecules capable of binding peptide from compartments containing class II molecules bound to processed antigen. This finding also serves as an important control, showing that the functional, processed antigen–class II complexes detected in fractions 19–22 are not the result of postfractionation binding of peptides. If this were the case, functional complexes should have formed in fractions 13–15.

### *Distribution of Ii and Class II Molecules in Subcellular Fractions*

CH27 cells were labeled with $^{35}$S-Met for 3–4 h, harvested, and fractionated, as above. The fractions were immunoprecipitated using an Ii-specific mAb, IN-1, and the precipitates displayed on SDS-PAGE under reducing and denaturing conditions. The major form of Ii immunoprecipitated from the CH27 cells, which we assume to be the p31 form, migrates in this gel system at ~36 kD. There is little, if any, detectable p41 form. Ii was concentrated in fractions 5–9 which contain the ER and PM (Fig. 8). This is expected as all class II molecules are associated with Ii in the ER and excess Ii is synthesized and present in the ER. Fractions 13–15, which contained class II molecules capable of presenting peptide in vitro, contained Ii but at significantly lower levels than ER/PM fractions. The lysosome-containing fractions, 19–21, which contain processed antigen–class II complexes contain no detectable Ii.

The same fractions derived from $^{35}$S-Met-labeled CH27 cells were immunoprecipitated using an I-E$^k$–specific mAb, 17.3.3s. The immunoprecipitates displayed on SDS-PAGE under reducing and denaturing conditions show αβ and Ii proteins migrating in the 30–40-Mr range (Fig. 9). A protein which appears at ~45 kD is nonspecifically immunoprecipitated and is seen in immunoprecipitates using an mAb isotype control (see Figs. 10 and 11). The majority of the I-E$^k$ was present in the lighter fractions 4–9 which contain endosomes, Golgi, PM, and ER (Fig. 9). Small but detectable amounts of I-E$^k$ are present in fractions 10 through the end of the gradient. The strong antigen-presenting activity associated with the denser fractions, namely 13–15 and 19–22 (Figs. 5 and 7) suggests that the class II contained in these

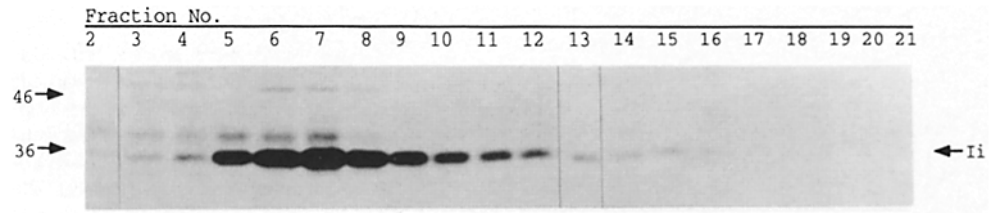


*Figure 8.* Subcellular distribution of Ii. CH27 cells were labeled with $^{35}$S-Met for 4 h, washed, homogenized, and fractionated by Percoll density gradient centrifugation. Ii was immunoprecipitated from fractions using the Ii-specific mAb IN-1. Immunoprecipitates were boiled and subjected to SDS-PAGE under reducing conditions. A composite photo is shown from two gels containing 13 samples each. The molecular weight markers have been removed.

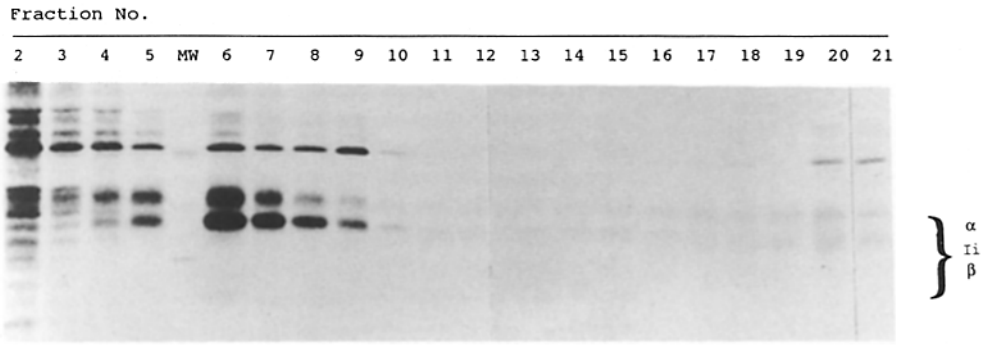


*Figure 9.* The subcellular distribution of I-E$^k$. CH27 cells were labeled with $^{35}$S-Met for 4 h, washed, homogenized, and fractionated on Percoll density gradient as in Fig. 8. Fractions were immunoprecipitated with the I-E$^k$-specific mAb 17.3.3s and the immunoprecipitates were boiled and subjected to SDS-PAGE under reducing conditions. Molecular weight markers (*MW*) are shown for the 31- and 46-kD markers. A composite photo is shown from two gels. One molecular weight marker was removed between fractions 20 and 21.

fractions is highly efficient at presenting antigen as compared to the bulk of the class II. However, the amount of class II detected in these compartments is likely significant given that we and others (57, 18) have shown that as few as 500 processed antigen–class II molecules per APC are sufficient for T cell activation.

As functional, processed antigen–class II complexes and class II molecules capable of stimulating T cells when provided with peptide in vitro were detected in fractions 19–22 and 13–15, respectively, it was of significant interest to determine if the class II molecules in these compartments were in the so-called floppy or compact forms. As discussed above, existing evidence indicates that $\alpha\beta$ chains form compact dimers following stable peptide binding. Immunoprecipitates of I-E$^k$ were subjected to SDS-PAGE without reducing or boiling the samples, conditions under which the $\alpha\beta$ chains remain as dimers. Comparing Figs. 9 and 10 there is an appearance of two higher molecular weight forms of class II molecules under nonreducing nonboiling conditions. The higher forms correspond to the floppy and compact forms of class II molecules. In a background analysis not shown here, Western blotting of immunoprecipitates of class II molecules run under nonreducing, nonboiling conditions showed class II molecules migrated at these approximate positions. The endosome and PM/ER fractions appeared to contain both floppy and compact forms. A small amount of the floppy form appeared in fractions 13–15 and the compact form was detectable in fractions 19–21. To better visualize the compact and floppy forms in fractions 13–15 and 19–21, gradient fractions were again subjected to SDS-PAGE under nonreducing conditions without boiling. Upon longer exposure of the gel there was a striking difference in the I-E$^k$ in these fractions (Fig. 11). The compact form was only detected in the lysosome-containing fractions 19–21 while the floppy form was only detected in fractions 13–15. The higher molecular weight forms of class II molecules migrate as $\alpha\beta$ chain when boiled and reduced, and no class II molecules are immunoprecipitated using an isotype control antibody (Fig. 11). Additional analyses showed that the floppy and compact forms are present in B cells regardless of whether the B cells have bound and processed the antigen DM*c*–anti-Ig (Fig. 11). Thus, the vesicles containing the floppy and compact forms of class II molecules appear to be constitutively present and not induced in B cells undergoing Ig-mediated processing. Thus, the floppy and compact forms of class II molecules reside in discrete subcellular compartments of the B cell which correspond to compartments in which class II molecules are receptive to peptide binding or in which class II molecules have bound processed antigen.

## *Discussion*

The helper T cell recognition of antigen requires the processing of antigen and presentation of the processed antigen

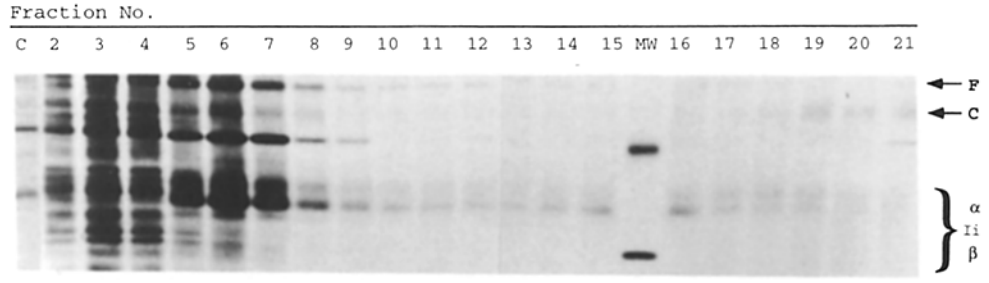


*Figure 10.* Subcellular distribution of the floppy and compact forms of I-E$^k$. CH27 cells were labeled with $^{35}$S-Met for 3–4 h, washed, homogenized, and fractionated by Percoll gradient centrifugation. Samples were immunoprecipitated with the I-E$^k$–specific mAb 17.3.3s and eluted with 2× nonreducing cocktail at room temperature. Samples were subjected to SDS-PAGE under nonreducing conditions. Molecular weight markers (MW) are shown for the 31- and 46-kD markers. A composite photo is shown from two gels. A molecular weight marker was removed between fractions 2 and 3. In addition an immunoprecipitate of pooled fractions 7–9 using an isotype-matched mAb is shown (*C*).

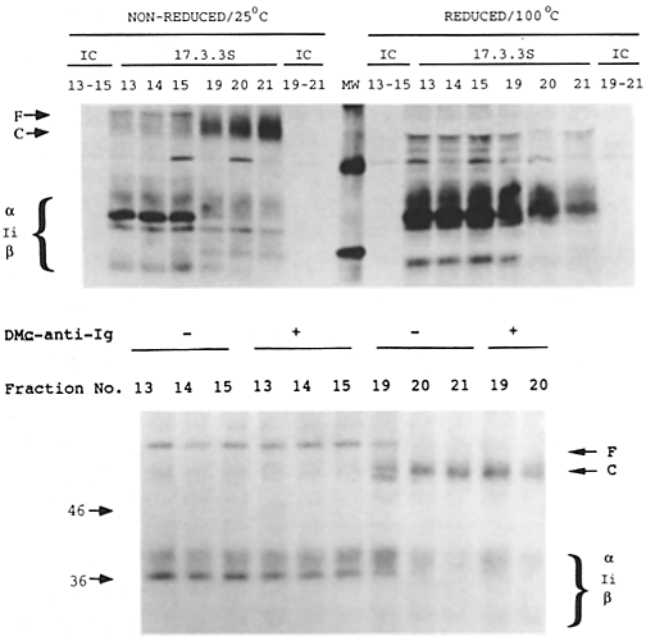


*Figure 11.* Distribution of the floppy and compact forms of I-E$^k$ in fractions 13–15 and 19–21. (*A*) CH27 cells were incubated with $^{35}$S-Met for 3 h, washed, homogenized, and fractionated by Percoll gradient centrifugation and fractions or pools of fractions were immunoprecipitated with the I-E$^k$-specific mAb 17.3.3s or isotype control antibody (*IC*). Samples were subjected to SDS-PAGE under reducing conditions after boiling the sample (reduced, 100°C) or under nonreducing conditions without boiling the sample (nonreduced, 25°C). (*B*) CH27 cells were incubated in media alone or in media containing DMc-anti-Ig for 4 h at 37°C in the presence of $^{35}$S-Met. Cells were washed, homogenized, fractionated by Percoll gradient centrifugation and fractions were immunoprecipitated with the I-E$^k$-specific mAb 17.3.3s. Samples were eluted with 2× nonreducing cocktail at room temperature and subjected to SDS-PAGE under nonreducing conditions.

bound to the major histocompatibility complex class II molecules. Recently, a considerable amount has been learned concerning the nature of processed antigens bound to the class II molecules (14, 31, 53) and the structure of the processed antigen–class II complex (8). In addition, the binding of peptides to class II has been studied in vitro in considerable detail (3, 9, 63, 66). Taken together, these studies have contributed greatly to our understanding of the assembly of peptide–class II complexes. However, a significant gap in our understanding of antigen processing remains, namely: where in the cell do critical events in the assembly of processed antigen–class II complexes occur? This is a particularly important consideration as the kinetics of peptide–class II binding measured in vitro, are extremely slow both for association and dissociation (9, 66) and do not reflect the relatively rapid assembly of processed antigen–class II complexes in antigen-presenting cells (17, 37, 42). The microenvironments of individual compartments differ significantly and may dramatically influence the assembly events. Indeed, the results of Jensen (33) and others (28, 54, 65, 66) showed that the formation of peptide–class II complexes is favored by acidic conditions that mimic the pH microenvironment of the endocytic pathway.

Current evidence indicates that class II molecules are distributed throughout the endocytic pathway (6). However, it is not known where processed antigen–class II complexes form. Here we address that question using a combination of subcellular fractionation and assays for functional, processed antigen–class II complexes. We show that these complexes are first formed in vesicles which cosediment with lysosomes in B lymphocytes and not in early endosomes or in the bulk of late endosomes. Within 15 min after antigen is bound to the B cell surface Ig, dense vesicles contain class II molecules capable of activating antigen-specific T cells in vitro. The activation of T cells by the class II molecules is dependent on the addition of antigen to the B cells and is blocked by antibodies to the class II molecules, indicating that T cell activation indeed reflects the presence of processed antigen–class II complexes. The Percoll gradient fractions in which T cell stimulatory activity is detected are hexosaminidase and LAMP-1 positive and contain HRP following a 25-min chase period. Rab 7, a marker for late endosomes, is also detected in these fractions, however, rab 5, a marker for early endosomes is absent. Class II molecules but not Ii are present in these fractions. From the experiments described here it is not possible to know if antigen is processed in a new compartment dedicated to this function in B cells or to well characterized compartments such as rab 7$^+$ late endosomes or lysosomes. By immunoelectron microscopy, Peters, et al. (50) have previously described a compartment, termed MIIC, which contains class II molecules but not Ii, is β-hexosaminidase and LAMP-1 positive and contains HRP 60 min after a short pulse. The vesicles we describe here may be the MIIC. Immunoelectron microscopy of the vesicles cosedimenting with lysosomes will address this possibility.

In the continued presence of antigen, processed antigen–class II complexes are observed in vesicles cosedimenting with lysosomes for at least 3 h. After 2 h, functional complexes are also detected in vesicles which cosediment with endosomal compartments and after 4 h are detected in the fractions which contain PM. The sequential appearance of the functional complexes in these fractions suggests that they are initially assembled in a compartment which cosediments with lysosomes and are then transported through the endosomes to the PM. Perhaps one of the most interesting findings presented here is that the processed antigen–class II complexes do not persist in the dense compartment beyond 4 h. This is found to be the case despite the fact that the antigen is continuously present in the B cell culture. Moreover, the concentration of antigen present in culture (~2 nM) is considerably less than would be required to saturate the surface Ig. We have observed a similar phenomenon when measuring the time dependence of the processing and presentation of antigen by splenic B cells. Unlike the presentation of pinocytosed antigen which persists as long as antigen is present (37), the peak presentation of antigen internalized bound to the surface Ig is maximal between 4 and 6 h. Presentation declines thereafter and by 12 to 24 h B cells no longer present the antigen. A similar time course was observed for a single round of antigen processing using a biochemical assay measuring the binding of radiolabeled antigen to class II (42). It is possible that binding of antigen to the surface Ig both triggers the initiation of processing and precludes processing of antigen which subsequently binds to surface Ig. Indeed, the surface Ig is a signal-transducing

receptor and our earlier studies showed that binding antigen to the surface Ig influenced the processing of antigen independent of internalization of the antigen bound to Ig (10). The impact of antigen binding to Ig on subsequent events in the processing pathway is of significant future interest.

In addition to identifying a subcellular compartment in which processed antigen–class II complexes are formed, the studies presented here also identify a compartment which is less dense than lysosomes and contains class II molecules which are capable of presenting a peptide antigen when provided in vitro. The class II molecules in this compartment have not yet bound processed antigen but are competent to bind peptide. This suggests that this compartment may not have access to processed antigen, consistent with the observation that HRP pinocytosed into cells does not enter this compartment. Ii is also contained within this compartment, but is clearly absent from the vesicles cosedimenting with lysosomes which contain processed antigen–class II complexes. We do not know if all class II molecules in this compartment are associated with Ii or whether there is a mixture of class II molecules, only some of which are bound to Ii. Since Ii blocks binding of peptides to class II molecules, the ability of the class II molecules to present peptide would suggest that Ii is not bound to at least a portion of the class II molecules. Strikingly, the class II molecules are in an SDS-stable conformation but migrate as a floppy form, slower than the compact form observed in the vesicles which sediment with lysosomes. The floppy form of class II is proposed to be an intermediate form between free $\alpha$ and $\beta$ chains and compact dimers (55). The class II molecules assume the compact form when peptides are bound (58) but whether floppy forms of class II molecules have peptide bound is not known. It is possible that the floppy form of class II molecules identified here has bound a peptide which does not induce the compact form. However, if so, this is not a peptide derived from the exogenous antigen.

It is interesting to speculate that the compartment which contains the floppy form of class II molecules is a precursor to the compartment which cosediments with lysosomes in which class II molecules bind processed antigen. Class II molecules may enter this compartment directly from the TGR. Alternatively, class II molecules could be retrieved from the cell surface and delivered to this compartment. Here the conditions may be appropriate for the dissociation of Ii from class II molecules. Once Ii dissociation is complete, class II molecules would be free to move into the lysosome-like compartment in which processed antigen is present. In this model, Ii would function to retain class II in a preloading compartment until conditions are appropriate for processed antigen loading to occur. Having entered the dense compartment, class II molecules may bind processed antigen, forming compact, functional dimers.

An alternative possibility for the floppy-class II–containing compartment is suggested by the recent studies of Germain and Rinker (23). They showed that class II dimers which fail to bind peptide form aggregates and are not delivered to the cell surface and likely represent a dead-end in assembly. Thus, it is formally possible that the floppy-class II–containing compartment corresponds to class II aggregates. The addition of peptide in culture would presumably result in deaggregation and account for the observed presentation of the peptide. However, the presence of Ii argues against this possibility as the aggregated dimers described by Germain and Rinker contain no Ii. Moreover, if the compartment containing the floppy form of class II molecules is not a precursor to the compartment containing compact class II molecules, it is difficult to explain the sequential disappearance of Ii described here. Pulse–chase experiments coupled with cell fractionation will be necessary to address this issue.

Regardless of the order of events in the processing pathway, the results presented here show that the class II molecules in floppy and compact forms reside in physically separable compartments. Significantly, these two compartments also define the peptide-binding states of the class II molecules. This finding suggests that these two forms of the class II molecules exist in discrete subcellular environments and may have distinct physiological roles in the assembly pathway. Lastly, our results do not support a key role for early endosomes or the bulk of late endosomes in the assembly process. However, our experiments only address the processing of the model antigen cytochrome *c* in B lymphocytes following receptor-mediated endocytosis. It is certainly possible that in other antigen-presenting cell types the processing compartments may not be identical and that other types of antigens may have different processing requirements. Future studies comparing processing compartments taking these potential variables into account should be of interest.

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