

Invariant Chain Cleavage and Peptide Loading in Major Histocompatibility Complex Class II Vesicles

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Summary

B lymphocytes contain a novel population of endocytic vesicles involved in the transport of newly synthesized major histocompatibility complex (MHC) class II $\alpha\beta$ chains and $\alpha\beta$ peptide complexes to the cell surface. We now present evidence that these class II-enriched vesicles (CIIV) are also likely to be a site for the loading of immunogenic peptides onto MHC molecules. We used the serine protease inhibitor leupeptin to accumulate naturally occurring intermediates in the degradation of $\alpha\beta$ -invariant chain complexes and to slow the intracellular transport of class II molecules. As expected, leupeptin caused an accumulation of Ii chain and class II molecules (I-A^d) in endosomes and lysosomes. More importantly, however, it enhanced the selective accumulation of a 10-kD invariant chain fragment associated with sodium dodecyl sulfate (SDS)-labile (empty) $\alpha\beta$ dimers in CIIV. This was followed by the dissociation of the 10-kD fragment, formation of SDS-stable (peptide-loaded) $\alpha\beta$ dimers, and their subsequent appearance at the cell surface. Thus, CIIV are likely to serve as a specialized site, distinct from endosomes and lysosomes, that hosts the final steps in the dissociation of invariant chain from class II molecules and the loading of antigen-derived peptides onto newly synthesized $\alpha\beta$ dimers.

The formation of ligands for antigen receptors on CD4⁺ T lymphocytes reflects a complex series of events that occur during intracellular transport of newly synthesized MHC class II from the endoplasmic reticulum (ER)¹ to the plasma membrane of APCs (1–3). These events begin with the assembly of nonameric complexes of three $\alpha\beta$ dimers associated with one invariant (Ii) chain trimer, and they end with their transformation into immunogenic $\alpha\beta$ dimers bound to peptides derived from internalized antigens (1–3). Completion of this process involves the diversion of newly synthesized class II molecules from the secretory to the endocytic pathway, the internalization and processing of extracellular antigens, transfer of peptides to MHC molecules, and the transport of the peptide–class II complexes to the cell surface (1–3).

Recent work has defined the general cellular mechanisms underlying the conversion of $\alpha\beta$ -Ii nonamers to peptide-loaded $\alpha\beta$ dimers. Transport of newly synthesized class II molecules to the endocytic pathway is determined, at least initially, by the Ii chain, whose cytoplasmic domain contains signals that

mediate transport from the *trans*-Golgi network (TGN) to endosomes (4–8). After arrival in endosomes, the Ii chain's luminal domain is cleaved, rendering the $\alpha\beta$ dimers competent to bind antigenic peptides that are derived from internalized antigens and that are also delivered to endosomes (9). However, the precise pathway taken by $\alpha\beta$ -Ii complexes to endosomes remains unknown (10).

Several groups recently showed that a single region of Ii chain luminal domain (designated CLIP) both prevents the binding of immunogenic peptides and is involved in the association of Ii chain with $\alpha\beta$ dimers (11–13). CLIP-associated $\alpha\beta$ dimers isolated from cells do not contain immunogenic peptides; moreover, synthetic CLIP inhibits peptide binding to human MHC class II *in vitro* (14). Deletion or truncation of the region of the Ii chain containing CLIP results in a failure of association of Ii and $\alpha\beta$ dimers (11–13). CLIP is also found as a soluble peptide constitutively associated with $\alpha\beta$ dimers in normal (15) and HLA-DM-deficient antigen presentation-defective cell lines, suggesting that CLIP plays a physiological role in the loading of peptides onto $\alpha\beta$ dimers (14, 16, 17).

An important hint as to the role of the Ii chain in antigen presentation may come from the observation that its degradation and dissociation involve several discrete steps, indicating the existence of intermediates in the formation of peptide-loaded $\alpha\beta$ dimers (3). While these intermediates are insufficiently long lived to be easily detected under normal con-

¹ Abbreviations used in this paper: CHAPS, 3-[(3-cholamidopropyl)-dimethylammonio]-1-propane sulfonate; CIIV, class II-enriched vesicles; CLIP, Ii chain luminal domain fragment; EM, electron microscopy; ER, endoplasmic reticulum; FFE, free-flow electrophoresis; HPSEC, high pressure size exclusion chromatography; LDM, low density membrane; mIg, membrane Ig; MIIC, MHC class II vesicles; TGN, *trans*-Golgi network.

ditions, inhibitors of endosome-lysosome proteolysis cause the transient accumulation of naturally occurring fragments of Ii chain bound to class II molecules. In human B cells, the serine protease inhibitor leupeptin causes the accumulation of 22- and 10–12-kD Ii fragments (leupeptin-induced peptides) (18, 19). Leupeptin also blocks peptide loading and delivery of MHC class II to the cell surface (3, 18, 20), suggesting that the dissociation of these Ii chain fragments from $\alpha\beta$ dimers must precede these two events. It also causes an accumulation of class II molecules and Ii chain in endosomes (20). Since dissociation of a CLIP-containing Ii fragment and peptide loading are likely to be coordinated events, they probably occur in the same endocytic compartment. However, endosomes comprise structurally and functionally distinct compartments, and the actual organelles involved in class II delivery, Ii chain cleavage, antigen degradation, or peptide loading, remain unknown.

Determining the actual site(s) in which the Ii chain is cleaved and peptide-class II complexes form has proved a major challenge. Recently, an attractive candidate has emerged after the identification of a novel intracellular compartment selectively enriched in class II molecules (21–24). These class II-containing vesicles serve as intermediates in the transport of newly synthesized class II to the cell surface, contain several markers of the endocytic pathway, but are distinct from conventionally defined endosomes and lysosomes. It is not yet clear whether the class II vesicles identified thus far comprise one or more structurally or functionally distinct subpopulations; thus, they have been termed either class II-enriched vesicles (CIIV) or MHC class II vesicles (MIIC) in the various murine and human cells where they have been observed. In general, MIIC appear more lysosome-like than do CIIV. In mouse A20 B-cells, we have found that CIIV are selectively enriched in newly synthesized class II molecules devoid of intact Ii chain, indicating that Ii chain is removed before or just after class II molecules reach CIIV. They also transiently contain peptide-loaded and immunogenic $\alpha\beta$ dimers (21, 25). However, it remains unknown whether CIIV (or MIIC in human cells) serve as a site for peptide loading or if they simply accumulate immunogenic complexes formed elsewhere in the endocytic pathway. By using leupeptin to slow Ii chain degradation and class II transport, we now provide direct evidence that class II molecules can bind peptide and be rendered stable to dissociation in SDS after their delivery to CIIV.

Materials and Methods

Cells and Antibodies. All the experiments were performed using the A6B9 clone of IIA1.6 cells (an Fc γ RII⁻ mutant of the A20 B-lymphoma cell line) transfected with a cDNA encoding the endocytosis-competent murine splice product Fc γ RII-B2 (26). For convenience, these cells are referred to throughout as A20 cells. The anti-MHC class II β chain mAb MKD6 (27) and the anti-Ii chain cytoplasmic tail mAb IN1 (28) were used for immunoprecipitation experiments. A polyclonal rabbit serum against MHC class II molecules (generously provided by R. Kubo, Cytel, San Diego, CA) was used for immunocytochemistry (21).

Pulse-Chase Labeling. This was performed as previously de-

scribed (21). Briefly, the cells were metabolically labeled with [³⁵S]methionine-cysteine (1 mCi/ml) and chased in the absence of label for various times. The leupeptin treatment was performed by including 2 mM leupeptin (Sigma Chemical Co., St. Louis, MO) in the labeling and the chase media.

Cell Surface Biotinylation and Immunoprecipitation. These were performed as previously described (21). The precipitation of the biotinylated MHC class II procedure was 15% efficient, judged from the ability of the procedure to recover class II molecules accessible to cell surface radioiodination. Quantification was performed using a phosphorimager (Molecular Dynamics, Sunnyvale, CA). To ensure that only intact $\alpha\beta$ dimers were scored when using the β -specific antibody MKD6 mAb, quantitative data were expressed as amounts of coprecipitated α chain.

Western Blot. Low density membranes from sucrose gradients were solubilized in Laemmli sample buffer, the proteins were analyzed by 12% SDS-PAGE as described above, and then transferred to membranes (Immobilon-P; Millipore Corp., Bedford, MA). The proteins recognized by the anti-Ii chain cytoplasmic tail IN1 were visualized using goat anti-rat antibodies coupled to horseradish peroxidase and enhanced chemiluminescence (ECL; Amersham Corp., Arlington Heights, IL).

Subcellular Fractionation. Subcellular fractionation was performed as described (21). Briefly, the cells were washed twice and homogenized in a ball-bearing homogenizer. The resulting postnuclear supernatant was first fractionated by centrifugation in a sucrose density gradient to enrich class II-containing membranes. The low density membrane (LDM) fraction, contained >90% of the α and β chains (21). The LDM were further fractionated by free flow electrophoresis (FFE) using a modified Bender and Hobein Elphor VaP 21/22 (Dr. Weber, GmbH, Munich) (29). The FFE fractions were pooled pairwise, and the membranes were pelleted and lysed in 1 ml lysis buffer (as before) for immunoprecipitation.

Protein Concentration and β -Hexosaminidase Activity Assays. The activity of the lysosomal enzyme β -hexosaminidase was determined as previously described (21). The protein concentration in the FFE fractions was determined using a protein assay kit (Bio-Rad Laboratories, Richard, CA).

Immunocytochemistry and Electron Microscopy. Immunocytochemical analysis of CIIV isolated by FFE was performed as previously described (21). Briefly, FFE fractions containing CIIV were glutaraldehyde fixed and pelleted by centrifugation. The vesicles were then embedded in 10% gelatin and cryoprotected in 2.1 M sucrose before freezing and sectioning. Contrasted sections were prepared using 2% uranyl acetate in methyl cellulose. Single and double labeling and preparation of protein A-gold were accomplished using conventional methods.

Size Exclusion Chromatography. High pressure size exclusion chromatography (HPSEC) separation was performed as previously described for human MHC class II (30). Briefly, 4×10^7 cells were pulse labeled for 20 min and chased for 3 h, or labeled for 1 h and chased for 1.5 h in the presence of 2 mM leupeptin. The cells were then homogenized using a ball bearing homogenizer as described above, and the homogenates were centrifuged to eliminate nuclei and cell debris. The membranes were then pelleted by a 60 min centrifugation at $10^5 g$ and solubilized in 2% C₁₂E₉ containing lysis buffer, clarified by centrifugation, and equilibrated in 0.6% 3-([3-cholamidopropyl]-dimethylammonio)-1-propane sulfonate (CHAPS). The cell lysates were injected over tandem KW-804 columns (Waters Chromatography, Milford, MA) in 0.6% CHAPS. Fractions were collected and used for immunoprecipitation as described above.

Results

Effect of Leupeptin on MHC Class II and Ii Chain Biosynthesis. Because of the rapid kinetics of class II transport, Ii chain cleavage, and peptide association in B cells, it has proved difficult to define the site or sites in which peptide is loaded onto $\alpha\beta$ dimers. Accordingly, we studied the transport and processing of class II molecules in cells treated with leupeptin, a serine protease inhibitor well known to delay class II transport in human cells by interfering with Ii chain degradation (3, 18).

A20 murine B lymphoma cells (A6B9 subline) were metabolically labeled for 20 min and then chased in the absence of label for various periods of time with or without leupeptin. The cells were then biotinylated with a membrane-impermeable reagent (NHS-SS-biotin) to detect molecules arriving at the plasma membrane (21, 31). Total MHC class II was immunoprecipitated using the I-A^d-specific mAb MKD6, which binds $\alpha\beta$ dimers more efficiently than $\alpha\beta$ -Ii complexes (see Fig. 1). The portion of molecules present at the surface after each incubation time was determined by reprecipitating biotinylated class II molecules.

The effect of leupeptin on MHC class II and Ii biosynthesis in murine cells was less dramatic but similar to that previously reported using human cells (20). As shown in Fig. 1 A, leupeptin treatment increased the lag before the first labeled class II molecules appeared at the surface (~ 30 vs 60 min) and increased the time of chase to obtain maximum surface transport (~ 120 vs 180 min). Leupeptin treatment did not, however, significantly affect the total amount of MHC class II detected by this antibody (Fig. 1 B, upper panels) or decrease the fraction of $\alpha\beta$ dimers that ultimately reached the surface (Fig. 1 B, lower panels). In both treated and untreated cells, we found that virtually 100% of the newly synthesized dimers detected by this antibody had become accessible to biotinylation by 3 h of chase (21). Neither the kinetics nor efficiency of transport of endogenous membrane IgG in A20 cells was affected by leupeptin (not shown); thus, the effect of leupeptin on MHC class II did not reflect an overall decrease in membrane protein transport (20). Little biotinylated Ii chain ($<0.5\%$ of the total signal) was detected at the cell surface in either the presence or absence of leupeptin (not shown).

In addition to MHC class II $\alpha\beta$ dimers, the anti-class II antibody MKD6 also precipitated two low molecular weight proteins (Fig. 1 B, upper panels; a longer exposure of the low molecular weight regions of the gels is shown under each panel). A 10-kD protein (p10) was transiently detected after 1–2 h of chase, while a 12-kD band (p12) appeared after 2 h and accumulated thereafter. Leupeptin significantly increased the amount of p10, but not of p12, that was coprecipitated with MHC class II. Small amounts of p12, but not p10, were detected at the cell surface (Fig. 1 B, lower panels).

We next sought to determine the origin of these coprecipitating proteins. Since leupeptin increased the amount of p10 observed, we reasoned that at least this protein might be derived from the Ii chain. We first performed Western

blot analysis using IN1, a monoclonal antibody to the cytoplasmic tail of murine Ii chain. A20 cells were incubated with or without leupeptin for 2.5 h, homogenized, and a low density membrane fraction was probed using IN1. As shown in Fig. 2 A, the presence of a 10-kD IN1-reactive band was significantly increased by leupeptin. Similar results were obtained by immunoprecipitation with IN1 from metabolically labeled A20 cells (not shown). Moreover, the kinetics of appearance of p10 as detected using anti- β (MKD6; Fig. 1 B) or anti-Ii chain antibodies were similar. Thus, p10 (Ii-p10) is derived from the NH₂ terminus of the Ii chain since it contains the cytoplasmic domain epitope IN1.

In contrast, Ii-p10 was not precipitated using a mAb to the Ii luminal domain (P4H5) (not shown). Yet given its size, Ii-p10 must include, in addition to the Ii cytoplasmic tail, the membrane spanning domain and a portion of the Ii chain luminal domain. The fact that Ii-p10 remains associated with $\alpha\beta$ dimers suggests that it is likely to include the region of Ii that mediates the association to MHC class II, a region including the so-called CLIP peptide (11–13). The absence of Ii-p10 reactivity with P4H5 mAb is not inconsistent with this expectation. P4H5 was raised against the 98–115 peptide of murine Ii (32), and the CLIP peptide eluted from murine MHC class II molecules corresponds to amino acids 85–99 (15) (Fig. 2 A). Neither IN1 nor P4H5 detected a protein corresponding to p12, suggesting that it was unrelated to the Ii chain; we did not explore its identity further given that the expression of p12 was not affected by leupeptin.

Ii-p10 coprecipitated with $\alpha\beta$ dimers both with and without previous leupeptin treatment (Fig. 1 and not shown). This indicated that the $\alpha\beta$ -Ii-p10 complex is likely to be a normal intermediate in the processing of the Ii chain in murine B cells. The fact that it accumulated at higher levels in the presence of leupeptin suggests that the proteases responsible for Ii-p10 degradation are leupeptin sensitive. The accumulation of $\alpha\beta$ -Ii-p10 also correlated with the increased delay in the transport of $\alpha\beta$ dimers to the cell surface, suggesting that its appearance slowed one or more steps in intracellular transport.

Selective Accumulation of $\alpha\beta$ -Ii-p10 Complexes in CIIV. Since the $\alpha\beta$ -Ii-p10 complex was likely to represent a more direct precursor of peptide-loaded $\alpha\beta$ dimers than $\alpha\beta$ dimers associated with intact Ii, it was of interest to determine the localization of the partially processed complexes. We first sought conditions of labeling that maximized the amount of Ii-p10, thus optimizing our ability to detect this Ii chain fragment after subcellular fractionation. A 1-h pulse and 1.5-h chase in the presence of leupeptin resulted in a maximum amount of labeled $\alpha\beta$ -Ii-p10 complexes (not shown). Since Ii-p10 is expected to have one-third the number of methionine residues as the intact Ii chain, the IN1 precipitates suggested that the amount of Ii-p10 that accumulated under these conditions corresponded to at least 50% the amount of the intact Ii chain.

To determine the intracellular localization of intact and

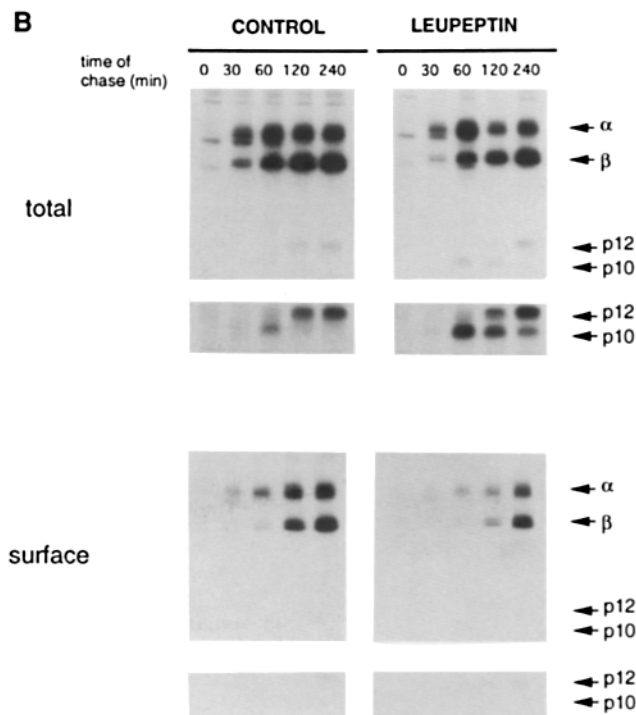
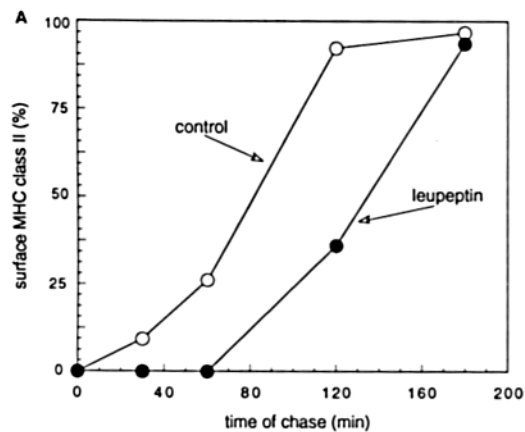


Figure 1. Leupeptin delays the transport of MHC class II molecules to the cell surface and causes the accumulation of class II-associated low molecular weight proteins. (A) Effect of leupeptin on the kinetics of appearance of $\alpha\beta$ dimers on the plasma membrane. A20 cells were pulsed with [35 S]methionine and chased at 37°C in the presence or absence of 2 mM leupeptin. At the indicated time points, the cells were surface biotinylated. After detergent lysis, MHC class II molecules were immunoprecipitated. To determine the amount of protein that had reached the surface, class II molecules were then eluted from the immunoabsorbents with SDS and reprecipitated using streptavidin-agarose beads. The amounts of α and β chains in the corresponding SDS gels (shown in B) were quantified by phosphorimaging. The percentage of MHC class II at the plasma membrane was corrected for the efficiency of the biotinylation procedure using established procedures (judged at 15% of total; see Materials and Methods) (21). Leupeptin increased by >1 h both the initial lag and the completion of transport of $\alpha\beta$ dimers to the surface. (B) Leupeptin induces the transient accumulation of a 10-kD MHC class II-associated polypeptide. The upper panels (*total*) show the total amount of MHC class II immunoprecipitated using the β chain-specific antibody MKD6 at the indicated times of chase after a 20-min pulse. The amount of labeled $\alpha\beta$ dimers increases as a function of time since MKD6 does not efficiently recognize $\alpha\beta$ -Ii complexes in the ER. The lower panels show the propor-

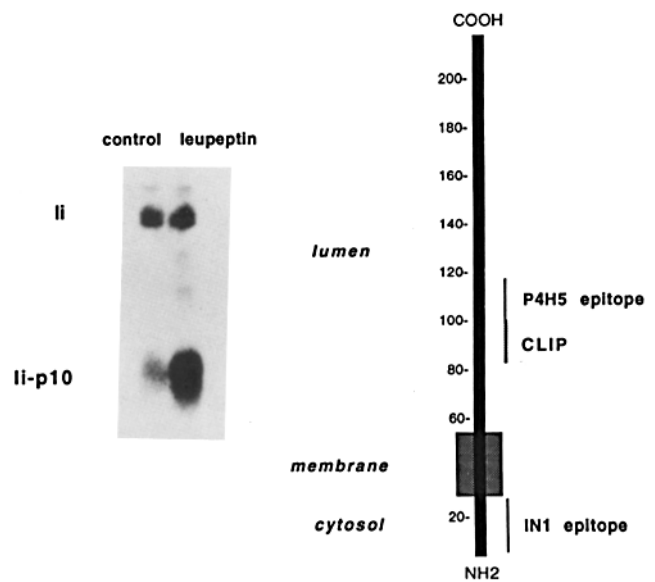


Figure 2. Ii-p10 is an NH₂-terminal fragment of Ii chain. Low density membranes from leupeptin-treated or untreated (control) cells were analyzed by SDS-PAGE and Western blot using the mAb to the Ii chain cytoplasmic IN1. In addition to the 31-kD intact Ii chain band, this mAb recognized a major 10-kD Ii chain fragment that was increased in the presence of leupeptin. A schematic representation of the Ii chain, a type II membrane protein, is also shown. The position of the epitopes recognized by IN1 and P4H5 mAbs are indicated. The region of the Ii chain corresponding to the CLIP peptide is also illustrated. Given its size and the presence of the IN1 epitope, it is likely that Ii-p10 is derived from the Ii chain NH₂ terminus and includes the CLIP region (see text for details).

processed Ii, labeled A20 cells were homogenized and fractionated by sucrose gradient centrifugation and FFE, a technique that allows the separation of CIIV from endosomes, lysosomes, as well as from other organelles (21). As found previously, the major peak of membrane protein was found in fractions 6–7, a region that also contained markers of plasma membrane and ER (21, 29, 33). Most of the lysosomal marker β -hexosaminidase was shifted towards the anode (fractions 11–13, Fig. 3A, both in the presence and absence of leupeptin). This also marked the position of endosomes on the FFE profile (21). Class II-containing CIIV migrated as a distinct peak

tion of MHC class II molecules that had reached the plasma membrane and were biotinylated (*surface*). Longer exposures of the low molecular weight regions of both gels are shown under each panel. Leupeptin did not significantly affect the expression or turnover of $\alpha\beta$ dimers. In contrast, it increased the amounts of a 10 kD protein (Ii-p10) that coimmunoprecipitated with MHC class II. The reversal of labeling ratios for α and β chains seen at early and late time points in the “total” immunoprecipitates probably reflects the difference in the rates of ER degradation for the two chains. Since the β chain is more stable than the α chain (48), it would be expected to have a slightly larger pool in the ER. Thus, dimers containing unlabeled β and labeled α chains might predominate at early times of pulse. Since the mAb used (MKD6) preferentially recognizes mature $\alpha\beta$ dimers, these “mixed” dimers would also predominate at early chase times since they would be among the first to exit the ER.

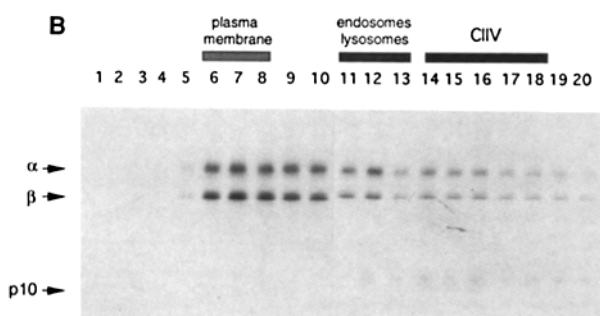
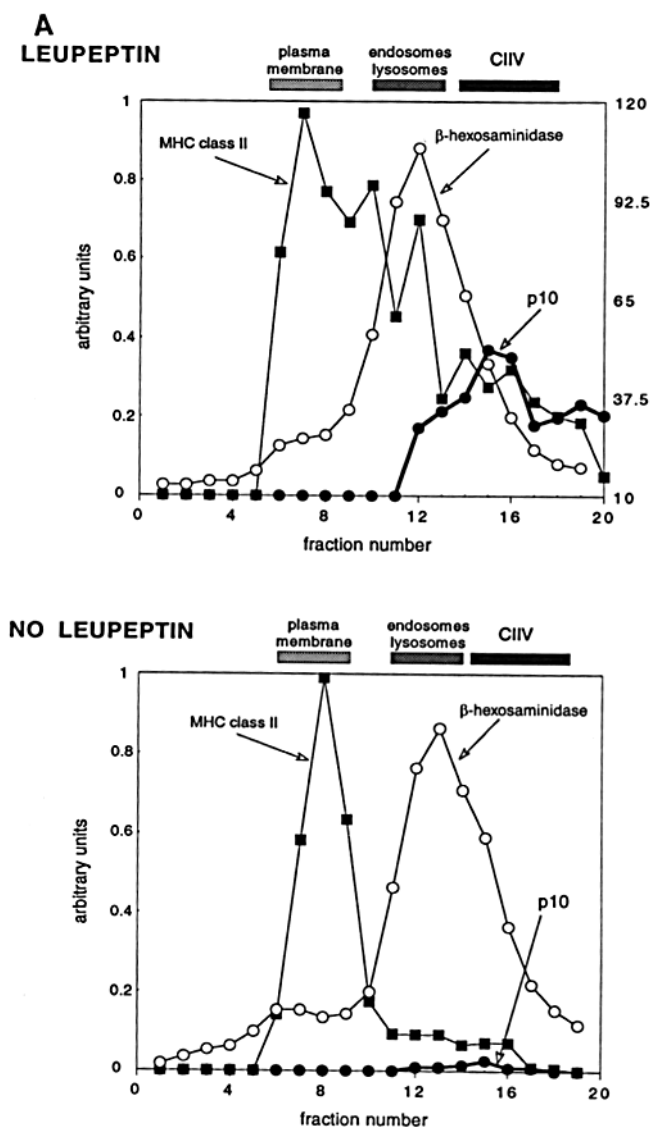


Figure 3. Subcellular distribution of the Ii chain fragment Ii-p10 in leupeptin-treated cells. (A) Quantitative distribution of $\alpha\beta$ dimers and Ii-p10 in leupeptin-treated cells. (Top) The bands corresponding to the coprecipitated MHC class II α chain and Ii-p10 shown in panel B were quantified by phosphorimaging and plotted relative to β -hexosaminidase activity. MHC class II $\alpha\beta$ dimers were found in three major peaks around fractions 7–9 (plasma membrane, Golgi complex, and ER), fraction 12 (β -hexosaminidase peak, containing endosomes and lysosomes), and fractions 14–18 (containing CIIV). Ii-p10 was found in a single major peak (fractions 14–18) corresponding to the position of CIIV. A fraction of the

that was more anodally shifted than endosomes and lysosomes (21). The migrations of these and other organelle markers were not changed by leupeptin.

After the fractionation of leupeptin-treated cells by FFE, MHC class II and Ii-p10 were detected by immunoprecipitation using MKD6. Labeled MHC class II was found in the unshifted fractions (containing plasma membrane and ER), the partially shifted endosome/lysosome fractions, as well as in CIIV (Fig. 3 A, top, shows the quantification of the gels shown in Fig. 3 B). Although, as expected, the amount of class II in endosome/lysosome fractions was increased by leupeptin, the amount of class II in CIIV was not significantly increased. Remarkably, however, almost all of the coprecipitating Ii-p10 was found in fractions containing CIIV. Relatively little Ii-p10 was detected in FFE fractions containing endosomes and lysosomes, and none in fractions containing plasma membrane, Golgi, or ER (Fig. 3) (21). Therefore, the $\alpha\beta$ -Ii-p10 complexes that accumulated in the presence of leupeptin were selectively localized in CIIV-containing fractions.

The accumulation of Ii-p10-containing complexes in CIIV was also reversible. Upon removal of leupeptin and incubation for an additional 1 h before fractionation, virtually all Ii-p10 and $\alpha\beta$ chains disappeared from CIIV fractions (Fig. 3 A, bottom). Since there was no loss of total labeled α and β chains due to degradation during this chase period, it is very likely that the $\alpha\beta$ dimers were transferred from CIIV to the plasma membrane.

One limitation of these results concerns the possible mistargeting of $\alpha\beta$ -Ii-p10 complexes in the presence of leupeptin. Ii-p10 was observed at 1 h of chase, even in the absence of leupeptin (Fig. 1 B). Since this was the time when a maximum amount of MHC class II molecules accumulate in CIIV before delivery to the plasma membrane (21), we attempted to detect Ii-p10 in CIIV under these conditions. Because of

Ii-p10 also overlapped with the endosome-lysosome peak. (Bottom) A20 cells metabolically labeled for 1 h and chased for 1.5 h in the presence of leupeptin were incubated in the absence of leupeptin for an additional 1 h before fractionation by FFE. MHC class II molecules were immunoprecipitated from the FFE fractions as before. The bands corresponding to the MHC class II α chain (coprecipitated by the anti- β chain mAb MKD6) and Ii-p10 were quantified by phosphorimaging and plotted relative to β -hexosaminidase activity. $\alpha\beta$ dimers were found in a single major peak around FFE fraction 8; this corresponded to the migration of the major peak of membrane protein and reflected class II on the plasma membrane (not shown). The two other class II peaks, as well as the Ii-p10 peak, were strongly reduced. Only small amounts of Ii-p10 were detected after washing out the leupeptin; these were limited to the CIIV region of the fractionation profile. (B) Ii-p10 is selectively localized in CIIV fractions. A20 cells metabolically labeled for 1 h and chased for 1.5 h in the presence of leupeptin before fractionation by FFE. The distribution of $\alpha\beta$ dimers and $\alpha\beta$ -Ii-p10 complexes were determined from FFE fractions by immunoprecipitation using MKD6 (as in Fig. 2); the positions of α , β , and Ii-p10 chains are indicated. Ii-p10 was detected only in the most anodally shifted CIIV fractions, while $\alpha\beta$ dimers were found in both unshifted and shifted fractions. This gel was used for the phosphorimager quantitation illustrated in A.

the lower amounts of Ii-p10 in the absence of leupeptin, all the CIIV-containing FFE fractions were pooled before immunoprecipitation of MHC class II with MKD6 mAb. As shown in Fig. 4, even in the absence of leupeptin, Ii-p10 was found in CIIV, but not in the unshifted plasma membrane fractions. Therefore, Ii-p10 presence in CIIV was not artefactually induced, but only increased, by the presence of leupeptin.

While the fraction of $\alpha\beta$ dimers that were associated with Ii-p10 could not be determined precisely, the ratio of labeled Ii-p10 to labeled α (or β) chain in CIIV fractions was 3–5-fold higher than in whole cell lysates (Fig. 3 B) and 10–25-fold higher than in CIIV of untreated cells (Fig. 4). Since Ii-p10 should include approximately the same number of methionine and cysteine residues as either α or β chains, quantitation of the Ii-p10 vs $\alpha\beta$ bands in CIIV fractions by phosphorimaging suggested that at least 50% of the $\alpha\beta$ dimers in CIIV existed as $\alpha\beta$ -Ii-p10 complexes.

We next analyzed the effect of leupeptin on the distribution of the intact Ii chain. Because the MKD6 mAb binds preferentially to Ii chain-free $\alpha\beta$ dimers, the Ii chain was immunoprecipitated from FFE fractions using IN1. In untreated cells, the Ii chain was absent from endosomal fractions and instead it was found in a less anodally shifted peak that overlapped with markers of the Golgi complex and ER (21 and not shown). In leupeptin-treated cells, however, >50% of the total intact Ii chain was found in fractions containing endosomes and lysosomes (Fig. 5). Despite this increase of Ii chain in endosomes and/or lysosomes, little, if any, intact Ii was detected in fractions containing CIIV.

To confirm that the vesicles containing the $\alpha\beta$ -Ii-p10 complexes were actually CIIV, the anodally shifted CIIV fractions from leupeptin-treated cells were pelleted and analyzed by electron microscopy (EM) immunocytochemistry. Isolated vesicles were stained with both the anti-MHC class II and the anti-Ii chain cytoplasmic domain (IN1) antibodies. CIIV from both leupeptin-treated and untreated cells were morphologically similar: 300–500 nm vesicles with multiple characteristic membrane infoldings or internal vesicles that stained strongly for MHC class II (Fig. 6). In the case of leupeptin-

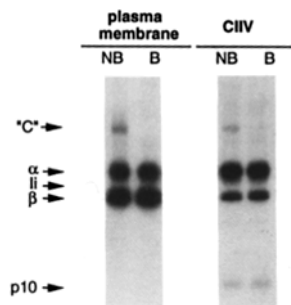


Figure 4. Intracellular distribution of Ii-p10 in the absence of leupeptin. A20 cells were pulse labeled for 20 min and then chased for 1 h, conditions previously shown to result in the maximum accumulation of MHC class II molecules in CIIV (21). The cells were then fractionated by FFE as before. The fractions corresponding to the protein peak (unshifted fractions containing plasma membrane, ER, and Golgi complex-derived membranes) and the fractions more shifted to the anode than the β -hexosaminidase peak

(CIIV-containing fractions) were independently pooled. The membranes in those pools were pelleted by centrifugation, lysed, and immunoprecipitated using MKD6 mAb. The samples were boiled (B) or not (NB) before separation by SDS-PAGE. MHC class II $\alpha\beta$ dimers in the compact (SDS-stable) peptide-loaded conformation are marked C. Ii-p10 coprecipitated with MHC class II molecules only in the anodally shifted CIIV fractions.

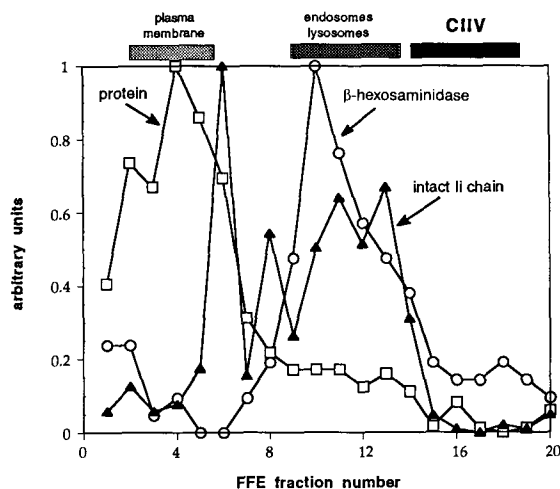


Figure 5. Intracellular distribution of intact Ii chain in leupeptin-treated cells. A20 cells metabolically labeled for 1 h and chased for 1.5 h in the presence of leupeptin before fractionation by FFE. Ii chain was immunoprecipitated from FFE fractions pools using the anti-Ii chain cytoplasmic tail antibody IN1 and then subjected to SDS-PAGE. The gels were quantified by phosphorimaging, and the distribution of intact Ii chain was plotted relative to total cell protein and β -hexosaminidase. In control cells, Ii chain was found in a single broad peak that overlapped but was more anodally deflected than the major class II and protein peaks (which marked the positions of the plasma membrane and ER) (21) (not shown). In leupeptin-treated cells, much of the Ii chain was now found in fractions that comigrated with markers of both endosomes and lysosomes. As previously found for control cells (21), little, if any, intact Ii chain was detected in CIIV fractions, even after leupeptin treatment. Cathode, left; anode, right.

treated cells, isolated CIIV were also positive for the cytoplasmic domain of Ii chain as visualized using the IN1 mAb (arrows, large gold particles). In contrast, very little Ii chain was detected in CIIV isolated from untreated cells (Fig. 6). No labeling of CIIV (from untreated or leupeptin-treated cells) was found using P4H5, the mAb against a luminal domain epitope of Ii chain that is not present in Ii-p10 (data not shown), confirming the absence of intact Ii chain from CIIV even from leupeptin-treated cells.

Together, these results show that leupeptin causes the reversible accumulation of partially cleaved $\alpha\beta$ -Ii-p10 complexes in CIIV. Given that $\alpha\beta$ -Ii-p10 must be derived from the intact $\alpha\beta$ -Ii complex, these results are consistent with the possibility that intact $\alpha\beta$ -Ii complexes are first delivered to endosomes, where they are processed, and then transferred to CIIV as partially cleaved $\alpha\beta$ -Ii-p10 complexes. We determined whether $\alpha\beta$ -Ii-p10 complexes in CIIV had yet acquired peptide, or if they were precursors of peptide-loaded $\alpha\beta$ dimers.

$\alpha\beta$ -Ii-p10 Complexes Are Nonameric. It seems likely that the same general region of Ii chain both stabilizes its interaction with and blocks peptide binding to $\alpha\beta$ dimers (11–14), suggesting that as long as MHC class II molecules remain as nonamers, the peptide-binding groove on the $\alpha\beta$ dimer should remain inaccessible. Accordingly, the dissociation of $\alpha\beta$ dimers from the nonameric complexes is likely to precede the loading of peptides onto MHC class II molecules.

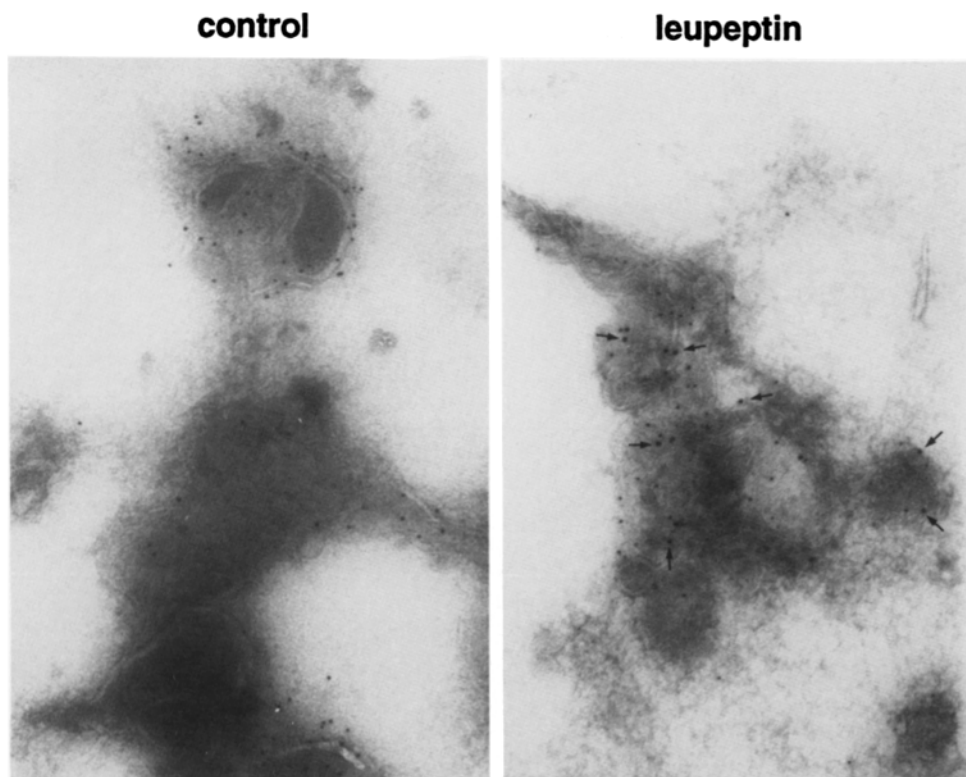


Figure 6. Morphological characterization of CIIV isolated from leupeptin-treated cells. Leupeptin-treated (2.5 h of leupeptin treatment at 37°C) or untreated A20 cells were fractionated by FFE. CIIV fractions were fixed, collected by centrifugation, cryosectioned, and double labeled with rabbit anti-MHC class II (5 nm protein A-gold) and anti-Ii chain cytoplasmic tail antibody IN1 (10 nm protein A-gold, *arrows*). As found previously, the fractions contained a population of vesicles with characteristic membrane infoldings. Most of the vesicles were positive for MHC class II. While very few class II⁺ structures were labeled with IN1 in the absence of leupeptin (*left*), most of the CIIV contained both class II and Ii chain molecules in leupeptin-treated cells (*right*).

It was therefore important to define the oligomeric structure of murine $\alpha\beta$ -Ii-p10 complexes.

A20 cells were labeled with [³⁵S]methionine, chased with or without leupeptin under conditions that maximized the accumulation of $\alpha\beta$ -Ii complexes, $\alpha\beta$ dimers, or $\alpha\beta$ -Ii-p10 complexes. The cells were lysed in CHAPS and the lysates were analyzed by HPSEC, a technique previously used to demonstrate the nonameric structure of human $\alpha\beta$ -Ii complexes (30). MHC class II was immunoprecipitated from the HPSEC fractions using either MKD6 (anti-I A^d β chain), IN1 (anti-Ii chain cytoplasmic tail), or P4H5 (anti-Ii chain luminal domain) antibodies.

After a 20-min pulse in the absence of leupeptin, intact $\alpha\beta$ -Ii complexes immunoprecipitated by IN1 (or P4H5, not shown) eluted from the HPSEC column as high molecular weight complexes consistent with a nonameric structure (30). On the other hand, but as expected, most of the β chain and coprecipitating α chains detected after a 3-h chase using the dimer-selective mAb MKD6 chromatographed as lower molecular weight species in elution volumes expected for Ii chain-free $\alpha\beta$ dimers (Fig. 7) (30).

We next determined the oligomeric structure of $\alpha\beta$ -Ii-p10 complexes by chromatographing CHAPS lysates from leupeptin-treated cells. As detected by immunoprecipitation with IN1 (or MKD6, not shown), most of the Ii-p10 eluted in the high molecular weight region of the elution profile close to intact Ii chain, indicating that Ii-p10 was also likely to be contained in a nonameric complex (Fig. 7). The $\alpha\beta$ -Ii-p10 complexes had a slightly higher elution volume than intact

$\alpha\beta$ -Ii complexes, consistent with the slightly lower molecular weight expected for Ii-p10-containing nonamers (intact Ii chain trimers vs the putative Ii-p10 trimers would be ~ 93 vs ~ 30 kD, respectively). Taken together, these results indicate that as long as Ii or Ii-p10 are associated with $\alpha\beta$ dimers, the resulting complexes behaved as high molecular weight oligomers. Therefore, in addition to being able to associate with $\alpha\beta$ dimers, Ii-p10 must also retain the capacity to form homotrimers since the formation of nonamers requires the trimerization of Ii chain (34, 35).

$\alpha\beta$ -Ii-p10 was not detected in lysates of leupeptin-treated cells immunoprecipitated with P4H5, an antibody that binds the Ii chain luminal domain whose determinant is not present in Ii-p10 (Fig. 2). Thus, the $\alpha\beta$ -Ii-p10 detected in the high molecular weight fractions of the HPSEC columns did not represent mixed complexes containing both Ii-p10 and intact Ii chain. Moreover, when the chromatography was performed in octylglucoside instead of CHAPS, intact Ii chain, Ii-p10, and $\alpha\beta$ dimers were found in low molecular weight fractions (not shown), indicating that, as shown for human MHC class II molecules, octylglucoside dissociated nonameric complexes (30).

$\alpha\beta$ Dimers in $\alpha\beta$ -Ii-p10 Complexes Are Not in the Compact Peptide-loaded Conformation. The Ii chain fragment Ii-p10 thus appeared to stabilize nonamers in a manner similar to intact Ii chain. Given this property, as well as the size of Ii-p10 itself, it appeared likely that Ii-p10 should contain sequences at or near its NH₂ terminus corresponding to the CLIP peptide previously identified in human cells. In human Ii chain,

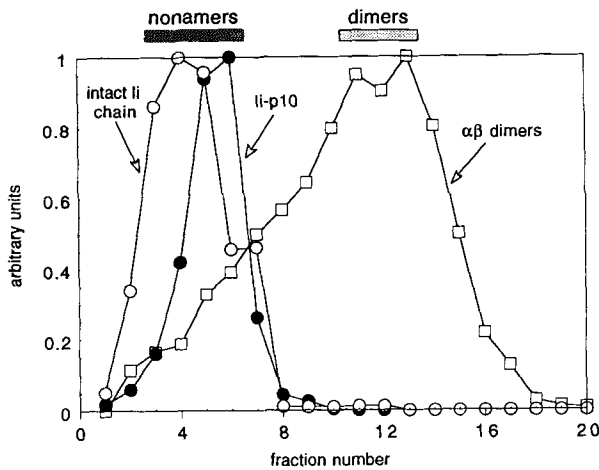


Figure 7. $\alpha\beta$ -Ii-p10 complexes are high molecular weight nonamers. Detergent lysates from metabolically labeled A20 cells were separated by gel filtration (HPSEC) before immunoprecipitation to determine the size of $\alpha\beta$ -Ii, $\alpha\beta$, and $\alpha\beta$ -Ii-p10 complexes. To analyze $\alpha\beta$ -Ii complexes, cells were pulse-labeled for 20 min, detergent extracts were separated by HPSEC, and the complexes were immunoprecipitated from the resulting fractions with the anti-Ii chain antibody IN1. As expected, intact $\alpha\beta$ -Ii complexes chromatographed as high molecular weight species corresponding to nonamers of three $\alpha\beta$ dimers and a single Ii chain trimer (30). To analyze the $\alpha\beta$ -Ii-p10 complexes, the cells were labeled for 1 h and chased for 1.5 h in the presence of 2 mM leupeptin. The complexes were immunoprecipitated using IN1. The $\alpha\beta$ -Ii-p10 complexes chromatographed in a position only slightly smaller than that of nonameric complexes containing intact Ii chain. The gels were quantified by phosphorimaging, and the corresponding plots are shown. The elution volume of $\alpha\beta$ -Ii complexes was 17.5 ml, which corresponds to the predicted elution volume of nonameric complexes composed of one Ii chain trimer associated with three $\alpha\beta$ dimers (30). The elution volume of $\alpha\beta$ dimers was 19.5 ml. It was similar to the elution volume of human $\alpha\beta$ dimers (30). The elution volume of $\alpha\beta$ -Ii-p10 complexes was 17.75 ml. This elution volume corresponds to high molecular weight nonameric complexes composed of a trimer of Ii-p10 and three $\alpha\beta$ dimers. To analyze $\alpha\beta$ dimers, the cells were pulsed for 20 min and chased for 3 h, under which conditions most of the Ii chain had been degraded and/or dissociated from newly synthesized class II molecules. $\alpha\beta$ chains were immunoprecipitated from the HPSEC fractions using MKD6, and they were found to chromatograph in the positions expected for class II dimers.

CLIP corresponds to amino acids 81–104, residues that are 71% conserved in murine Ii chain; antibodies to human CLIP do not cross-react with murine CLIP, nor are specific antimurine antibodies yet available. As discussed earlier, CLIP is thought to serve a dual purpose: stabilizing the interaction between the Ii chain trimer and three $\alpha\beta$ dimers and simultaneously preventing the binding of peptide to α and β chains (11–13, 36). Therefore, we next determined whether $\alpha\beta$ dimers in Ii-p10-containing nonamers were likely to be empty or peptide loaded using as a criterion stability to dissociation of $\alpha\beta$ dimers by SDS at room temperature (37). Since leupeptin treatment did not completely prevent the formation of peptide-loaded compact dimers (see below), it remained possible that $\alpha\beta$ -Ii-p10 complexes were peptide loaded.

We addressed the stability of $\alpha\beta$ -Ii-p10 complexes in SDS in two independent ways. First, we determined whether the SDS-stable dimers present in leupeptin-treated cells were in-

cluded in high molecular weight nonamers (as $\alpha\beta$ -Ii-p10 complexes). All of the SDS-stable dimers generated in the presence of leupeptin eluted in the low molecular weight fractions containing $\alpha\beta$ dimers that were well separated from Ii-p10, which eluted as high molecular weight complexes (not shown).

Second, the anti-Ii chain cytoplasmic domain antibody IN1 was used to precipitate $\alpha\beta$ -Ii-p10 complexes from leupeptin treated cells and to test the stability of those $\alpha\beta$ dimers in SDS at room temperature. As shown in Fig. 8, although both IN1 and MKD6 mAb precipitated Ii-p10, only MKD6 recovered the small amount of SDS-stable “compact dimers” found after leupeptin treatment; SDS-stable dimers were never coprecipitated using IN1 mAb. Therefore, the $\alpha\beta$ dimers in Ii-p10-containing complexes are not stable in SDS, suggesting that Ii-p10-associated class II molecules were not tightly associated with peptides.

$\alpha\beta$ -Ii-p10 Complexes Are Precursors of SDS-stable $\alpha\beta$ Dimers. Since most of the $\alpha\beta$ -Ii-p10 complexes were not SDS stable, it seemed likely that they represented an intermediate between intact Ii complexes and mature SDS-stable $\alpha\beta$ dimers. If true, the fact that $\alpha\beta$ -Ii-p10 complexes were selectively localized to CIIV would strongly suggest that all of the final steps in acquisition of SDS stability could occur in CIIV. Such a situation would provide functional evidence that CIIV are a site of peptide loading in the pathway of antigen processing and presentation. To determine whether the $\alpha\beta$ -Ii-p10 complex was a precursor of peptide-loaded $\alpha\beta$ dimers, we analyzed the effect of leupeptin on the kinetics of appearance of Ii-p10 and the formation of SDS-stable MHC class II $\alpha\beta$ dimers.

A20 cells were labeled for 20 min with [³⁵S]methionine, chased in the presence or absence of 2 mM leupeptin for up to 4 h, and class II complexes were immunoprecipitated at various time points using the anti- β chain antibody MKD6. Fig. 9 shows the quantitation of SDS-stable $\alpha\beta$ dimers and Ii-p10 detected in the gels. Small amounts of Ii-p10 were transiently detected in untreated control cells beginning at ~45 min of chase; this was also about the time when SDS-stable dimers first began to appear. A likely precursor-product relationship between Ii-p10 and peptide-loaded $\alpha\beta$ dimers was much more clearly illustrated in the case of leupeptin-treated cells. Leupeptin greatly increased the amount of Ii-p10 that transiently appeared at 60–90 min of chase and also caused

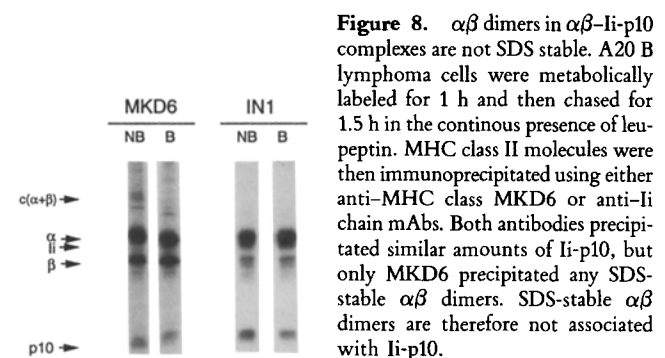


Figure 8. $\alpha\beta$ dimers in $\alpha\beta$ -Ii-p10 complexes are not SDS stable. A20 B lymphoma cells were metabolically labeled for 1 h and then chased for 1.5 h in the continuous presence of leupeptin. MHC class II molecules were then immunoprecipitated using either anti-MHC class MKD6 or anti-Ii chain mAbs. Both antibodies precipitated similar amounts of Ii-p10, but only MKD6 precipitated any SDS-stable $\alpha\beta$ dimers. SDS-stable $\alpha\beta$ dimers are therefore not associated with Ii-p10.

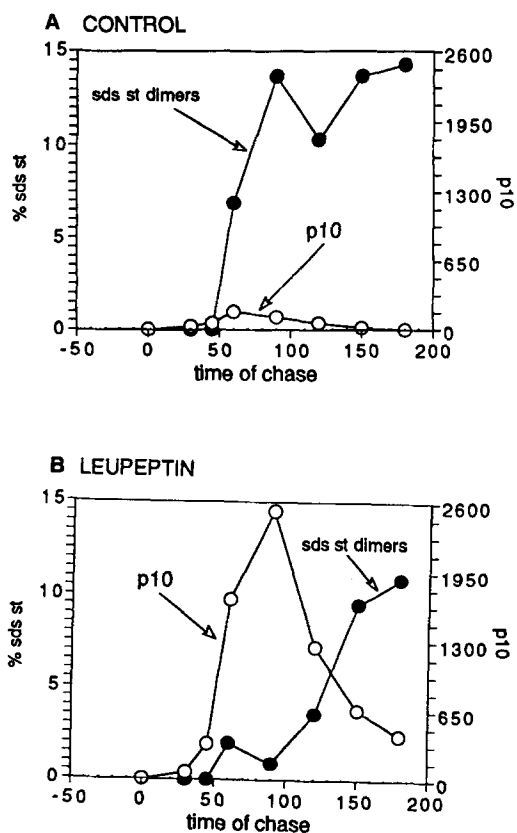


Figure 9. $\alpha\beta$ -li-p10 complexes are precursors of MHC class II-peptide complexes. A20 cells were pulse labeled for 20 min, chased for the indicated time periods with or without 2 mM leupeptin, and MHC class II (I-A^d) molecules were immunoprecipitated using the β chain-specific mAb MKD6. The samples were not boiled before SDS-PAGE. SDS-stable (*sds st*) compact dimers ($c[\alpha\beta]$) exhibited an electrophoretic mobility slower than free α or β chains. The fraction of SDS-stable class II dimers reached a maximum of 16% of the total precipitated $\alpha\beta$ dimers after 90 min of chase. The bands corresponding to SDS-stable dimers and li-p10 in the gels were quantified by phosphorimaging. In the absence of leupeptin, a small amount of li-p10 was transiently detected and then degraded; SDS-stable class II dimers began to appear after 60 min of chase, reaching a maximum by 90 min. Leupeptin markedly delayed the degradation and/or dissociation of li-p10 and caused a corresponding delay in the appearance of SDS-stable compact dimers. The amount of li-p10 reached a maximum at 90 min and decreased rapidly thereafter. Compact dimers did not begin to appear until after li-p10 began to disappear (120 min) and did not reach a maximum until 180 min.

a corresponding delay in the formation of SDS-stable complexes (not detectable until 120 min). The appearance of most of the peptide-loaded $\alpha\beta$ dimers was delayed until after li-p10 began to disappear, that is, at ~ 120 min of chase. The amount of SDS-stable compact dimers that had accumulated by 4 h of chase was comparable to the maximum amount that had accumulated in untreated cells (10.4 vs 14.3% of total class II) by 1.5 h of chase. As expected, the compact dimers ($c[\alpha + \beta]$) detected both in control and leupeptin-treated cells were abolished upon boiling the 240-min samples before SDS-PAGE (not shown).

MHC class II molecules were not detected at the plasma membrane until li-p10 disappeared and SDS-stable dimers

formed (see Fig. 1 A). Thus, as long as li-p10 remained associated with $\alpha\beta$ dimers, both SDS stability and transport to the cell surface were inhibited. Together, these results strongly suggest that the $\alpha\beta$ -li-p10 complexes were nonameric SDS-unstable precursors of SDS-stable $\alpha\beta$ dimers. Since most of $\alpha\beta$ -li-p10 complexes were found in CIIV, conversion of these peptide-free $\alpha\beta$ -li-p10 complexes into peptide-loaded $\alpha\beta$ dimers was likely to have occurred within CIIV.

Discussion

B lymphoblasts and other MHC class II-expressing cells have been found by several groups to transiently sequester newly synthesized class II molecules in novel organelles that are related to but distinct from conventionally defined endosomes and lysosomes (21-24). Although direct evidence is lacking, it seems reasonable to presume that the vesicle populations identified by each group are related to each other as well as to the MIIC originally observed by EM immunocytochemistry (38). However, there is also significant heterogeneity among the class II vesicles described thus far. CIIV isolated from mouse cells are low density structures that contain markers of the receptor recycling pathway (e.g., transferrin receptor, membrane Ig [mIg]), making them more closely related to early endosomes (21, 25; Drake, J., P. Webster, J. C. Cambier, and I. Mellman, manuscript in preparation). On the other hand, MIIC from human cells are of somewhat higher buoyant density than CIIV and they contain markers also found in lysosomes (e.g., lgp/LAMPs) (22, 23, 38). CIIV may also be distinguished from MIIC by the presence of a novel 50-kD glycoprotein related to the mIg-associated signaling molecule Ig- α (Drake, J., P. Webster, J. C. Cambier, and I. Mellman, manuscript in preparation).

Although it will be important to resolve the biogenetic relationship between CIIV and MIIC, an even more critical unknown relates to the function of these structures in antigen processing. They clearly serve as intermediates during the transport of newly synthesized class II molecules from the TGN to the plasma membrane. They also appear to transiently accumulate peptide-loaded $\alpha\beta$ dimers (21, 23, 25). These observations are consistent with at least two possibilities. The formation of immunogenic complexes could occur within these vesicles, suggesting that CIIV or MIIC may represent a specialized site for peptide loading. Alternatively, or in addition, peptides could be loaded onto MHC class II before delivery to these vesicles, in which case CIIV or MIIC may serve to regulate the transport of immunogenic class II molecules to the plasma membrane.

Where Do SDS-stable Dimers Form? Our conclusion that SDS-stable dimers can form in CIIV is based on the identification of an intermediate in the peptide loading reaction ($\alpha\beta$ -li-p10) and its subcellular localization to CIIV. Several considerations indicate that $\alpha\beta$ dimers in $\alpha\beta$ -li-p10 complexes were not associated with peptides, but rather represented precursors of peptide-loaded MHC class II. First, even in the absence of leupeptin, li-p10 was transiently detected before SDS-stable complexes formed. Leupeptin did not affect the kinetics of li-p10 appearance, but delayed its dissociation

from $\alpha\beta$ dimers and caused a corresponding delay in the formation of SDS-stable compact dimers. Thus, as long as Ii-p10 was present, peptide loading onto $\alpha\beta$ dimers did not occur. Second, although most of the $\alpha\beta$ -Ii-p10 complexes were high molecular weight oligomers similar to nonameric $\alpha\beta$ -Ii complexes, SDS-stable dimers were only found in low molecular weight fractions containing free $\alpha\beta$ dimers. Finally, after releasing the leupeptin block (by washing out the drug or by allowing accumulated intermediates to pass through the block after extended incubation in leupeptin), the $\alpha\beta$ -Ii-p10 complexes disappeared, an event that was immediately followed by the formation of SDS-stable $\alpha\beta$ dimers and transport to the plasma membrane. Thus, there was a kinetic precursor-product relationship between $\alpha\beta$ -Ii-p10 complexes and SDS-stable, presumably peptide-loaded, $\alpha\beta$ dimers.

If $\alpha\beta$ -Ii-p10 complexes are precursors of peptide-loaded $\alpha\beta$ dimers and $\alpha\beta$ -Ii-p10 complexes are found predominantly in CIIV, then the peptide-loading reaction itself must occur either within CIIV or in an as yet to be identified compartment existing between CIIV and the cell surface. Loading within CIIV is the more likely possibility since these structures, and their possible equivalents in other cell types, have been found to contain SDS-stable $\alpha\beta$ dimers (21–24). The fact that peptide loading can occur in CIIV does not necessarily mean that it cannot also occur somewhere else. However, the fact that CIIV represent a novel vesicle population on the pathway of class II transport strongly implies that they have a novel functional role as well.

While we cannot completely eliminate the possibility that our results at least in part reflected an unspecified effect of leupeptin on class II transport or processing, such an effect appears unlikely. $\alpha\beta$ -Ii-p10 was found to be a normally occurring intermediate in the conversion of $\alpha\beta$ -Ii nonamers to peptide-loaded $\alpha\beta$ dimers and, even in the absence of leupeptin, localized to CIIV. Leupeptin simply served to increase their concentration by reducing the rate of Ii-p10 cleavage and/or dissociation. The leupeptin-induced accumulation of $\alpha\beta$ -Ii-p10 complexes was needed to generate sufficient amounts of the precursor to analyze its biochemical features and precise intracellular localization. Despite its effects on transport (39), leupeptin itself had no effect on the fractionation profile of A20, unlike many lysosomotropic agents, which are well known to affect the morphology and properties of endocytic organelles. Final proof of the peptide-loading activity of CIIV, however, must await its reconstitution *in vitro*.

By following the formation of SDS-stable $\alpha\beta$ dimers rather than that of single antigen-derived peptides, our conclusions concern the pool of naturally occurring peptides. Several peptides eluted from MHC class II molecules in A20 cells have been sequenced (40). They were derived from various proteins having access to the endocytic pathway, including transferrin receptor, a secreted protease inhibitor (cys-C), or MHC class II itself. Our conclusions only apply to the peptides that confer SDS stability to I-A^d $\alpha\beta$ dimers. It remains possible, if not likely, that other peptides that do not induce stability in SDS behave differently. Preliminary results show that the

transient association of MHC class II with a 10-kD fragment also applies to other class II molecules, such as I-A^k and I-A^b, which were expressed by transfection in A20 cells or expressed endogenously in other B cell lines (unpublished observations). In the case of I-A^b, >75% of which is converted by endogenous peptides to SDS stability, leupeptin induces an almost complete block of both peptide loading and transport. The block is reversed only upon leupeptin removal and again, the dissociation of Ii-p10 kinetically precedes the formation of compact dimers, albeit at a slower rate than for I-A^d molecules (Amigorena, S., and I. Mellman, unpublished observations).

The notion that progressive degradation of Ii chain from its COOH-terminal luminal end exposes the class II peptide-binding domain derives from experiments showing that Ii chain inhibits peptide binding both *in vitro* and in intact cells (9, 41, 42). Recently, a small Ii chain-derived peptide, CLIP, was shown to be sufficient to inhibit peptide binding to human class II molecules (14). Furthermore, any Ii chain fragment that contains CLIP remains associated with $\alpha\beta$ dimers and inhibits peptide binding (11–13). Since Ii-p10 remained associated with $\alpha\beta$ dimers and since these dimers were not SDS stable, one would predict that Ii-p10 includes CLIP, a possibility supported by the apparent molecular weight of Ii-p10 and the position of the CLIP-homologous domain in the murine Ii chain sequence (residues 85–99). We are preparing antibodies to murine CLIP to test this possibility directly.

The proteolytic removal of the CLIP region of Ii is therefore likely to have at least two important consequences. First, it exposes the peptide-binding groove in $\alpha\beta$ dimers, providing MHC class II molecules a chance to bind peptides. Second, it induces the dissociation of the remaining portion of Ii, which includes Ii cytoplasmic tail and the intracellular retention signals found there, allowing transport to the plasma membrane. It is not clear yet whether peptide association is needed for efficient cell surface expression, despite some suggestions that peptide loading protects MHC class II from aggregation and degradation (43).

Is There an Endosomal Intermediate in the Transport of MHC Class II to CIIV? Until recently, it seemed clear that MHC class II molecules en route from the ER to the cell surface transiently reside in endosomes (4–6). However, the discovery of CIIV and perhaps other specialized class II compartments requires a redefinition of this long-held understanding. Newly synthesized class II molecules may be targeted directly from the Golgi complex to CIIV, perhaps bypassing conventional early and/or late endosomes entirely. Such a mechanism would be difficult to reconcile with our finding that intact Ii chain is never observed in CIIV. Thus, at present, it seems more likely that the bulk of class II molecules reach CIIV after leaving endosomes. Several lines of evidence, both biochemical and immunocytochemical, suggest that class II transits through conventional endosomes before reaching the cell surface. For example, it has long been known that newly synthesized $\alpha\beta$ -Ii complexes can be digested by transferrin-coupled or free neuraminidase before reaching the cell sur-

face (44, 45). Thus, the newly synthesized class II molecules become accessible to endocytic tracers before dissociating from the Ii chain and appearing in CIIV.

In control cells, we have found that class II molecules and Ii chain are largely absent from both early and late endosomes isolated from A20 cells (21). Thus, if there is an endosomal intermediate in these potent APCs, class II molecules must reside there only very briefly. It thus may be significant that leupeptin also appeared to induce the accumulation of $\alpha\beta$ dimers and intact Ii chain in FFE fractions containing endosomes and lysosomes. Unless leupeptin causes a significant alteration in the pathway of intracellular transport, this accumulation of $\alpha\beta$ -Ii complexes in endosomes suggests an endosomal intermediate between the TGN and arrival in CIIV. It is unlikely that leupeptin causes a nonselective alteration in either the endocytic or secretory pathways since the drug had no effect on the transport of other non-class II-related membrane proteins or the internalization of endocytic tracers (Amigorena, S., and I. Mellman, unpublished observations). Our results also suggest the reason why so little new MHC class II is normally found in endosomes. Not only do newly synthesized $\alpha\beta$ dimers reside only briefly in endosomes, but

this compartment normally mediates a very rapid degradation of Ii chain.

If MHC class II molecules are selectively transported first from the TGN to endosomes and then from endosomes to CIIV, at least two independent sorting events may occur before peptide-loaded $\alpha\beta$ dimers are found at the cell surface. Sorting in the TGN is likely to be determined by the cytoplasmic domain of the Ii chain, which bears targeting and/or retention signals inducing endosomal localization in transfected fibroblasts (7, 8). On the other hand, it is unlikely that these signals also determine endosomal sorting and transport from endosomes to or retention in CIIV. In this instance, we would have expected to find $\alpha\beta$ dimers bound to the intact Ii chain in CIIV, at least in leupeptin-treated cells. Accordingly, $\alpha\beta$ -Ii-p10 must bear some type of sorting determinant that is absent from its $\alpha\beta$ -Ii progenitor. One attractive possibility is that Ii chain cleavage to form Ii-p10 unmasks a sorting signal in the ectodomain of MHC class II molecules (46, 47). As long as $\alpha\beta$ dimers remain associated with the intact Ii chain, this determinant may be hidden and the Ii chain-containing complexes would be retained in endosomes.

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References

1. Germain, R.N., and D.H. Margulies. 1993. The biochemistry and cell biology of antigen processing and presentation. *Annu. Rev. Immunol.* 11:403-450.
2. Germain, R.N. 1994. MHC-dependent antigen processing and peptide presentation: providing ligands for T lymphocyte activation. *Cell.* 76:287-299.
3. Cresswell, P. 1992. Chemistry and functional role of the invariant chain. *Curr. Opin. Immunol.* 4:87-92.
4. Bakke, O., and B. Dobberstein. 1990. MHC class II-associated invariant chain contains a sorting signal for endosomal compartments. *Cell.* 63:707-716.
5. Lotteau, V., L. Teyton, A. Peleraux, T. Nilsson, L. Karlsson, S.L. Schmid, V. Quaranta, and P.A. Peterson. 1990. Intracellular transport of class II MHC molecules directed by invariant chain. *Nature (Lond.)* 348:600-605.
6. Lamb, C.A., J.W. Yewdell, J.R. Bennink, and P. Cresswell. 1991. Invariant chain targets HLA class II molecules to acidic endosomes containing internalized influenza virus. *Proc. Natl. Acad. Sci. USA.* 88:5998-6002.
7. Odorizzi, C.G., I.S. Trowbridge, L. Xue, C.R. Hopkins, C.D. Davis, and J.F. Collawn. 1994. Sorting signals in the MHC class II invariant chain cytoplasmic region determine trafficking to an endocytic processing compartment. *J. Cell Biol.* 126:317-330.
8. Pieters, J., O. Bakke, and B. Dobberstein. 1993. The MHC class II-associated invariant chain contains two endosomal targeting signals within its cytoplasmic tail. *J. Cell Sci.* 106:831-846.
9. Roche, P.A., and P. Cresswell. 1991. Proteolysis of the class II-associated invariant chain generates a peptide binding site in intracellular HLA-DR molecules. *Proc. Natl. Acad. Sci. USA.* 88:3150-3154.
10. Roche, P.A., C.L. Teletski, E. Stang, O. Bakke, and E.O. Long. 1993. Cell surface HLA-DR-invariant chain complexes are targeted to endosomes by rapid internalization. *Proc. Natl. Acad. Sci. USA.* 90:8581-8585.
11. Freisewinkel, I.M., K. Scenck, and N. Koch. 1993. The segment of invariant chain that is critical for association with major

- histocompatibility complex class II molecules contains the sequence of a peptide eluted from class II polypeptides. *Proc. Natl. Acad. Sci. USA.* 90:9703-9706.
12. Bijlmakers, M.J., P. Benaroché, and H.L. Ploegh. 1994. Assembly of HLA DR1 molecules translated in vitro: binding of peptide in the endoplasmic reticulum precludes association with invariant chain. *EMBO (Eur. Mol. Biol. Organ.) J.* 13: 2699-2707.
 13. Romagnoli, P., and R.N. Germain. 1994. The CLIP region of invariant chain plays a critical role in regulating major histocompatibility complex class II folding, transport, and peptide occupancy. *J. Exp. Med.* 180:1107-1113.
 14. Riberdy, J.M., J.R. Newcomb, M.J. Surman, J.A. Barbosa, and P. Cresswell. 1992. HLA-DR molecules from an antigen-processing mutant cell line are associated with invariant chain peptides. *Nature (Lond.)* 360:474-477.
 15. Rudensky, A., H.P. Preston, S.C. Hong, A. Barlow, and C.J. Janeway. 1991. Sequence analysis of peptides bound to MHC class II molecules. *Nature (Lond.)* 353:622-627.
 16. Mellins, E., P. Cameron, M. Amaya, S. Goodman, D. Pious, L. Smith, and B. Arp. 1994. A mutant human histocompatibility leukocyte antigen DR molecule associated with invariant chain peptides. *J. Exp. Med.* 179:541-549.
 17. Fling, S.P., B. Arp, and D. Pious. 1994. HLA-DMA and -DMB genes are both required for MHC class II/peptide complex formation in antigen-presenting cells. *Nature (Lond.)* 368:554-558.
 18. Blum, J.S., and P. Cresswell. 1988. Role for intracellular proteases in the processing and transport of class II HLA antigens. *Proc. Natl. Acad. Sci. USA.* 85:3975-3979.
 19. Maric, M.A., M.D. Taylor, and J.S. Blum. 1994. Endosomal aspartic proteinases are required for invariant-chain processing. *Proc. Natl. Acad. Sci. USA.* 91:2171-2175.
 20. Neefjes, J.J., and H.L. Ploegh. 1992. Inhibition of endosomal proteolytic activity by leupeptin blocks surface expression of MHC class II molecules and their conversion to SDS resistance alpha beta heterodimers in endosomes. *EMBO (Eur. Mol. Biol. Organ.) J.* 11:411-416.
 21. Amigorena, S., J.R. Drake, P. Webster, and I. Mellman. 1994. Transient accumulation of new class II molecules in a novel endocytic compartment in B lymphocytes. *Nature (Lond.)* 369:113-120.
 22. Tulp, A., D. Verwoerd, B. Dobberstein, H.L. Ploegh, and J. Pieters. 1994. Isolation and characterization of the intracellular MHC class II compartment. *Nature (Lond.)* 369:120-126.
 23. West, M.A., J.M. Lucocq, and C. Watts. 1994. Antigen processing and class II MHC peptide-loading compartments in human B-lymphoblastoid cells. *Nature (Lond.)* 369:147-151.
 24. Qiu, Y., X. Xu, A. Wandinger-Ness, D.P. Dalke, and S.K. Pierce. 1994. Separation of subcellular compartments containing distinct functional forms of MHC class II. *J. Cell Biol.* 125: 595-605.
 25. Barnes, K.A., and R.N. Mitchell. 1995. Detection of functional class II-associated antigen: role of a low density endosomal compartment in antigen processing. *J. Exp. Med.* 181:1715-1727.
 26. Amigorena, S., C. Bonnerot, J.R. Drake, D. Choquet, W. Hunziker, J.G. Guillet, P. Webster, C. Sautes, I. Mellman, and W.H. Fridman. 1992. Cytoplasmic domain heterogeneity and functions of IgG Fc receptors in B lymphocytes. *Science (Wash. DC)* 256:1808-1812.
 27. Braunstein, N.S., R.N. Germain, K. Loney, and N. Berkowitz. 1990. Structurally interdependent and independent regions of allelic polymorphism in class II MHC molecules. *J. Immunol.* 145:1635-1645.
 28. Peterson, M., and J. Miller. 1990. Invariant chain influences the immunological recognition of MHC class II molecules. *Nature (Lond.)* 345:172-174.
 29. Schmid, S.L., R. Fuchs, P. Male, and I. Mellman. 1988. Two distinct subpopulations of endosomes involved in membrane recycling and transport to lysosomes. *Cell.* 52:73-83.
 30. Newcomb, J.R., and P. Cresswell. 1993. Structural analysis of proteolytic products of MHC class II-invariant chain complexes generated in vivo. *J. Immunol.* 151:4153-4163.
 31. Harter, C., and I. Mellman. 1992. Transport of the lysosomal membrane glycoprotein Igpl20 (Igp-A) to lysosomes does not require appearance on the plasma membrane. *J. Cell Biol.* 117: 311-325.
 32. Mehringer, J.H., M.R. Harris, C.S. Kindle, D.W. McCourt, and S.E. Cullen. 1991. Characterization of fragments of the murine Ia-associated invariant chain. *J. Immunol.* 146:920-927.
 33. Marsh, M., S. Schmid, H. Kern, E. Harms, P. Male, I. Mellman, and I. Helenius. 1987. Rapid analytical and preparative isolation of functional endosomes by free flow electrophoresis. *J. Cell Biol.* 104:875-886.
 34. Lamb, C.A., and P. Cresswell. 1992. Assembly and transport properties of invariant chain trimers and HLA-DR-invariant chain complexes. *J. Immunol.* 148:3478-3482.
 35. Roche, P.A., M.S. Marks, and P. Cresswell. 1991. Formation of a nine-subunit complex by HLA class II glycoproteins and the invariant chain. *Nature (Lond.)* 354:392-394.
 36. Riberdy, J.M., and P. Cresswell. 1992. The antigen-processing mutant T2 suggests a role for MHC-linked genes in class II antigen presentation. *J. Immunol.* 148:2586-2590.
 37. Sadegh, N.S., and R.N. Germain. 1991. A role for peptide in determining MHC class II structure. *Nature (Lond.)* 353: 167-170.
 38. Peters, P.J., J.J. Neefjes, V. Oorschot, H.L. Ploegh, and H.J. Geuze. 1991. Segregation of MHC class II molecules from MHC class I molecules in the Golgi complex for transport to lysosomal compartments. *Nature (Lond.)* 349:669-676.
 39. Zachgo, S., B. Dobberstein, and G. Griffiths. 1992. A block in degradation of MHC class II-associated invariant chain correlates with a reduction in transport from endosome carrier vesicles to the prelysosome compartment. *J. Cell Sci.* 103: 811-822.
 40. Hunt, D.F., H. Michel, T.A. Dickinson, J. Shabanowitz, A.L. Cox, K. Sakaguchi, E. Appella, H.M. Grey, and A. Sette. 1992. Peptides presented to the immune system by the murine class II major histocompatibility complex molecule I-Ad. *Science (Wash. DC)* 256:1817-1820.
 41. Teyton, L., D. O'Sullivan, P.W. Dickson, V. Lotteau, A. Sette, P. Fink, and P.A. Peterson. 1990. Invariant chain distinguishes between the exogenous and endogenous antigen presentation pathways. *Nature (Lond.)* 348:39-44.
 42. Newcomb, J.R., and P. Cresswell. 1993. Characterization of endogenous peptides bound to purified HLA-DR molecules and their absence from invariant chain-associated α/β dimers. *J. Immunol.* 150:499-507.
 43. Germain, R.N., and A.J. Rinker. 1993. Peptide binding inhibits protein aggregation of invariant-chain free class II dimers and promotes surface expression of occupied molecules. *Nature (Lond.)* 363:725-728.
 44. Cresswell, P. 1985. Intracellular class II HLA antigens are accessible to transferrin-neuraminidase conjugates internalized

- by receptor-mediated endocytosis. *Proc. Natl. Acad. Sci. USA*. 82:8188-8192.
45. Neefjes, J.J., V. Stollorz, P.J. Peters, H.J. Geuze, and H.L. Ploegh. 1990. The biosynthetic pathway of MHC class II but not class I molecules intersects the endocytic route. *Cell*. 61: 171-83.
 46. Griffith, I.J., N. Nabavi, Z. Ghogawala, C.G. Chase, M. Rodriguez, D.J. McKean, and L.H. Glimcher. 1988. Structural mutation affecting intracellular transport and cell surface expression of murine class II molecules. *J. Exp. Med.* 167: 541-555.
 47. Chervonsky, A.V., L. Gordon, and A.J. Sant. 1994. A segment of the MHC class II b chain plays a critical role in targeting class II molecules to the endocytic pathway. *Int. Immunol.* 6:973-982.
 48. Bonnerot, C., M.S. Marks, P. Cosson, E.J. Robertson, E.K. Bikoff, R.N. Germain, and J.S. Bonifacino. 1994. Association with BiP and aggregation of class II MHC molecules synthesized in the absence of invariant chain. *EMBO (Eur. Mol. Biol. Organ.) J.* 13:934-944.