

Biochemical Evidence for the Rapid Assembly and Disassembly of Processed Antigen–Major Histocompatibility Complex Class II Complexes in Acidic Vesicles of B Cells

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Summary

Helper T cell recognition of antigen requires that it be processed within antigen-presenting cells (APC) to peptide fragments that subsequently bind to major histocompatibility complex (MHC) class II molecules and are displayed on the APC surface. Heretofore, processed antigen–MHC class II complexes have been detected by functional assays, measuring the activation of specific T cells. We now report direct, biochemical evidence for the assembly of processed antigen–MHC class II complexes within splenic B cells as APC. The I-E^k MHC class II molecules were immunoprecipitated from B cells that had processed the model protein antigen cytochrome *c* radiolabeled across its entire length by reductive methylation of lysine residues and covalently coupled to Ig-specific antibodies, allowing internalization after binding to surface Ig. Our previous studies showed that I-E^k immunoaffinity purified from B cells that had processed cytochrome *c* contains functional processed antigen–MHC class II complexes and that approximately 0.2% of the I-E^k molecules are specifically associated with one of two predominant processed antigenic fragments. Here we show that these complexes are rapidly assembled, within 30–60 min after antigen binding to surface Ig on splenic B cells. Maximal numbers of complexes are assembled by 2 h in a process that is sensitive to acidic vesicle inhibitors but not to inhibitors of protein synthesis. The processed antigen–I-E^k complexes have a relatively short half-life of 2–4 h and are disassembled or degraded within 8 h after antigen is first internalized. The disassembly or degradation of the processed antigen–I-E^k complexes requires acidic vesicle function, and in the presence of an acidic vesicle inhibitor the complexes are long lived. Thus, using a biochemical assay to monitor processed antigen–I-E^k complexes, we find that, in B cells, processed antigen is relatively rapidly associated in acidic vesicles with preexisting MHC class II molecules, and the complexes are disassembled 4–6 h later in processes that also require acid vesicle function.

It is well established that the recognition of protein antigens by helper T lymphocytes requires the processing and presentation of the antigen by APC (1). Most of our understanding of the events in the processing pathway comes from studies of the effects of cellular inhibitors on the ability of an APC provided with a native antigen to stimulate specific T cells, and from analyses of the ability of APC to stimulate T cells when provided with synthetic peptides that mimic processed antigen. Results of such studies have lent valuable insights to the antigen processing pathway, indicating that antigen is taken into an acidic compartment within APC where proteolysis proceeds, producing peptide fragments that ultimately bind to the MHC class II molecules and are subsequently displayed on the APC surface for recognition by specific T cells. Here we describe a biochemical assay for the assembly of processed antigen–MHC class II complexes and show that in B cells, as APC, the formation of such com-

plexes does not require new protein synthesis and both the assembly and disassembly of complexes requires acidic vesicle function.

APC internalize antigen either by fluid phase pinocytosis or, in a more efficient fashion, by receptor-mediated endocytosis, which for B cells is accomplished by antigen binding to specific surface Ig (2). The discrete compartments in which antigen is degraded and in which peptides bind to MHC class II have not yet been identified. As for most membrane proteins, the α and β chains of the MHC class II molecules are synthesized in the endoplasmic reticulum (ER).¹ Within minutes of synthesis, they associate with invariant chains (Ii) and are transported through the Golgi apparatus with a half-

¹ Abbreviations used in this paper: DMc, ³H-Drosophila melanogaster cytochrome *c*; ER, endoplasmic reticulum; Ii, invariant chains; Pt, pigeon cytochrome *c*.

time of 30–60 min (3–5). The MHC class II resides in a post-Golgi compartment, during which time the Ii chain dissociates (4, 6). This compartment is able to fuse with pinocytotic vesicles carrying antigen into the cell (4, 7). Recent studies indicate that MHC class II bound to Ii cannot bind peptide (8, 9), suggesting that Ii functions to insure that peptides do not bind to the MHC class II until it reaches a post-Golgi compartment that intersects with exogenous antigen. Brodsky and coworkers (10) have shown by immunoelectron microscopy that within minutes of binding to surface Ig, antigen is internalized into compartments that contain MHC class II, Ii, and the proteases cathepsin B and D. As this compartment contains all the cellular machinery presumed necessary for antigen processing, it has been suggested as a processing site, although functional data remain to be provided. Harding et al. (11) have recently shown that in macrophages, for the antigen lysozyme, processing requires a lysosomal/late endosomal function, and have proposed that processed peptides are transported from the lysosomes to vesicles containing MHC class II. Protein synthesis inhibitors have been shown to block antigen presentation (12–14), as has treatment with brefeldin A (13, 14), which blocks transport of proteins from the ER, suggesting that newly synthesized MHC class II molecules are required for assembly of peptide–MHC class II complexes. However, the possibility that cell surface MHC class II recycles into the cell and binds peptide has not been ruled out. Indeed, recent evidence indicates that MHC class II in B cells rapidly recycles, providing the opportunity for binding to processed antigen (15–17).

The assembly of processed antigen–MHC class II complexes has not been studied directly in the APC, most likely due to the fact that only a small number of processed antigen–MHC class II complexes are assembled in APC. Indeed, recent estimates indicate that this number is between 50 and 1,000/APC (18–20). Synthetic antigenic peptides have been shown to bind to purified MHC class II molecules in detergent solution (21, 22) and in planar membranes (23) *in vitro*. The binding kinetics *in vitro* are unusual in that the association rate is extremely slow and, once bound, the off rate is negligible, making peptide binding to MHC class II nearly irreversible (22). Indeed, Lee and Watts (24) have shown that the release of peptide from MHC class II *in vitro* requires extreme conditions (pH 2 for 2 h). At present, it is not known if the measurements of peptide binding to mature MHC class II molecules *in vitro* are a true reflection of the assembly of newly processed antigen and MHC class II complexes inside the cell. Indeed, recent studies by Jensen (25) and Wettstein et al. (26) showed that the binding of peptide to MHC class II *in vitro* is facilitated by acidic pH, suggesting that the microenvironment in which peptide encounters MHC class II may dramatically affect assembly.

Here we report the kinetics of the assembly and disassembly of processed antigen–MHC class II complexes in splenic B cells. The work to be described is based on our previous results, which showed that the I-E^k MHC class II molecules immunoaffinity purified from APC that have processed the antigen cytochrome *c* are stimulatory to specific T cells when

incorporated into liposomes in the absence of any additional antigen (27). This unequivocally demonstrated that functional, naturally processed, antigenic peptide–I-E^k complexes are stable during immunoaffinity purification. Subsequent studies, in which I-E^k was immunoaffinity purified from cells that had processed radiolabeled cytochrome *c*, allowed the quantitation and characterization of the naturally processed radiolabeled peptides bound to the I-E^k molecules (20). These results showed that ~0.2% of I-E^k contains processed antigen composed of two predominant peptides and that the association is highly specific in that the processed antigen bound to I-E^k was not identical to that bound to I-A^k and no processed antigen bound to class I K^k/D^k. We describe now the assembly of processed antigen–I-E^k complexes in splenic B cells after antigen binding to surface Ig. The antigen monitored is radiolabeled cytochrome *c* covalently coupled to antibodies specific for mouse Ig, allowing efficient internalization into B cells (28). Processed antigen–MHC class II complexes were detected by immunoprecipitating the MHC class II and monitoring radioactivity. We found that antigen bound to surface Ig is rapidly internalized and degraded into small molecular weight products within an hour. Processed antigen–MHC class II complexes form 1 h after antigen is internalized and are transiently observed over a 4-h period. Processed antigen–MHC class II complexes are not assembled in the presence of NH₄Cl, a documented inhibitor of antigen presentation to T cells (1). However, the assembly of the peptide–MHC class II complexes is not blocked by inhibitors of protein synthesis which also have been shown to inhibit antigen presentation (12–14), distinguishing the assembly of the complexes from their presentation to T cells. By 6–8 h, the processed antigen–MHC class II complexes are no longer detectable even though there is no appreciable turnover of the MHC class II molecules during this time. In the presence of NH₄Cl, the processed antigen–MHC class II complexes are found to be long lived, demonstrating that the disassembly or degradation of these complexes requires acidic vesicle function. Thus, in contrast to the kinetics of peptide–MHC class II complex formation *in vitro*, the kinetics *in vivo* reported here show that splenic B cells rapidly associate processed antigen with MHC class II in an acid vesicle–dependent manner, and disassemble or degrade these complexes in an acid vesicle–dependent pathway 4–6 h later.

Materials and Methods

Preparation of B Cells. B cells were obtained from mouse spleens as described (29). Briefly, spleens were isolated from CBA/J mice (The Jackson Laboratory, Bar Harbor, ME) and perfused with DME to release cells into a single cell suspension. The spleens were teased to remove any residual cells. The single cell suspension was washed once with DME and resuspended at 2 ml/spleen. The single cell suspension was transferred to vacutainer tubes (Becton Dickinson & Co., Mountain View, CA) at 4 ml per tube, underlaid with 4 ml of Histopaque 1119 (Sigma Chemical Co., St. Louis, MO) and spun at 1,200 *g* for 15 min to remove red blood cells. The cells at the interface were harvested, washed twice with DME, and counted. The cells were depleted of T cells by complement lysis

using antibodies specific for CD4(GK1.5), CD8 (243.5), and Thy-1.2 (AT83A) and rabbit complement. The cells were then washed to remove cell debris and resuspended in complete medium to the desired concentration.

Reductive Methylation of Pigeon Cytochrome *c* (Pc). Pc was methylated according to Jentoft and Dearborn (30). Pc was dissolved at 2 mg/ml in 10 mM PO₄, pH 7.0, and either sodium cyanoborohydride (Aldrich Chemical Co., Milwaukee, WI) or ³H-sodium cyanoborohydride (Amersham Corp., Arlington Heights, IL). Formaldehyde (1 M in 10 mM PO₄, pH 7.0) was added such that the molar ratio of the reactants was 1:64:64 respectively. The reaction was allowed to proceed for 4 h at room temperature. The methylated Pc was separated from excess reactants by gel filtration on a Sephadex G-25 column. Methylated Pc products were separated using a mono S HR 5/5 column (Pharmacia Fine Chemicals, Piscataway, NJ) equilibrated in 10 mM PO₄, pH 7.0, buffer. The sample was eluted using a linear gradient over 60 min with 10 mM PO₄, 250 mM NaCl. Fractions (1 ml) were collected. For ³H-Pc, an aliquot of each fraction was removed for liquid scintillation counting. For nonradioactive methylated Pc, fractions were buffer changed into PBS for use in tissue culture experiments.

Preparation of Methylated-Pc-Anti-Ig. Pc was thiolated using 2-iminothiolane (Pierce Chemical Co., Rockford, IL) at a molar ratio of 1:0.06, for 30 min at 4°C. The low 2-iminothiolane-to-Pc ratio was selected to produce monothiolated Pc so as to maintain the antigenicity of the thiolated Pc and to prevent polymerization. The thiolated Pc was separated from unmodified Pc by affinity chromatography on activated-thiol-propyl Sepharose 4B (Pharmacia Fine Chemicals) by incubating Pc overnight with the thiol gel, washing the gel free of unbound Pc, and then eluting thiolated Pc with 50 mM DTT, pH 8. Approximately 3% of the total Pc is bound to the thiol-propyl sepharose indicating that a low level of thiolation was achieved. The eluted thiol-Pc was buffer changed into 10 mM PO₄, 1 mM EDTA, 1.5 mM DTT, pH 7.0, using a Sephadex G-25 column, and concentrated to 2 mg/ml in a Centricon 10 (Amicon Corp., Danvers, MA). The thiolated-Pc was reductively methylated using either sodium cyanoborohydride (Aldrich Chemical Co.) or ³H-sodium cyanoborohydride and concentrated as described above. Rabbit anti-mouse F(ab')₂ (Pierce Chemical Co.) was reacted with a 10-fold molar excess of *N*-succinimidyl 3-(2-pyridyl-dithio) propionate (SPDP) (Pierce Chemical Co.) for 30 min at room temperature. The modified antibody was buffer changed into 10 mM PO₄, 150 mM NaCl pH 8.0 using a Sephadex G-25 column, and then concentrated to ~1 ml using a Centricon 30. The methylated-thiolated-Pc was reacted with SPDP-modified antibody for 16 h at room temperature. The resulting methylated-Pc-anti-Ig was separated from methylated-Pc by gel filtration chromatography on a Sephadex G-75 column. The ratio of ³H-Pc to anti-Ig in the conjugates ranged in different preparations from 1:3 to 1:5. The specific activity of the ³H-Pc was 4–6 × 10¹⁶ cpm/mol Pc.

Assay for Antigen Internalization and Degradation. B cells were incubated with 0.2 μM ³H-Pc-anti-Ig on ice for 1 h in the presence of 25% normal rabbit serum, pelleted, and washed twice with FCS. The washed cells were resuspended at 2 × 10⁷ cells/ml, and 1 ml of the cell suspension was aliquoted for each time point. The cells were rapidly warmed to 37°C, incubated for the desired time, and then pelleted. The supernatant was removed and designated the released fraction. The cells were resuspended in 0.2 mM HCl, 150 mM NaCl and incubated for 15 min on ice. The cells were again pelleted, and the supernatant was removed and designated the surface fraction. The cell pellet was resuspended and designated the internal fraction. A portion of each fraction was counted by liquid scintillation. The remainder of each fraction was precipitated

with TCA (10% final concentration) for 90 min. The precipitate was pelleted and a portion of the supernatant removed for counting.

Immunoprecipitation of Peptide-I-E^k Complexes. B cells (10⁸) were incubated as above with ³H-Pc-anti-Ig for 1 h on ice, washed and incubated at 37°C for varying lengths of time, washed, and pelleted. The cell pellet was lysed in 0.15 M NaCl containing 0.05 M Tris, 5 mM EDTA, 0.5% NP-40, 200 μg/ml PMSF (Boehringer Mannheim Biochemicals, Indianapolis, IN), and 25 μg/ml Aprotinin (Boehringer Mannheim Biochemicals), pH 7.6, for 45 min on ice with frequent vortexing (31). The lysate was cleared of debris by centrifuging, first at 1,200 g for 30 min, then at 100,000 g for 1 h. The lysate was incubated twice at 4°C for 1.5 h with 800 μl of a 1:1 protein A-Sepharose slurry (Pharmacia Fine Chemicals). The lysate was incubated for 15 h at 4°C with 30 μg of mAbs specific for either I-E^k, 14.4.4S (32), or for B220, 14.8 (33), that had been preabsorbed to 120 μl of a 1:1 protein A-Sepharose slurry. After incubation, protein A-Sepharose was washed with 0.15 M NaCl, 0.05 M Tris, 5 mM EDTA, 0.2% NP-40 until the washes contained no tritium as assayed by liquid scintillation counting. The protein A-Sepharose was resuspended in 62.5 mM Tris, pH 6.8, 2% SDS buffer for 15 min to release the bound material. The protein A-Sepharose was pelleted and the radioactivity in the supernatant and on the Sepharose beads determined. No radioactivity was detected associated with the beads indicating all bound material had been eluted.

When indicated B cells were surface iodinated (34) before immunoprecipitation of I-E^k. B cells (1.5 × 10⁸) were washed three times with 10 mM PO₄, 150 mM NaCl, pH 7.4 (PBS), resuspended at 3 × 10⁸ cell/ml in PBS, and chilled on ice. 20 μl of 10 μM Na₂SO₃ and 40 μl 100 mM PO₄, pH 7.0, was added to 1 mCi NaI (ICN Biomedicals, Irvine, CA) for a total volume of 78 μl. Lactoperoxidase (Calbiochem-Behring Corp., San Diego, CA) (20 U) and the NaI solution were sequentially added to the cells and mixed. Hydrogen peroxide (1.8 mM) was added in six 50-μl aliquots at 2-min intervals. Cells were washed twice with PBS, then four times with DME. The cells were 93% viable by trypan blue exclusion. Immunoprecipitation was carried out as above.

Treatment of B Cells with Inhibitors. B cells were incubated with NH₄Cl (10 mM) for 1 h before the addition of ³H-Pc-anti-Ig and maintained in NH₄Cl throughout the completion of the experiment. The concentrations of NH₄Cl used are sufficient to block antigen presentation (35). B cells were treated with 10 μg/ml cycloheximide for 3 h before the addition of ³H-Pc-anti-Ig and maintained in cycloheximide thereafter. B cells were treated with 100 μM anisomycin for 1 h before addition of ³H-Pc-anti-Ig and maintained in anisomycin thereafter. To determine the degree of protein synthesis inhibition by cycloheximide and anisomycin, B cells (10⁷) were treated as described above followed by a 4-h incubation with 44 μCi of ³⁵S-methionine in the presence of cycloheximide or anisomycin. The cells were washed with complete media until the washes no longer contained detectable ³⁵S as measured by liquid scintillation counting. Treatment reduced ³⁵S incorporation by >90%.

Measurement of APC Function. Either B cells or cells of the CH27 B cell lymphoma line (5 × 10⁴) as APC (36) and TPr9.1 hybridoma cells (5 × 10⁴) (37) were cocultured overnight with graded doses of antigen. Culture supernatants were removed and tested for their IL-2 content by the ability to maintain the growth of the IL-2-dependent T cell line, CTLL (38). CTLL growth was measured by the conversion of 3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) to a blue formazan product having an absorbance at 570 nm (39) or by the incorporation of [³H]thymidine (38). To follow the processing of Pc-anti-Ig, B cells

were incubated with methylated Pc-anti-Ig at a Pc concentration of 0.2 μM , at 4°C, washed, and assayed for the ability to activate TPc9.1 cells to IL-2 secretion. IL-2 was measured by the ability of culture supernatants to maintain the growth of CTLL cells assayed by the incorporation of [^3H]thymidine.

Results

Characterization of Radiolabeled Cytochrome *c*-Antibody Conjugates. Pc was reductively methylated at lysine residues using ^3H -sodium cyanoborohydride (^3H -Pc). Pc, a 104-amino acid protein, contains 18 lysine residues (Fig. 1) spaced relatively evenly across the length of the polypeptide chain (40). Thus, after methylation to introduce ^3H , most peptide fragments of Pc resulting from processing will be radiolabeled and therefore can be detected. Significantly, the major antigenic determinant of Pc recognized by T cells and monitored here (minimally residues 95–104) contains two lysine residues, ensuring its detection. Micro-sequencing of a 23-residue COOH-terminal peptide fragment generated by CNBr cleavage of ^3H -cytochrome *c* confirms that all lysine residues in this peptide are tritiated (data not shown).

The antigenicity of methylated Pc was compared with unmodified Pc. It was predicted that methylation of Pc would have little effect on the protein's antigenicity as the charge on the lysine residues would not be effected nor would the modification significantly increase the size of the residue. Indeed, recently published results show that ^3H -*Drosophila melanogaster* cytochrome *c* (DMc) and unmodified DMc were antigenically comparable with respect to pulsing the B cell lymphoma CH27, thereby indicating that for CH27 cells methylation of DMc does not greatly affect its antigenicity (20, 27). Pc was modified using cold cyanoborohydride, chromatographed on an ion exchange resin, resolving three populations, designated A, B, and C (Fig. 2). Similar chromatography of ^3H -Pc (Fig. 2) indicates that each population contains Pc modified on an average at six lysine residues, and thus the populations likely represent ^3H -Pc modified at different subsets of lysine residues. Methylated and unmodified Pc were tested for the ability to be processed and presented by B cells to a Pc-specific I-E^k-restricted T cell hybrid, TPc 9.1 (35). The methylated pools of Pc were nearly equivalent to unmodified Pc in their ability to be processed and presented to T cells (Fig. 2). Thus, methylated Pc is processed to a relevant antigenic peptide fragment and associated with I-E^k in B cells.

To study the processing of antigen internalized after binding to the surface Ig, ^3H -Pc was covalently coupled to affinity-purified rabbit antibodies specific for mouse F(ab')₂ of Ig

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1  GDVEKGGKKIFVOKCSQCHTVEKGGKHKKTGPNLHG
34  LFGRKGTGQAEGRFSYTDANKNKGITWGEDTLMEYL
69  ENPKKYIPGTKMIFAGIKKKAERADLIAYLKQATAK

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Figure 1. The amino acid sequence of Pc using standard one-letter amino acid abbreviations. The 18 lysine residues have been highlighted and are the sites of tritiation. The minimal antigen peptide, Pc 95–104, is underlined.

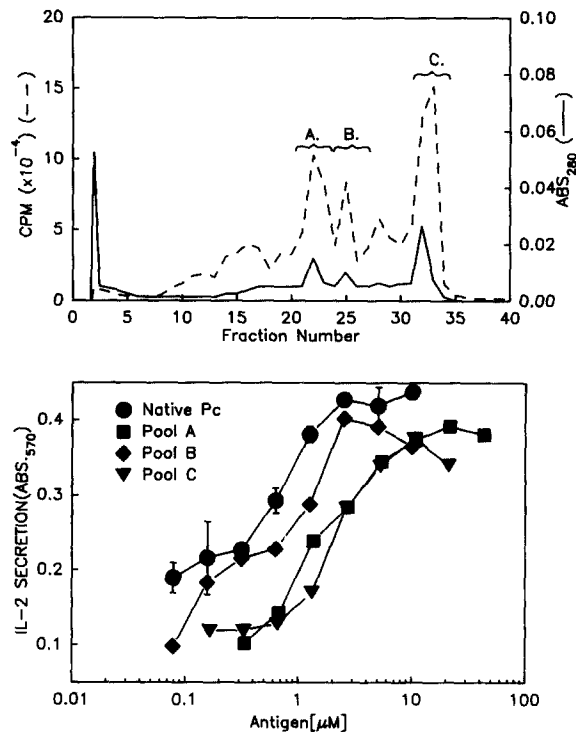


Figure 2. Antigenicity of methylated Pc. (Top) Pc was reductively methylated using ^3H -sodium cyanoborohydride as described in the Materials and Methods, and the resulting reaction mixture was fractionated by ion exchange chromatography. Three pools were formed from the fractions and designated A, B, and C containing Pc modified at 6.9, 5.6, and 5.8, lysines respectively. The unfractionated mixture contained Pc modified at 5.8 lysines on average. (Bottom) Pc was reductively methylated using cold sodium cyanoborohydride and chromatographed on an ion exchange resin as above. Pools A, B, and C were buffer changed into PBS and graded doses of each pool were compared with respect to unmodified Pc for their ability to stimulate TPc9.1 cells to secrete IL-2.

(^3H -Pc-anti-Ig). Ig-mediated internalization of antigen was considered desirable both because it likely represents the major route of antigen entry into B cells under physiological conditions and because it would allow concentration of antigen into the processing pathway facilitating its detection. Indeed, earlier studies showed that coupling Pc to Ig-specific antibodies greatly increases the efficiency with which the antigen is processed (28), allowing formation of maximal numbers of processed antigen-MHC class II complexes using $\sim 0.1\%$ the concentration required for Pc alone. ^3H -Pc-anti-Ig binds to B cells at 4°C in a concentration-dependent fashion and can be competed by unmodified Ig-specific antibodies, indicating specific binding to surface Ig (Fig. 3). To compare the incorporation of Pc and Pc-anti-Ig during processing, B cells were incubated for 6 h at 37°C with ^3H -Pc-anti-Ig or ^3H -Pc, washed, and cell-associated radioactivity was measured (Fig. 3). Radioactivity is detectable in B cells incubated with as little as 0.005 μM ^3H -Pc-anti-Ig, whereas >500 -fold as much ^3H -Pc is required to reach the same level of incorporation. The ability to measure radioactivity associated with B cells incubated with as little as 0.005 μM ^3H -Pc-anti-Ig indicates the technical feasibility of monitoring ^3H -Pc-anti-Ig as it is processed by the B cell, which would not be pos-

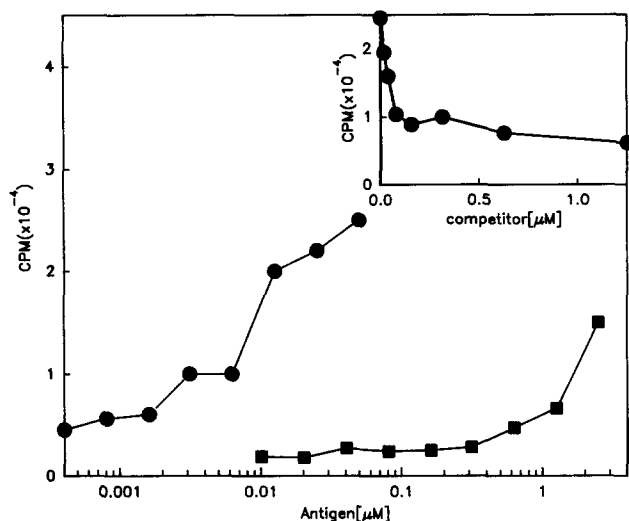


Figure 3. The binding of ^3H -Pc-anti-Ig to B cells. B cells (10^7) were incubated with graded concentrations of either ^3H -Pc (squares) or ^3H -Pc-anti-Ig (circles) for 6 h. The cells were pelleted, washed twice in FCS, resuspended in DME, and cell associated radioactivity was measured. (Inset) B cells (5×10^7) were incubated with ^3H -Pc-anti-Ig ($0.08 \mu\text{M}$) and graded doses of unlabeled rabbit anti-mouse F(ab')₂ for 1 h on ice. The cells were washed twice with FCS, and the amount of cell-associated radioactivity was measured.

sible relying on fluid phase uptake of the antigen. Furthermore, the amount of Pc-anti-Ig bound to B cells after incubation at 4°C with the conjugate at $0.2 \mu\text{M}$ Pc, a concentration used in all subsequent experiments, is sufficient for presentation to T cells. Indeed, B cells incubated at 4°C with the Pc-anti-Ig conjugate and washed are able to stimulate TPc9.1 cells to IL-2 secretion (data not shown).

Binding, Internalization, and Degradation of [^3H]Pc-Anti-Ig by B Cells. To further characterize the processing of ^3H -Pc-anti-Ig, B cells were incubated with ^3H -Pc-anti-Ig ($0.2 \mu\text{M}$) on ice for 1 h, washed, resuspended in complete media, and incubated at 37°C for varying lengths of time. Radioactivity was measured in three fractions: in the culture supernatant (released); associated at the cell surface, defined as that which is removed from cells by acid treatment (surface); and intracellular, defined as that which is not removed from cells by acid treatment (internal) (Fig. 4). At the initiation of the experiment, we calculate that $\sim 6 \times 10^3$ ^3H -Pc-anti-Ig conjugates are bound to each cell B lymphocyte considering a specific activity of the conjugate of 5×10^{16} cpm/mol ^3H -Pc. Upon warming, the majority of the ^3H -Pc-anti-Ig is internalized within 15–30 min. With continued incubation at 37°C , a small portion of ^3H -Pc-anti-Ig reappears on the surface, which may be recycled antigen bound to surface Ig. Over time at 37°C , the amount of cell surface and internal antigen gradually decreases while the amount in the supernatant increases.

To determine at what point the ^3H -Pc-anti-Ig is processed to peptide fragments, the released, surface, and internal fractions were each precipitated with TCA (Fig. 4). The radioactivity in solution, representing small peptides, was measured. Peptides are first detected in the released fraction between

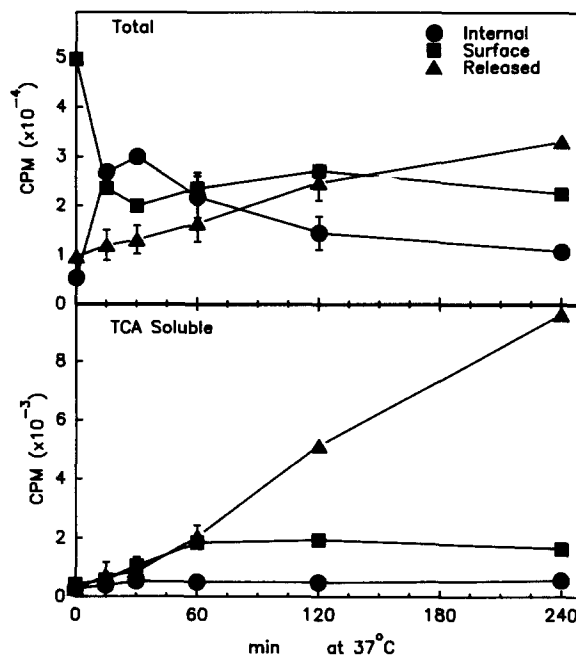


Figure 4. Time dependence of antigen internalization and degradation. (Top) B cells were assayed for the internalization and degradation of ^3H -Pc-anti-Ig as detailed in Materials and Methods. Briefly, B cells (1.2×10^8) were incubated with $0.2 \mu\text{M}$ ^3H -Pc-anti-Ig in 25% normal rabbit serum in complete medium for 1 h on ice, washed twice with FCS, resuspended in 6 ml of complete media, and aliquoted and incubated for the times indicated. The radioactivity in the released, cell surface, and internal fractions was measured. Shown are the cpm in each fraction versus time at 37°C . (Bottom) Each fraction was brought to 10% TCA and incubated on ice for 90 min. The precipitate was pelleted by centrifugation at $16,000 g$ for 45 min. An aliquot of the TCA-soluble supernatant was removed and counted by liquid scintillation. The data are expressed as the total cpm contained in each fraction versus the time at 37°C .

30 and 60 min, and the amount increases with continued incubation at 37°C . A small but detectable amount of radioactivity appears on the cell surface by 60 min. This amount remains on the surface for 1–2 h and gradually declines returning to background levels by 8 h. As will be shown below, a portion of the cell surface-associated radiolabeled antigen is bound to the I-E^k molecules. There is no detectable TCA-soluble material inside the cell at any time, indicating that there is not a significant intracellular pool of free antigenic peptides.

The released, surface, and internal fractions were analyzed by molecular sieve chromatography to gain additional information concerning the size of the processed antigen (Fig. 5). The time course of the appearance of degraded antigen is similar to that obtained monitoring TCA-soluble material. The first degradation products are detected in the released fraction and on the cell surface 30–60 min after warming to 37°C , while no free peptides are detected at any time in the internal fraction. The bulk of the first processed peptides detected both in the supernatant and on the cell surface are small (<25 residues). It is striking that no intermediate degradation products between the whole conjugate or Pc and small peptides are detectable at any time, suggesting that processing

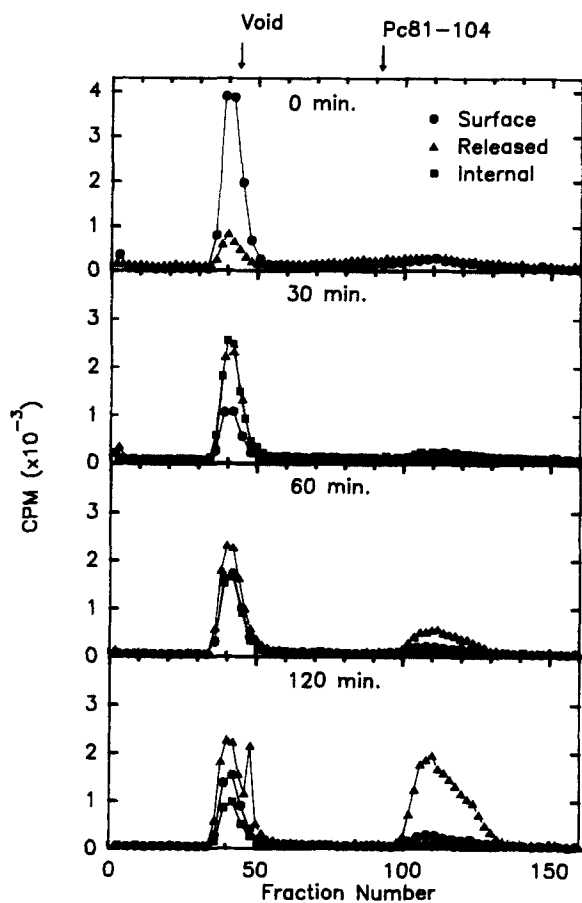


Figure 5. Molecular sieve chromatography of processed antigen. B cells (1.2×10^8) were incubated with ^3H -Pr-anti-Ig for 1 h at 4°C , washed, aliquoted, and incubated at 37°C for the times indicated, as described in Fig. 4. The released, cell surface, and internal fractions were collected and then chromatographed on a Sephadex G-50sf column (1×33 cm) equilibrated in 7% formic acid. The eluate was collected in $200\text{-}\mu\text{l}$ fractions and counted by liquid scintillation.

occurs in a highly degradative compartment where proteolysis goes to completion unless peptides are protected in some fashion.

To relate the observed patterns of internalization and degradation (Fig. 4) to antigen presentation, we determined the effects of documented inhibitors of APC function in this assay. In three separate experiments we evaluated the effects of reduced temperature, NH_4 , and cycloheximide on the internalization and degradation of ^3H -Pr anti-Ig. Reduced temperature (4°C) blocks both the internalization of the antigen by B cells and degradation of antigen bound to B cell surface Ig (Fig. 6), consistent with its effect on the ability of APC to stimulate T cells at reduced temperature. Treatment of B cells with the lysosomotropic agent NH_4Cl for 1 h before incubation with ^3H -Pr-anti-Ig completely blocks the degradation of the antigen, as shown by the complete absence of any TCA-soluble material in the released, cell surface, and internal fractions at any time (Fig. 6). NH_4Cl has little effect on the binding and internalization of the conjugate, however, there is a marked decrease in the amount of antigen

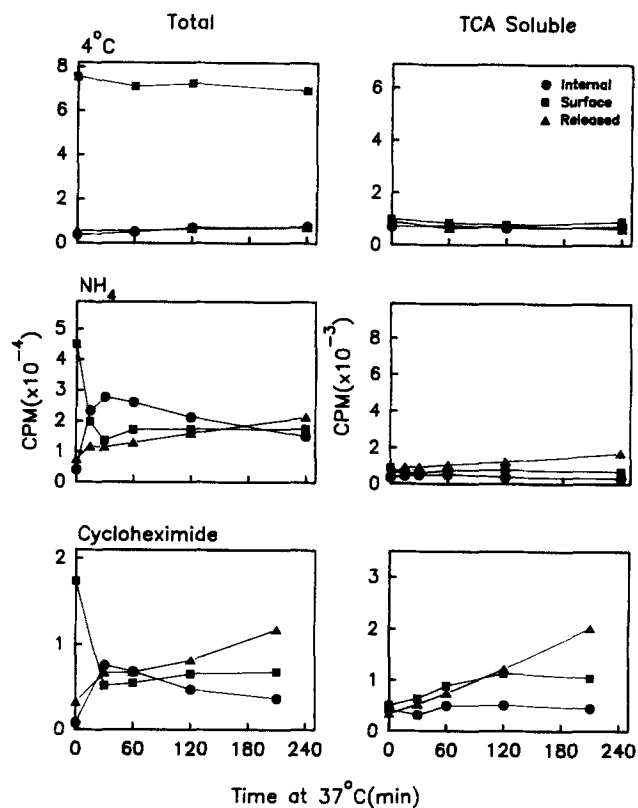


Figure 6. The effect of inhibitors on antigen internalization and degradation. Shown is a composite of the results of three separate experiments for which untreated cell controls were carried out for each. (Top) The internalization and degradation assay was carried out on B cells as described in Fig. 4 except the cells were maintained at 4°C for the times indicated. In control experiments where cells were warmed to 37°C , after ^3H -Pr-anti-Ig binding at 4°C , internalization and degradation patterns were similar to those shown in Fig. 4. (Middle) B cells were incubated in complete medium alone or complete medium containing $10\text{ mM NH}_4\text{Cl}$ at 37°C for 1 h before and during the assay for antigen internalization and degradation as in Fig. 4. Shown are results for NH_4 -treated cells. Control, untreated cells initially bound 4.5×10^4 cpm ^3H -Pr anti-Ig, and the internalization and degradation pattern were similar to those shown in Fig. 4. (Bottom) B cells were incubated in complete medium alone or complete medium containing $10\text{ }\mu\text{g/ml}$ cycloheximide at 37°C for 3 h before and during the assay for antigen internalization and degradation as in Fig. 4. Shown are results for cycloheximide-treated cells. Control untreated cells initially bound 1.5×10^4 cpm ^3H -Pr anti-Ig and internalization and degradation patterns appeared similar to those shown in Fig. 4.

in the released fraction and consequently a large portion of the antigen remains cell associated (Fig. 6). The inability of NH_4Cl -treated B cells to degrade the internalized antigen may block the release of the antigen, therefore resulting in its accumulation in the cell. The effect of NH_4Cl on antigen degradation in this assay is consistent with its documented ability to block APC function in T cell activation. Cycloheximide treatment has little effect on the binding, internalization, or degradation of antigen resulting in TCA-soluble material in the surface and released fractions (Fig. 6). Thus, cycloheximide's ability to block APC function in B cells must be due to a requirement for new protein synthesis in antigen presentation not measured in this assay.

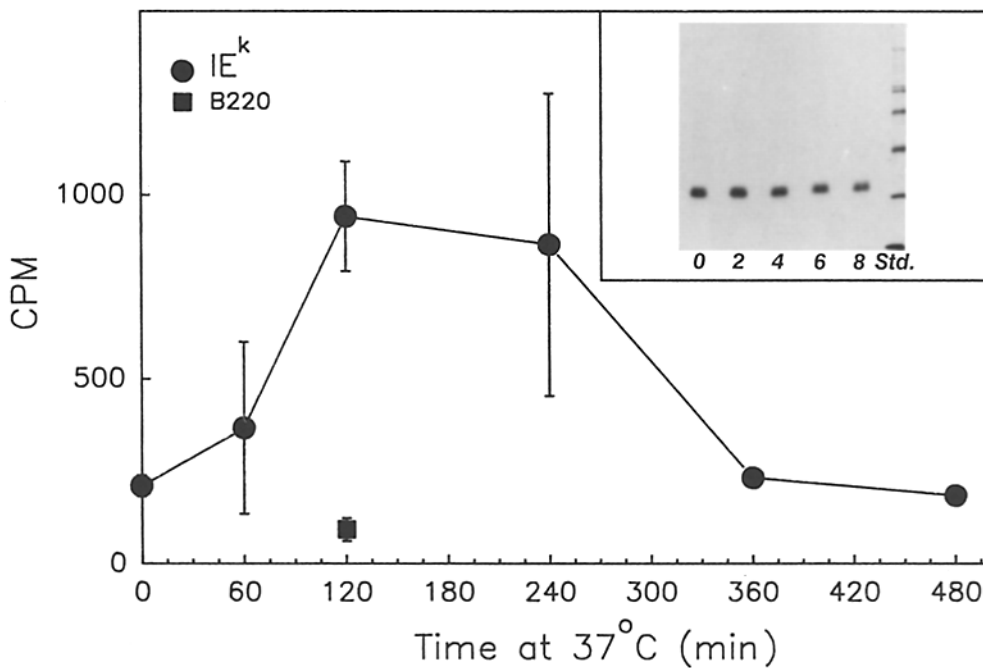


Figure 7. The kinetics of the assembly of processed antigen-MHC class II complexes in B cells. B cells (6×10^8) were incubated in complete media with $^3\text{H-Pc-anti-Ig}$ for 1 h on ice, washed thoroughly, and resuspended in complete media at 10^7 cells/ml. Aliquots of cells (10^8) were maintained at 37°C for the indicated times, pelleted, and frozen. Pellets were thawed, solubilized, and immunoprecipitated with either I-E^k- or B220-specific mAbs as described in Materials and Methods. The radioactivity in the precipitates is given with time at 37°C . Each data point represents between 2 and 10 immunoprecipitations carried out in independent experiments. (Inset) B cells were surface iodinated as described in Materials and Methods. The cells were resuspended in complete media at 10^7 cells/ml, and 1-ml aliquots were incubated at 37°C for varying lengths of time. The cells were pelleted and immunoprecipitated with I-E^k-specific

mAbs as described. Precipitates were analyzed by SDS-PAGE on a 10.5% polyacrylamide gel and shown is the autoradiograph of samples after 0, 2, 4, 6, and 8 h of incubation at 37°C . Surface iodination results in the labeling of only the β chain of I-E^k as previously shown which migrates at 35 kD (20).

The Assembly of Processed Antigen-MHC Class II Complexes in B Cells. To directly measure the binding of processed antigen to the I-E^k molecules, the kinetic experiments described above were repeated and at the end of each time point the I-E^k molecules were immunoprecipitated and the associated radioactivity was measured. As discussed above, previous studies showed that functional processed antigen-I-E^k complexes are stable to affinity purification, that the association of processed antigen with I-E^k in B cells is highly specific, and that ~ 800 – $1,000$ of the I-E^k molecules in each cell contained processed antigen (20, 27). Radiolabeled antigen is first immunoprecipitated with I-E^k ~ 1 h after internalization of $^3\text{H-Pc-anti-Ig}$ (Fig. 7). The amount of radioactivity precipitated is maximal at 2 h and remains at that level for the next 2 h. This time course is consistent with our previously published time course of the acquisition of the ability of B cells to process antigen so as to stimulate specific T cells (41). The radioactivity precipitated with I-E^k at 2 h represented $\sim 2\%$ of the $^3\text{H-Pc-anti-Ig}$ initially bound to the cell surface. From previous studies (20) in which the number of processed antigen-I-E^k complex was measured by direct biochemical methods, we estimate that this represents ~ 800 – $1,000$ complexes in the immunoprecipitate. The association of antigen with I-E^k is specific as radioactivity is not immunoprecipitated with proteins other than MHC class II. Indeed, no radioactivity is associated with B220 molecules (Fig. 7) under conditions where equivalent quantities of I-E^k and B220 are immunoprecipitated as measured by ^{35}S -methionine-labeled protein analyzed by SDS-PAGE (data not shown). The association of processed antigen with I-E^k is unlikely to be the result of released peptides binding to

surface I-E^k because processed antigen-I-E^k complexes begin to disappear after 4 h (Fig. 7), while the amount of processed antigen in the supernatant continues to increase up to 8 h (Fig. 4).

To assess the requirement for acidic vesicle function for the formation of processed antigen-MHC class II complexes, B cells were pretreated at 37°C with NH_4Cl for 1 h before incubation with $^3\text{H-Pc-anti-Ig}$ for 1 h at 4°C . The cells were then washed, and incubated at 37°C for 2 h in the continued presence of NH_4Cl to allow time for maximal processed antigen-MHC class II complex formation. The I-E^k and B220 molecules were immunoprecipitated from cell lysates. Treatment with NH_4Cl completely blocks processed antigen-I-E^k complex formation (Fig. 8), consistent with the effect of NH_4Cl on presentation of antigen as measured by the ability of B cells to activate T cells (35). Indeed, as shown (Fig. 6), $^3\text{H-Pc-anti-Ig}$ is internalized but not degraded in NH_4Cl -treated B cells indicating that antigen-MHC class II complexes cannot form unless the internalized antigen is degraded.

To assess the requirement for new protein synthesis on the assembly of processed antigen-MHC class II complexes, B cells were incubated with either cycloheximide for 3 h or anisomycin for 1 h before incubation with $^3\text{H-Pc-anti-Ig}$ for 1 h at 4°C . The cells were washed and incubated at 37°C for 2 h in the continued presence of the inhibitors. In the presence of cycloheximide or anisomycin, B cells assemble $\sim 75\%$ of the complexes assembled in untreated B cells (Fig. 8). Thus, new protein synthesis for 4 h preceding antigen internalization is not required for assembly of the processed antigen-I-E^k complexes. Since newly synthesized I-E^k is not required for peptide association, the processed antigen-MHC

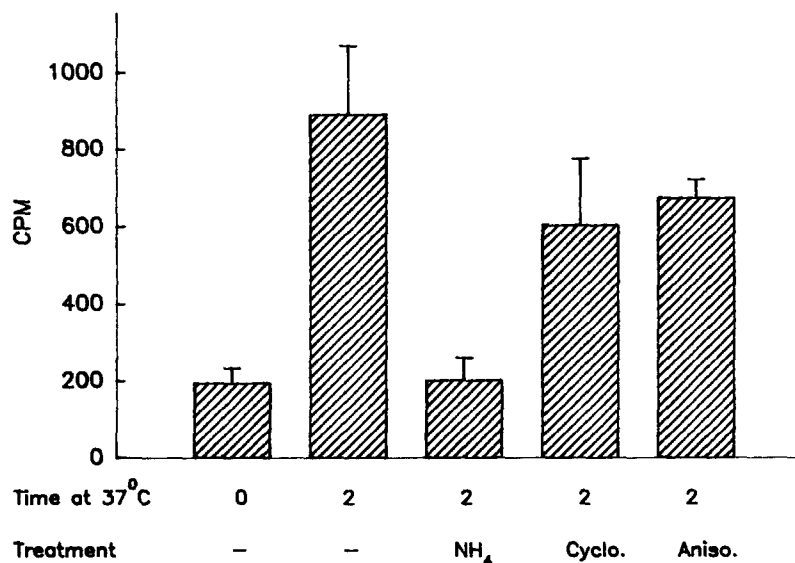


Figure 8. The effects of inhibitors of antigen processing on the assembly of processed antigen-MHC class II complexes. B cells (10^8) were preincubated with the indicated inhibitors as described in Materials and Methods, then incubated with ^3H -Pc-anti-Ig in the continued presence of the inhibitor at 4°C for 1 h. The cells were washed and resuspended at 10^7 cells/ml in complete media containing the inhibitor and incubated at 37°C for 2 h. The cells were pelleted and subjected to immunoprecipitation, as in Fig. 6. Shown is the cpm immunoprecipitated with I-E^k . In each case, insignificant cpm were immunoprecipitated using the the B220-specific antibody (90 ± 27 cpm).

class II complexes may either be derived from a long-lived intracellular pool of I-E^k or from a population of I-E^k recycling from the B cell surface.

The Disassembly or Degradation of Processed Antigen- I-E^k Complexes in B Cells. Processed antigen- I-E^k complexes gradually decrease with time 4 h after formation, such that by 6-8 h no peptide-MHC class II complexes are detected (Fig. 7). This time course is consistent with our previously published time course of the loss of the ability of B cells to activate specific T cells once antigen has been processed (41). There is considerable variability in the amount of processed antigen- I-E^k complexes detected after 4 h of incubation at 37°C . This appears to represent variation between B cell preparations in each of five independent experiments. Freshly isolated spleen B cell preparations in two experiments showed no loss of processed antigen- I-E^k complexes at 4 h ($1,192 \pm 236$ cpm), while in three experiments B cells showed significant loss of complex (490 ± 128 cpm).

The peptide- I-E^k complexes disappear from B cells within 6-8 h, even though there is no detectable decrease in the amount of I-E^k on the cell surface over the 8-h period (Fig. 7). B cell surface proteins were labeled with ^{145}I using lactoperoxidase, incubated for varying lengths of time at 37°C , and the I-E^k molecules immunoprecipitated. The cell surface I-E^k shows no decrease over 8 h, indicating that the loss of the processed antigen-MHC class II complexes does not reflect the turnover rate of the bulk of the I-E^k .

The disassembly or degradation of the processed antigen-MHC class II complexes requires acidic vesicle function (Fig. 9). B cells were incubated at 4°C with ^3H -Pc-antigen-Ig, washed, warmed to 37°C , and incubated for 2 h to allow maximal complex formation. At the end of 2 h, NH_4Cl (final concentration, 10 mM) was added to cultures, and the cells were incubated for additional periods of time. At the end of each period, I-E^k was immunoprecipitated from lysates. In the presence of NH_4Cl the complexes are stable and can be purified 8 h later at a time when no complexes can be detected in the untreated cells. Thus, in B cells the dis-

assembly and/or degradation of complexes requires functional acidic vesicles and does not correlate with the turnover time of the bulk of the I-E^k .

Discussion

Considerable progress has been made in understanding the discrete intracellular steps in the synthesis and assembly of the MHC class II molecules and in the binding of synthetic peptides to purified MHC class II in vitro. However, the molecular mechanisms by which antigen is degraded and peptide fragments associated with the MHC class II inside cells remain, for the most part, uncharacterized. Here we describe biochemical assays for the binding, internalization, and degradation of radiolabeled antigen initially bound to surface Ig and for the intracellular assembly of processed antigen-MHC

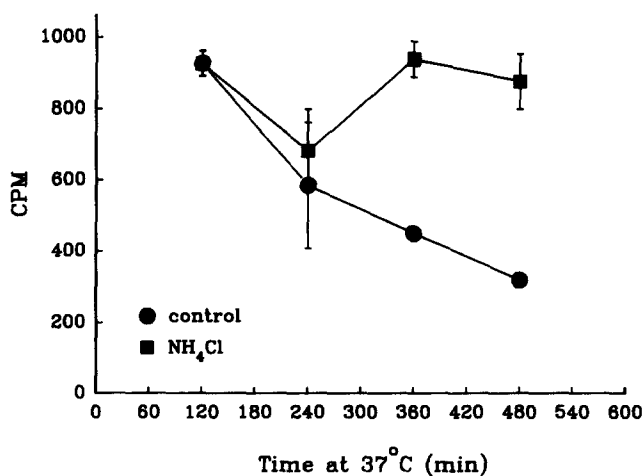


Figure 9. The loss of processed antigen- I-E^k complexes requires acidic vesicle function. B cells were incubated with ^3H -Pc-anti-Ig and treated as in Fig. 7. After a 2-h incubation at 37°C , NH_4Cl (10 mM) was added to cells and incubated at 37°C continued for varying lengths of time. I-E^k was immunoprecipitated as in Fig. 7.

class II complexes. The results of the studies presented here using these assays afford a number of insights into the mechanisms of antigen processing.

The assembly of processed antigen-MHC class II complexes is relatively rapid in that complexes are measurable by 2 h. Our earlier studies showed that splenic B cells require 4–6 h to process antigen so as to activate specific T cells (41). However, it was not known if the time requirement was for the formation of the complexes themselves or the transport and/or accumulation of processed antigen-MHC class II complexes on the B cell surface. Here we show that antigen bound to surface Ig is internalized within minutes, and degradation products detected \sim 1 h later, at the same time processed antigen-I-E^k complexes are first detected and \sim 2–4 h before T cells are activated. Thus, there is a short lag period between the time the first complexes are formed and the time T cells are activated. At present the rate-limiting steps in either the formation of processed antigen-MHC class II complexes or the display on the cell surface of such complexes are not known. Published studies show that the α and β chains of MHC class II and Ii chains are rapidly assembled and transported through the Golgi compartment and then reside for at least 1–2 h in a post-Golgi compartment, before cell surface expression of the α and β chains (4, 6). During this time the α and β chains dissociate from the Ii chain and the MHC class II molecules take on a final native form, presumably with processed antigen bound. The process of Ii chain dissociation and final assembly of α and β chains with processed antigen may account for the observed time requirement for formation of complexes. The time that elapses before the B cells are able to activate T cells may represent the time necessary to acquire a threshold number of processed antigen-MHC class II complexes on the B cell surface. The time requirement may also reflect the accumulation of other accessory molecules on the B cell surface that facilitate interaction with T cells. The recruitment of additional accessory molecules may be triggered by the binding of the antigen-antibody conjugate to the B cell surface Ig.

The rate of intracellular assembly of peptide-I-E^k complexes reported here is far more rapid than the rate of binding of peptide to MHC class II measured *in vitro*, which shows a $t_{1/2}$ of binding of 8 h (22). Several explanations may be offered to account for the observed differences that take into account the conformation of MHC class II molecules required for peptide binding and the microenvironment within the APC in which binding occurs. Jensen (25) and Wettstein et al. (26) have shown enhanced binding of peptide to MHC class II at reduced pH *in vitro*, presumably mimicking the environment within the acidic compartments of the APC. Although the effect of pH on the MHC class II molecules is not known, these findings suggest that conformation and/or chemical properties of the MHC class II are not identical at all pHs and are more favorable for peptide binding at reduced pH. In this regard, we have recently observed that MHC class II bound to newly processed antigen can be distinguished from the bulk of MHC class II in B cells by migration on SDS-PAGE (20). Indeed, the two forms of I-E^k observed in our studies are reminiscent of the compact and floppy forms

of class II described by Dornmair et al. (42). Although there are several explanations for such a change in the migration pattern, it is interesting to speculate that the class II may have two conformations: one that accommodates peptide and one that does not. Another possible explanation of the differences in results of peptide-MHC class II binding studies *in vitro* and in the APC may be found in the fact that studies *in vitro* have used model, minimal length synthetic peptides that may not represent the optimal length of peptides for binding to MHC class II in the cell. Preliminary amino acid sequence analysis indicates that the predominant processed cytochrome *c* peptide bound to I-E^k is considerably longer than the minimal length peptide (20). It is possible that the length of the peptide and thus its chemical composition affects binding to class II. Last, with regard to processed antigen binding to MHC, there may be accessory proteins within the APC that facilitate binding, such as the recently characterized peptide binding protein, PBP72/74 (43, 44), which has been colocalized to the endosomal compartments into which antigen bound to surface Ig is transported (45). PBP72/74 is a new member of the heat shock protein family (44). Members of this family share a common function of binding to newly synthesized, unfolded or degraded proteins and facilitating their refolding or transport in the cell (46). We have speculated that PBP72/74 may be involved in the transport of processed antigen to the MHC class II and/or in the assembly of the processed antigen with the MHC class II (47). Clearly, more information is needed concerning both the conformational flexibility of the MHC class II and the microenvironment in which processed antigen is bound to MHC class II before the reasons for the observed differences in peptide binding *in vitro* and in the APC are understood.

The results presented here show that once formed, the peptide-MHC class II complexes are only transiently expressed by B cells and that the disappearance of the processed antigen-I-E^k complexes requires acidic vesicle function. An acidic vesicle environment may be required either for the degradation or for the disassembly of the complexes. However, loss of complexes from B cells are more rapid than the measured rate of turnover of the bulk I-E^k. In fact, there was no measurable degradation of surface I-E^k over the time the processed antigen-MHC class II complexes disappear. If the processed antigen-MHC complexes are being degraded, the degradation mechanism must be selective, discriminating new processed antigen-MHC class II complexes from the bulk of the MHC class II. In this regard, as discussed above, we recently showed that the MHC class II purified from cells that have processed antigen and that are bound to processed antigen can be distinguished from the bulk of MHC class II molecules by their migration in SDS-PAGE (20). It is possible that such newly processed antigen-MHC class II complexes can be discriminated from the majority of MHC class II in B cells and be selectively targeted for degradation. Alternatively, the disappearance of processed antigen-MHC class II vesicles may represent the dissociation of the processed antigen from MHC class II in acidic vesicles. Although not all investigators have observed MHC class II cycling from the cell surface (4, 48), when reported, the cycling rates for

MHC class II are fast, with ~20% recycling every 30 min (15, 16). Processed antigen-MHC class II complexes may be taken into acidic compartments in which the release of the bound processed antigen is facilitated. It remains to be determined whether the acidic vesicles in which processed antigen binds to MHC class II molecules and in which disassembly or degradation occurs are one and the same.

As commented on above, an issue that has not been completely resolved is whether the MHC class II that binds processed antigen is from a newly synthesized pool that has not reached the cell surface, from a surface recycling pool, or from both. Experiments that show that inhibitors of protein synthesis block the ability of APC, when provided with native antigen, to stimulate T cells have been interpreted to indicate that newly synthesized MHC class II is required for presentation (12, 13). However, as is shown here, hours after new protein synthesis is blocked, peptide-MHC class II complexes are still assembled. This suggests that the requirement for new protein synthesis is for the presentation of these complexes to T cells. The MHC class II that binds peptide in the presence of protein synthesis inhibitors may be from a long-lived intracellular pool of MHC class II delayed in its transport to the cell surface (4, 6) or from recycling MHC class II molecules (15, 16).

Another observation to note is that the antigen internalized by B cells is degraded immediately to small peptides with no larger intermediates detected at any time. This suggests that the compartment in which proteolysis occurs is highly degradative and that degradation of the antigen would go to completion if peptides were not protected in some way. If MHC class II is indeed present in this compartment, it could serve this function. Recent evidence by Brodsky and coworkers (10) indicates that MHC class II, Ii chain, and proteases colocalize in the same compartment into which an-

tigen bound to Ig is transported. If this is the compartment in which degradation occurs, peptides may bind directly to MHC class II. Alternatively, other APC proteins such as PBP72/74, discussed above (43, 44), might be required to scavenge processed antigen in this or from other degradative compartments and deliver the peptides to the MHC class II for binding. In support of this possibility, PBP72/74 was colocalized to ~35% of the compartments described by Brodsky and coworkers (45), indicating that PBP72/74 may function at this site.

The kinetics of the assembly and disassembly of processed antigen-MHC class II complexes reported here are for normal splenic B cells. Whether similar kinetics will be observed for other APC or for transformed cell lines remains to be seen. An important aspect of the studies described here is that processing is initiated by B cells binding antigen to surface Ig. The surface Ig is a well-documented signal transducing receptor (49) and binding antigen has been shown to signal for enhanced antigen processing by an as yet undefined mechanism (37). The assembly and disassembly rates of processed antigen-MHC complexes may be regulated by antigen binding to surface Ig, allowing for regulation of the time during which T cells may interact with B cells. It will be of interest to determine if other regulators of B cells, such as interleukins, affect assembly and disassembly rates and whether other APC have mechanisms to regulate such processes.

In summary, the ability to directly measure the intracellular assembly of processed antigen-MHC class II complexes provides a link between the studies of the biosynthesis of MHC class II and the requirement for T cell activation. The results obtained thus far lend important insights into the process, and the assay may help further delineate discrete steps in the processing pathway.

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