Ii Chain Controls the Transport of Major Histocompatibility Complex Class II Molecules to and from Lysosomes

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Abstract. Major histocompatibility complex class II molecules are synthesized as a nonameric complex consisting of three αβ dimers associated with a trimer of invariant (Ii) chains. After exiting the TGN, a targeting signal in the Ii chain cytoplasmic domain directs the complex to endosomes where Ii chain is proteolytically processed and removed, allowing class II molecules to bind antigenic peptides before reaching the cell surface. Ii chain dissociation and peptide binding are thought to occur in one or more postendosomal sites related either to endosomes (designated CIIV) or to lysosomes (designated MIIC). We now find that in addition to initially targeting αβ dimers to endosomes, Ii chain regulates the subsequent transport of class II molecules. Under normal conditions, murine A20 B cells transport all of their newly synthesized class II I-A^b αβ dimers to the plasma membrane with little if any reaching lysosomal compartments. Inhibition of Ii processing by the cysteine/serine protease inhibitor leupeptin, however, blocked transport to the cell surface and caused a dramatic but selective accumulation of I-A^b class II molecules in lysosomes. In leupeptin, I-A^b dimers formed stable complexes with a 10-kD NH_2-terminal Ii chain fragment (Ii-p10), normally a transient intermediate in Ii chain processing. Upon removal of leupeptin, Ii-p10 was degraded and released, I-A^b dimers bound antigenic peptides, and the peptide-loaded dimers were transported slowly from lysosomes to the plasma membrane. Our results suggest that alterations in the rate or efficiency of Ii chain processing can alter the postendosomal sorting of class II molecules, resulting in the increased accumulation of αβ dimers in lysosome-like MIIC. Thus, simple differences in Ii chain processing may account for the highly variable amounts of class II found in lysosomal compartments of different cell types or at different developmental stages.

THE initiation of most immune responses requires antigen recognition by helper T lymphocytes. The antigen receptors on T cells can only recognize antigens as small peptides bound to major histocompatibility complex (MHC)[1] class II molecules at the surface of antigen presenting cells (Cresswell, 1994; Germain, 1994). The complexes between class II molecules and antigenic peptides are formed intracellularly somewhere along the endocytic pathway (Germain, 1994; Wolf and Ploegh, 1995). This process requires the internalization of protein antigen and its delivery to a site suitable for the generation of antigenic peptides. In addition, the peptides must be generated within, or transferred to, a site to which newly synthesized MHC class II molecules are delivered and rendered competent for peptide binding (Davidson et al., 1991).

Invariant (Ii) chain plays a central role in controlling the intracellular transport of MHC class II (Cresswell, 1996). In the ER, Ii chain is synthesized as a trimer that complexes with three αβ dimers of MHC class II (Roche et al., 1991). Its NH_2-terminal cytoplasmic domain contains a well-known targeting signal that directs class II–Ii chain complexes to endosomes after exit from the TGN (Bakke and Dobberstein, 1990; Lotteau et al., 1990; Neefjes et al., 1990; Odorizzi et al., 1994; Pieters et al., 1993). Once in endosomes, Ii chain is subjected to proteolysis by acid hydrolases (Roche and Cresswell, 1991). Degradation occurs in a stepwise fashion, resulting in the appearance of class II–bound NH_2-terminal intermediates containing the Ii chain cytoplasmic domain, membrane anchor, and parts of its luminal domain (Newcomb and Cresswell, 1993). The intermediates accumulate in the presence of protease inhibitors that interfere with Ii chain processing such as the serine-cysteine protease inhibitor leupeptin, treatment with which can also block the transport of at least some class II haplo-

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1. *Abbreviations used in this paper*: FFE, free flow electrophoresis; Ii, invariant; lamp, lysosomal-associated membrane protein; lgp, lysosomal glycoprotein; MHC, major histocompatibility complex; Tfn, transferrin.

types to the cell surface (Amigorena et al., 1995; Blum and Cresswell, 1988; Neefjes and Ploegh, 1992). How leupeptin inhibits surface appearance is unknown.

In human cells, Ii chain degradation intermediates include a 21–22-kD fragment (designated LIP [leupeptin-inducible peptide]) and a 10–12-kD fragment (designated SLIP [small leupeptin-inducible peptide]) (Blum and Cresswell, 1988; Maric et al., 1994). In murine cells, only a 10–12-kD fragment has been identified (Ii-p10) (Amigorena et al., 1995). Ii-p10 remains as a trimer associated with three αβ dimers and blocks the binding of antigenic peptides (Amigorena et al., 1995; Morton et al., 1995). It is thus likely that Ii-p10 includes a luminal region of Ii chain (designated CLIP) known to occupy the peptide binding groove of αβ dimers. Cleavage of Ii-p10 by a leupeptin-sensitive protease causes its dissociation from αβ dimers, while leaving CLIP in the peptide binding groove. The removal of CLIP is favored at acidic pH but is additionally catalyzed by a second MHC gene product, HLA-DM (Sloan et al., 1995; Denzin and Cresswell, 1995; Karlsson et al., 1994; Roche, 1995). In mutant cells lacking HLA-DM, there is defective loading of antigenic peptides and the appearance of CLIP-αβ dimers on the plasma membrane (Mellins et al., 1994; Riberdy et al., 1992).

The precise site(s) where these events occur remains unclear. In A20 B cells, a specialized population of endosome-like vesicles designated CIIV (for class II vesicles) represents a site through which a majority of newly synthesized class II molecules pass en route to the cell surface and a place where antigenic peptides bind αβ dimers of the I-A^d haplotype (Amigorena et al., 1994, 1995; Barnes and Mitchell, 1995). CIIV are physically distinct from the bulk of endosomes and lysosomes and contain at least some HLA-DM (Pierre et al., 1996). Despite the fact that most of the αβ dimers reaching CIIV are newly synthesized, CIIV contain little or no intact Ii chain (Amigorena et al., 1995). Thus, Ii chain–αβ complexes first may be delivered to endosomes where Ii chain is cleaved before being delivered to CIIV. That peptide loading can occur in CIIV has been demonstrated by experiments showing that leupeptin causes CIIV to transiently accumulate Ii-p10–containing complexes, which can then bind peptide (Amigorena et al., 1995).

In human Epstein-Barr virus–transformed B lymphoblasts, most class II molecules have been localized to structures collectively designated MIIC (for MHC class II compartment) (Peters et al., 1991; Tulp et al., 1994; West et al., 1994). MIICs differ from CIIVs in that the latter contain endosomal but not lysosomal markers, while MIICs have most or all of the features of lysosomes (Peters et al., 1991, 1995; Pierre et al., 1996). Interestingly, the distribution of class II between endosomal (CIIV) and lysosomal (MIIC) compartments varies widely among cell types. Since lysosomes are classically defined as terminal degradative organelles (Kornfeld and Mellman, 1989), such variations may reflect differences in the rates at which class II is turned over in different cell types. On the other hand, MIICs also contain the bulk of HLA-DM and can host the loading of antigenic peptides onto class II molecules (Sanderson et al., 1994). The extent to which these complexes escape degradation and reach the cell surface is unclear. Nor is it at all clear how different cell types regulate the intracellular distribution of class II molecules between early and late endocytic compartments.

We now show that murine A20 cells expressing endogenous I-A^d and transfected I-A^b normally localize little class II in lysosomes. Selective lysosomal accumulation of I-A^b αβ dimers can be induced after leupeptin treatment. Interestingly, I-A^b dimers, but not I-A^d dimers, are induced by leupeptin to form stable complexes with Ii-p10. Upon removal of the inhibitor, the Ii-p10 was removed and class II molecules were slowly transported from lysosomes to the cell surface. Thus, the rate of dissociation of Ii chain intermediates can regulate whether newly synthesized class II molecules are transported to the plasma membrane or to lysosomes.

Materials and Methods

Cells and Antibodies

All experiments were performed using A20 B lymphoma cell line transfected with a cDNA encoding I-A^b class II molecules (B4-14 cells; kindly provided by Avlin Barlow and Charles Janeway, Yale University, New Haven, CT). The antibodies used for these experiments were: mouse anti–mouse I-A^b mAb Y3P (Janeway et al., 1984), anti–mouse I-A^d mAb MKD6 (Braunstein et al., 1990), rat anti–mouse lgp-B/lamp-2 mAb GL2A7 (Granger et al., 1990), hamster anti–mouse Ii chain mAb IN1 (directed against the Ii chain cytoplasmic tail) (Peterson and Miller, 1990), FITC-conjugated rat anti–mouse transferrin receptor CD71 (Pharmingen, San Diego, CA), a rat mAb reactive against both I-A^b and I-A^d mouse MHC class II molecules (M5.114) (Bhattacharya et al., 1981), and a polyclonal rabbit serum against the conserved cytoplasmic tail of mouse I-A β chain.

Pulse-Chase Labeling

The experiments were performed as previously described (Amigorena et al., 1994). Briefly, the cells were metabolically labeled for 20 min with [^{35}S]methionine/cysteine labeling mix (500 μCi/ml; Amersham Corp., Arlington Heights, IL) and chased for various periods of time. Leupeptin treatments were performed by adding 2 mM leupeptin (Sigma Chemical Co., St. Louis, MO) to the labeling and the chase media.

Cell Surface Biotinylation and Immunoprecipitation

Cell surface biotinylation was performed as previously described (Amigorena et al., 1994). Cells were biotinylated with NHS-SS-biotin (Pierce Chemical Co., Rockford, IL) and lysed in detergent, and I-A^b class II molecules were precipitated with Y3P-coated protein A–Sepharose beads (Pharmacia Fine Chemicals, Piscataway, NJ). I-A^b molecules were eluted by a 30-min incubation at room temperature in 100 μl PBS containing 2% SDS. 80 μl was then used to recover the biotinylated surface proteins by adsorption to streptavidin-agarose (Bio Rad Laboratories, Hercules, CA). After washing, the beads were resuspended in 20 μl of sample buffer containing 0.5 M DTT and incubated for 30 min at room temperature (this treatment releases SDS-stable complexes from the beads without boiling and thus permits the visualization of the different conformational forms of class II molecules accessible to biotinylation at the cell surface). Parallel samples were boiled for 3 min before analysis on 12% acrylamide gels. After scanning the autoradiographs with a video camera (Bio print system; Vilber Lourmat, Marne-La-Vallée, France), the quantifications were performed using the Bio-1D software (Vilber Lourmat).

Free Flow Electrophoresis

Free flow electrophoresis (FFE) fractionation was performed as previously described (Amigorena et al., 1994). Briefly, after homogenization in a ball-bearing homogenizer, the postnuclear supernatant was first fractionated by flotation in a discontinous sucrose gradient. The low density membranes were then further fractionated by FFE using a modified Bender and Hobein Elphor VaP 21/22 or Octypus PZE (Dr. Weber, GmbH, Munich, Germany). Membranes in individual or pooled fractions were pelleted and lysed before immunoprecipitation.

Percoll Gradient Centrifugation

Percoll gradients were performed as described (Pierre et al., 1996). Briefly, the cells were washed twice and homogenized in a ball-bearing homogenizer. The resulting postnuclear supernatant was fractionated by centrifugation in a 25% Percoll gradient at 4°C for 45 min at 18,000 rpm in a 70.1 Ti rotor (Beckman Instruments, Inc., Palo Alto, CA). 1-ml fractions were collected. Endosomes were labeled before fractionation by incubating the cells for 30 min at 37°C in RPMI medium containing 35 μg/ml HRP-conjugated human transferrin (Pierce Chemical Co.); 50 μl of each fraction was mixed with 50 μl of PBS containing 0.1% TX-100, and HRP acivity was detected using *o*-phenylenediamine dihydrochloride (OPD; Sigma Chemical Co.). After 1:10 dilution, the membranes were pelleted by centrifugation and lysed before class II molecules or Ii chain were immunoprecipitated as described above.

Immunofluorescence Staining and Confocal Microscopy

Immunofluorescence was performed as previously described (Bonnerot et al., 1995). Briefly, cells were allowed to adhere on glass coverslips precoated with 0.2% poly-L-lysine, were fixed for 10 min in PBS containing 3% paraformaldehyde, and were permeabilized with 0.05% saponin in PBS. The cells were then incubated for 30 min with primary antibodies in PBS with 0.2% BSA and 0.05% saponin. After three washes, the cells were stained with FITC- or TRITC-conjugated streptavidin, $F(ab')_2$ fragments of donkey anti–rat IgG, donkey anti–mouse IgG, or donkey anti–rabbit IgG antisera for 30 min. The coverslips were then mounted in Mowiol (Calbiochem-Novabiochem Corp., La Jolla, CA). Immunofluorescence microscopy was performed using a TCS scanning laser confocal microscope (Leica Microscopy and Scientific Instruments, Inc., Heerbrugg, Switzerland).

Immunoelectron Microscopy

Control cells and cells treated with 2 mM leupeptin for 16 h were fixed in 2% paraformaldehyde in 0.2 M phosphate buffer, pH 7.4, for 1 h at room temperature and processed for ultracryomicrotomy as described (Liou et al., 1996; Kleijmeer et al., 1996). For the visualization of MHC class II molecules, ultrathin cryosections were immunolabeled with the rat mAb M5.114 followed by a rabbit polyclonal directed against rat IgG. Double labelings allowing the detection of lysosomal glycoprotein (lgp)–B/lysosome-associated membrane protein (lamp)–2 and MHC class II or Ii chain and MHC class II were performed with the rat monoclonal GL2A7 directed against lgp-B, the hamster monoclonal IN1 antibody directed against the cytoplasmic domain of the Ii chain, and a rabbit polyclonal antibody raised against the cytoplasmic domain of the I-A molecule. All the antibodies were visualized with protein A–gold conjugates (purchased from J.W. Slot, Utrecht University, The Netherlands). Ultrathin cryosections were performed with a Leica FCS (Leica Microscopy and Scientific Instruments, Inc.) ultracryomicrotome and analyzed with a CM120 electron microscope (Philipps Electronic Instruments, Inc., Mahwah, NJ).

Results

Leupeptin Blocks I-A^b Transport to the Cell Surface and Causes the Stable Association of αβ Dimers with Ii-p10

Treatment of A20 cells with the protease inhibitor leupeptin increases the fraction of endogenous I-A^d class II molecules normally found in a complex with the 10-kD Ii chain fragment Ii-p10 (Amigorena et al., 1995). The increase in I-A^d–Ii-p10 complexes was transient, however, since even in the continued presence of leupeptin Ii-p10 was released and followed by the transport of peptide-loaded I-A^d αβ dimers to the cell surface. Since αβ dimers of the I-A^b haplotype may have a higher affinity for Ii chain than I-A^d dimers (Sette et al., 1995), we asked whether leupeptin might more severely affect the processing and transport of class II molecules in A20 cells expressing I-A^b αβ dimers.

A20 cells stably transfected with cDNAs encoding I-A^b α and β chains were metabolically labeled for 20 min and chased for various times before lysis and immunoprecipitation of class II with the mAb Y3P. Immunoprecipitates were analyzed by SDS-PAGE without boiling to determine if the class II molecules had bound antigenic peptide, an event that renders some αβ dimers stable to SDS at room temperature, resulting in their migration as a 60-kD complex ("compact dimer") (Germain and Hendrix, 1991).

As shown in Fig. 1 *A* (*left*), control cells began to accumulate SDS-stable I-A^b compact dimers ("*C*") within 60 min of chase. The amount of the 60-kD dimer reached a plateau by 120 min and remained constant, accounting for ~90% of the transfected I-A^b recovered from these cells. This is in contrast with the endogenous αβ dimers of the I-A^d haplotype, a much smaller fraction of which (15%) can be rendered SDS stable by peptide binding (Germain and Hendrix, 1991; Amigorena et al., 1995). Little class II was immunoprecipitated at time points <60 min since the antibody used (Y3P) does not detect class II molecules bound to intact Ii chain. Using a polyclonal antibody that detects all forms of class II, equivalent recovery of class II was found at all chase times, indicating that there was no degradation during the chase period (0–240 min; not shown).

A strikingly different result was obtained in cells labeled in the presence of leupeptin. As shown in Fig. 1 *A* (*right*), leupeptin strongly inhibited the formation of 60-kD peptide-loaded, SDS-stable dimers and, instead, induced the accumulation of a 70-kD band (p70); a band of the same molecular mass appeared transiently in control cells (60–120 min). Leupeptin also enhanced the accumulation of a small amount of Ii-p10.

To better characterize the species induced by leupeptin, their mobilities were compared by SDS-PAGE with and without boiling before electrophoresis. As shown in Fig. 1 *B*, the 60-kD SDS-stable compact dimer in control cells was, as expected, converted into ~30-kD α and β monomers. In leupeptin-treated cells, the 70-kD band yielded not only the α and β chains but also Ii-p10, suggesting that Ii-p10 had formed a stable complex with the αβ dimers. This was confirmed by immunoprecipitation of the leupeptin lysates using an mAb to the Ii chain cytoplasmic tail (IN-1). As shown in Fig. 1 *C*, precipitation with IN-1 resulted in the recovery of Ii-p10, p70, and associated α and β chains (Fig. 1 *C*). As expected, intact Ii chain was also precipitated under these conditions. Therefore, p70 represents an SDS-stable, ternary complex of I-A^b αβ dimers together with Ii-p10. Since in I-A^b, αβ dimers bound to CLIP, but not to intact Ii chain, are also SDS stable (Miyazaki et al., 1996), Ii chain cleavage appears to induce a conformational change that confers SDS stability to the complex.

Although leupeptin was previously found to induce the transient accumulation of Ii-p10 in A20 cells expressing only class II of the I-A^d haplotype, the degree to which this Ii chain fragment was induced in I-A^b–expressing cells was more pronounced. To directly compare the ability of both haplotypes to complex with Ii-p10, A20 cells expressing I-A^d αβ dimers with or without I-A^b αβ dimers were pulse labeled and chased for various periods of time in the presence or absence of leupeptin, and then class II complexes were precipitated using mAbs specific for one or the other

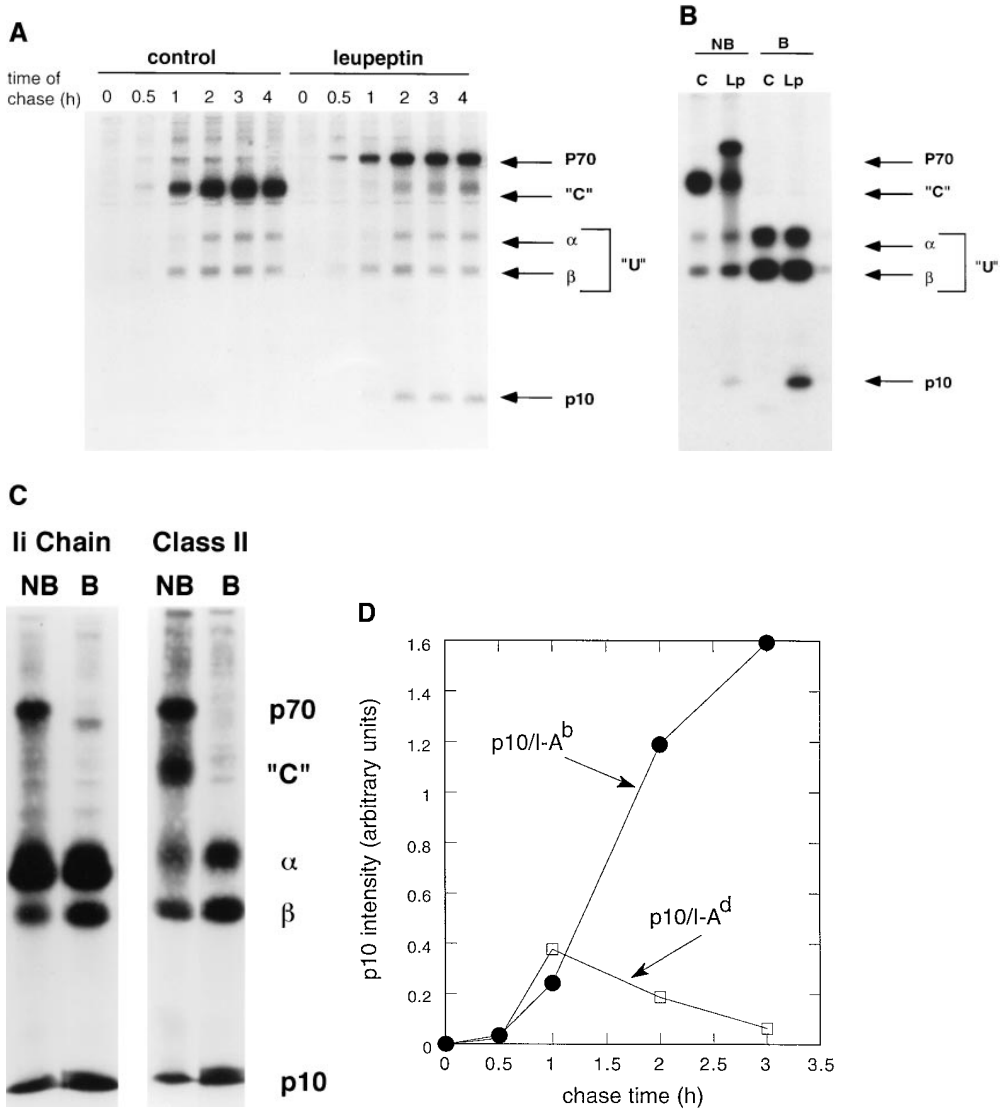


Figure 1. Leupeptin induces an accumulation of SDS-resistant I-A^b Ii-p10 complexes. (*A*) Leupeptin induces the accumulation of 10-kD (Ii-p10) and 70-kD (p70) proteins that coprecipitate with I-A^b. I-A^b–expressing A20 cells were pulsed for 20 min with [^{35}S]methionine and chased at 37°C for the indicated times in the presence or absence of 2 mM leupeptin. After lysis, the I-A^b molecules were immunoprecipitated using the Y3P mAb. The samples were not boiled before SDS-PAGE. Labeled class II molecules were not detected before 30 min of chase because the Y3P mAb used for immunoprecipitation does not detect immature αβ dimers complexed with intact Ii chain. (*B*) p70 represents SDS-stable complexes containing class II α and β chains and a 10-kD protein. After a 20-min pulse and 4-h chase with or without 2 mM leupeptin (lanes *Lp* and *C*, respectively), class II molecules were immunoprecipitated using the Y3P mAb, and the samples were boiled (*B*) or not boiled (*NB*) before SDS-PAGE. After boiling, p70 dissociated quantitatively into monomers corresponding to αβ and Ii-p10. (*C*) p70 represents SDS-stable I-A^b αβ–Ii-p10 complexes. After a 20-min pulse and 4-h chase in the presence of leupeptin, class II molecules were immunoprecipitated with either anti–I-A^b (Y3P) or anti–Ii chain cytoplasmic domain (IN-1) mAbs. While both antibodies precipitated the p70 complex, only anti–class II mAb precipitated the 60-kD SDS-stable compact dimer. Thus, p70 but not compact dimers are complexed with Ii chain or Ii chain fragments (i.e., Ii-p10) that contain the Ii chain cytoplasmic domain. (*D*) Kinetics of association between Ii-p10 and I-A^b or I-A^d. Pulse-chase experiments were performed as above using A20 cells expressing only I-A^d or expressing both I-A^d and I-A^b. I-A^d or I-A^b–containing complexes were then immunoprecipitated using specific mAbs (Y3P and MKD6, respectively), and the amounts of Ii-p10 associated to the class II molecules were quantified by phosphorimaging. The association of Ii-p10 with I-A^b persisted throughout the chase period, while Ii-p10–I-A^d complexes appeared only transiently.

haplotypes. As shown in Fig. 1 *D*, and as found previously, Ii-p10 bound to I-A^d αβ dimers could be coprecipitated only transiently, with a peak at 1 h of chase. In contrast, in the I-A^b–expressing cells, the amount of Ii-p10 recovered that bound to class II molecules increased steadily throughout the chase period. Thus, Ii-p10 formed a more stable complex with I-A^b than with I-A^d αβ dimers. This would account for why the Ii-p10–αβ complex (p70) was barely detected in cells expressing only I-A^d (Amigorena et al., 1995).

We showed previously that leupeptin delayed the cell surface delivery of I-A^d αβ dimers by 1–2 h (Amigorena et al., 1995). If association with Ii-p10 was responsible for retaining class II molecules intracellularly, the more stable association of I-A^b dimers with Ii-p10 suggested that leupeptin might more severely delay their delivery to the plasma membrane. To address this question, transfected A20 cells were pulse labeled, and then chased with or without leupeptin for various periods of time. At various intervals, class II molecules that had reached the surface were detected by derivatization with NHS-SS-biotin and total vs surface class II determined by immunoprecipitation.

In the absence of leupeptin, both SDS-stable and unstable I-A^b class II molecules rapidly formed SDS-stable compact dimers ("*C*") that were efficiently transported to the cell surface after a lag of just 1–2 h (Fig. 2, *top*). These kinetics were similar to those previously observed for I-A^d class II molecules (Amigorena et al., 1995). In the presence of leupeptin, most of the class II molecules recovered

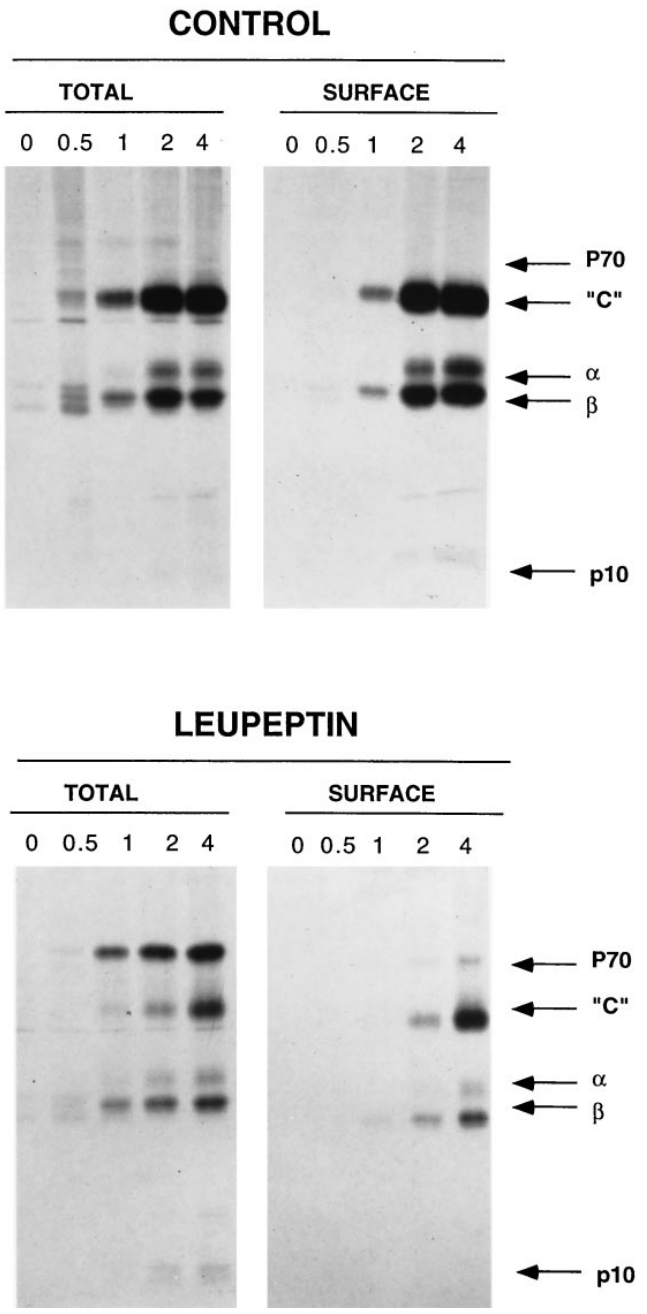


Figure 2. Intracellular retention of Ii-p10–associated class II molecules. Cells were pulsed for 20 min and chased for the indicated times (h) in the presence or absence of 2 mM leupeptin. At each time point, the cells were surface biotinylated before lysis. Total and cell surface biotinylated class II molecules were sequentially immunoprecipitated using the mAb Y3P and streptavidin-agarose. The samples were then analyzed by SDS-PAGE without boiling before electrophoresis. In untreated control cells (*top*), peptide-loaded, 60-kD compact αβ dimers ("*C*") began to appear both in total lysates and on the surface after 0.5 h of chase. A small amount of the p70 complex of Ii-p10–αβ dimers appeared transiently, beginning also at 0.5 h of chase. In leupeptin-treated cells (*bottom*), p70 began to accumulate in total lysates by 0.5 h, but little was recovered at the plasma membrane. Peptide-loaded compact dimers only began to appear after 2–4 h of chase both in lysates and at the cell surface.

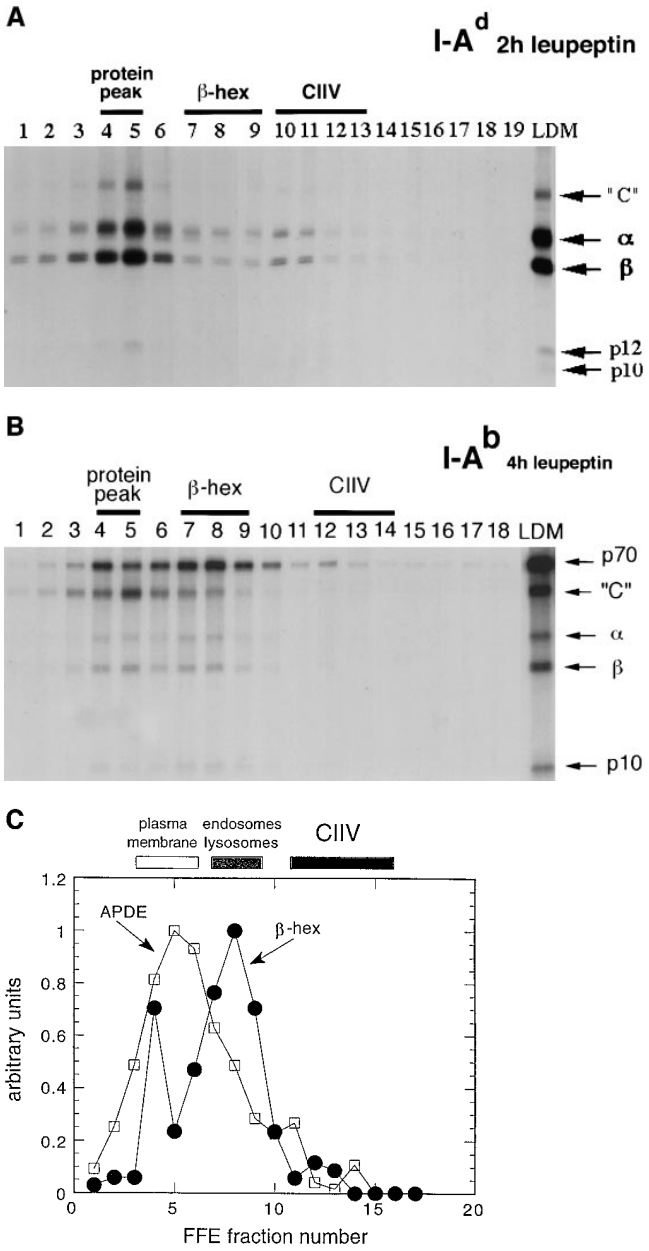


Figure 3. Fractionation of leupeptin-treated A20 cells by free flow electrophoresis. (*A*) A20 cells were pulse labeled for 20 min and chased for 2 h in the presence of leupeptin before fractionation by FFE. Membranes collected in each fraction were pelleted by centrifugation and lysed in Triton X-100, and I-A^b class II molecules were immunoprecipitated using mAb MKD6. The samples were then analyzed by SDS-PAGE without boiling. The positions of compact dimers ("*C*"), α, β chains, Ii-p10, and a p12 protein of unknown origin are indicated relative to the positions of markers for the major protein peak (plasma membrane), endosomes/lysosomes (β-hexosaminidase), and anodally shifted CIIV-containing fractions. (*Left*) Anode; (*right*) cathode. (*B*) I-A^b–expressing A20 cells were pulse labeled for 20 min and chased for 4 h in the presence of leupeptin before fractionation by FFE. The positions of p70 (Ii-p10–αβ complexes), 60-kD peptide-loaded compact dimers, free α and β chains, and Ii-p10 are indicated. (*C*) Positions of marker enzymes for plasma membrane (alkaline phosphodiesterase) and endosomes/lysosomes (β-hexosaminidase) in the FFE profile shown in *B*. p70 codistributed largely with the lysosomal marker β-hexosaminidase.

as 70-kD Ii-p10 complexes (p70) failed to reach the cell surface even after 4 h of chase (Fig. 2, *bottom*). A small amount of SDS-stable, peptide-loaded compact dimers did begin to appear after 2–4 h and were selectively transported to the plasma membrane. Thus, association of Ii-p10 with αβ dimers correlated with, and was probably responsible for, the intracellular retention of MHC class II molecules. Leupeptin did not affect the transport of class II molecules that were not bound to Ii-p10, indicating that the protease inhibitor did not inhibit MHC class II transport pathway per se. The kinetics of transport to the surface of membrane IgG was not affected by leupeptin (not shown), further indicating that the transport block was specific to the subset of class II molecules complexed with Ii-p10.

Ii-p10 Complexes of I-A^b Dimers Reach but Do Not Accumulate in CIIV

We previously found that in leupeptin-treated A20 cells, complexes of Ii-p10 and I-A^d dimers selectively accumulated in CIIV, a vesicle population that can be resolved from all other intracellular organelles by FFE (Amigorena et al., 1995). The intracellular sites of accumulation of I-A^d and I-A^b were compared by fractionating leupeptin-treated cells using FFE. The distribution of I-A^d molecules was determined after a 20-min pulse with [^{35}S]methionine and 2 h of chase in the presence of leupeptin. These conditions maximized the amount of I-A^d complexes that accumulate intracellularly because of leupeptin (Amigorena et al., 1995). As shown in Fig. 3 *A*, most Ii chain free I-A^d molecules (immunoprecipitated using mAb MKD6) were found in the nonshifted FFE fractions containing the bulk of cellular membranes including the plasma membrane. A second peak of class II was also detected in anodally shifted fractions corresponding to CIIV. Little I-A^d was detected in β-hexosaminidase–containing fractions, demonstrating that endosomes and lysosomes were not a major site of I-A^d accumulation after leupeptin treatment. The amounts of labeled I-A^d–bound Ii-p10 were too low to allow detection in FFE fractions (see above). As found previously, an unknown I-A^d–associated 12-kD protein was present at the plasma membrane and in FFE unshifted fractions, but this protein was not Ii chain derived (Amigorena et al., 1995). When the cells were fractionated after a 4 h chase in the presence of leupeptin or a 2-h chase in the absence of leupeptin, no I-A^d was detected in any FFE-shifted fraction (not shown; Amigorena et al., 1995).

To determine the distribution of the much larger quantity of I-A^b Ii-p10–αβ complexes that accumulated intracellularly after leupeptin treatment, I-A^b–expressing A20 cells were labeled for 20 min, chased for 4 h in leupeptin, and fractionated by FFE. As shown in Fig. 3 *B*, whatever SDS-stable compact dimers ("*C*") that formed despite leupeptin treatment were found in fractions 4–6, comigrating with plasma membranes (indicated by alkaline phosphodiesterase activity; Fig. 3 *C*). A small fraction of these peptide-loaded αβ dimers was also immunoprecipitated from endosome/lysosome-containing fractions (fractions 7–9; β-hexosaminidase), with even less in the more anodally

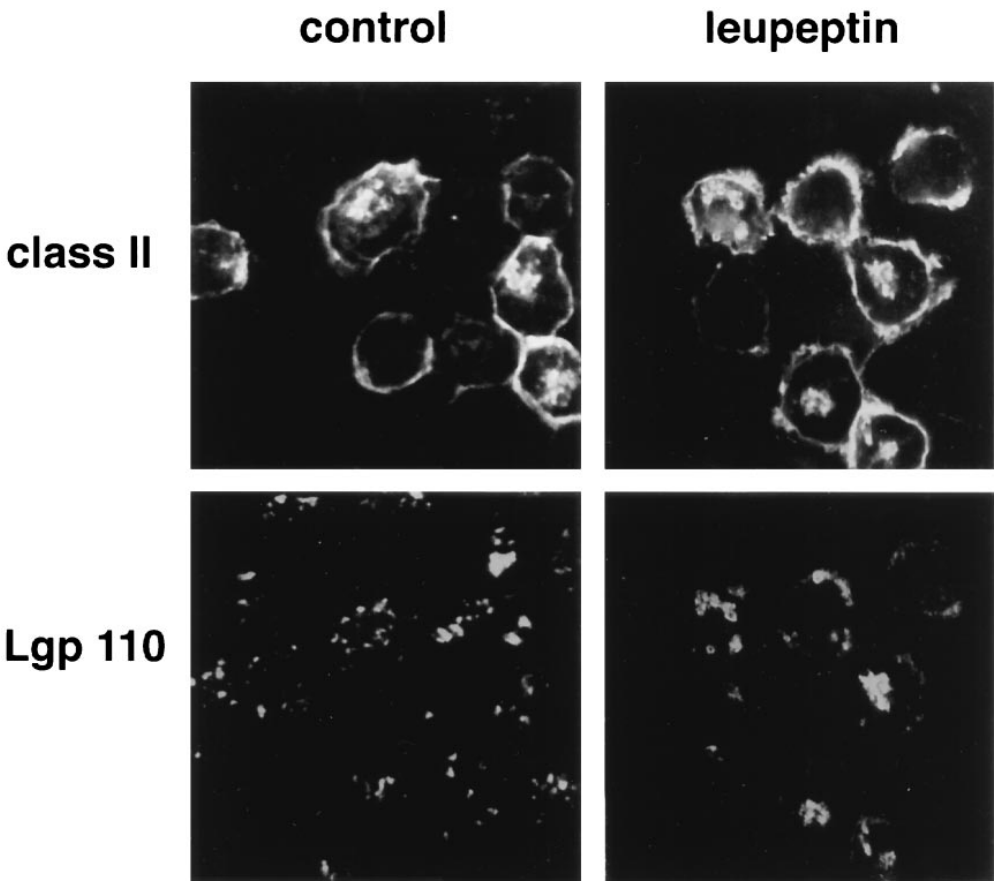


Figure 4. Leupeptin treatment causes MHC class II molecules to accumulate in lgp-containing structures by immunofluorescence microscopy. Control or leupeptin-treated (3 h, 2 mM leupeptin) I-A^b–expressing A20 cells were fixed, permeabilized, and then stained for MHC class II (FITC, using mAb Y3P, *two upper panels*) vs lgp-B (TRITC, using mAb GL2A7, *lower two panels*). In control cells, the small amount of intracellular MHC class II was localized to structures that were generally negative for lgp-B. These probably represented CIIVs and early endosomes. Leupeptin treatment, however, induced extensive colocalization of class II and lgp-B.

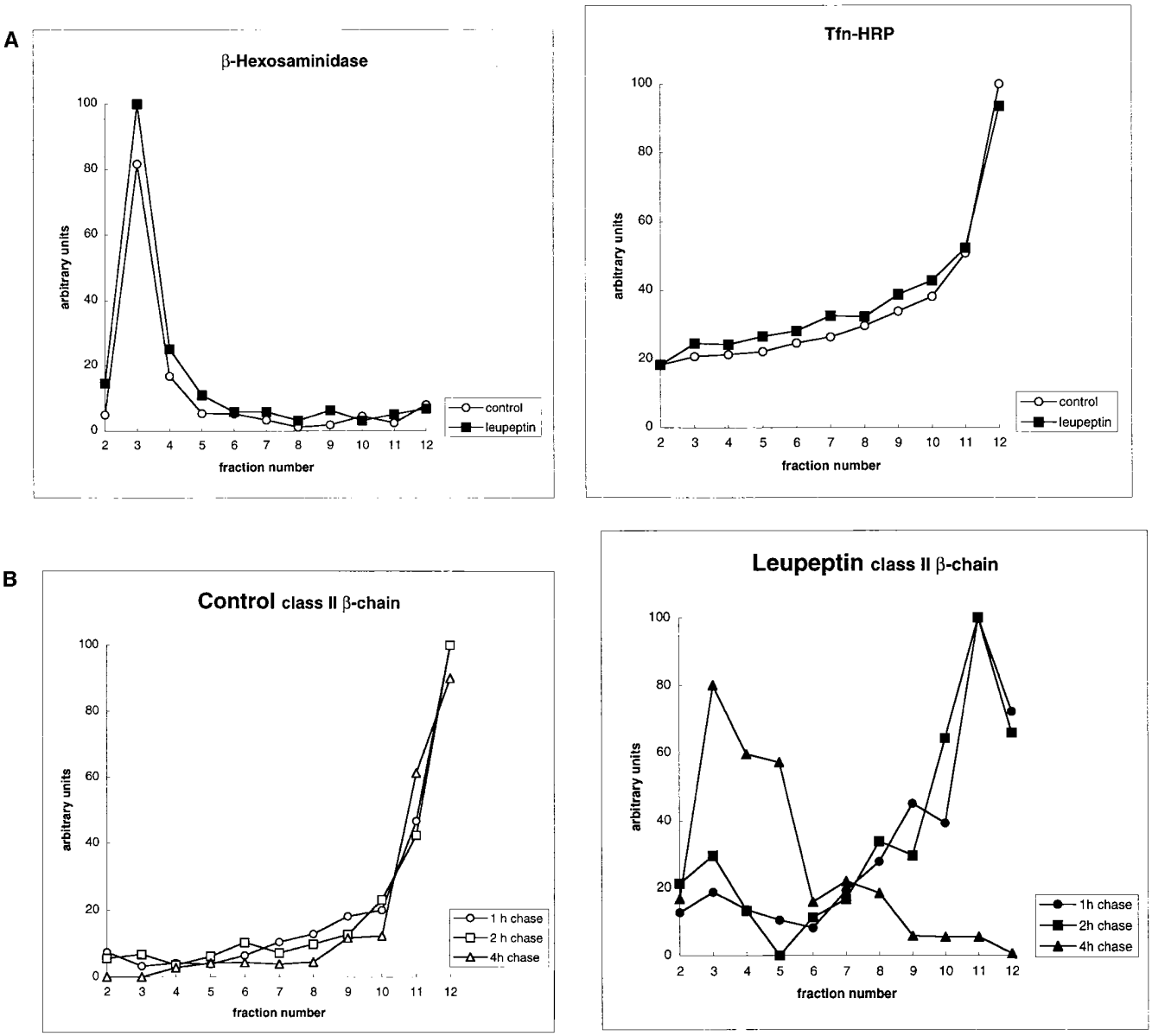


Figure 5. Selective redistribution of newly synthesized MHC class II molecules to lysosomes in leupeptin-treated cells analyzed by Percoll gradient centrifugation. (*A*) Leupeptin does not affect the density of compartments containing β-hexosaminidase and Tfn-HRP, markers of lysosomes and endosomes, respectively. After a 3-h incubation with or without 2 mM leupeptin, cells were permitted to internalize Tfn coupled to HRP for 30 min at 37°C (in the presence or absence of leupeptin) before homogenization and fractionation on Percoll density gradients. The β-hexosaminidase and HRP activities were determined in individual fractions. High density fractions (bottom of the gradient) contained most of the β-hexosaminidase activity, while Tfn-HRP was mainly present in low density fractions. No difference was observed between leupeptin-treated and control cells. (*B*) Redistribution of newly synthesized MHC class II molecules into high density fractions in leupeptin-treated cells. After a pulse of [^{35}S]methionine and a 1-, 2-, or 4-h chase in the absence (*left*) or presence (*right*) of leupeptin, I-A^b–expressing A20 cells were fractionated on Percoll gradients. The membranes in each of the gradient fractions were pelleted by centrifugation, lysed in Triton X-100, and then immunoprecipitated using mAbs to I-A^b (Y3P). The samples were analyzed by SDS-PAGE, and bands corresponding to class II β chains were quantified by phosphorimaging and optical densitometry. Ii-p10 intensity was also quantified after the 4-h chase in the presence of leupeptin (*bottom left*). Leupeptin caused a strong redistribution of class II into high density fractions. The majority of Ii-p10 was also found in lysosome-containing fractions. As expected, Ii-p10 was barely detectable in control cells and thus was not shown.

shifted CIIV fractions. This was consistent with the finding (Fig. 2) that most of the compact dimers reach the plasma membrane rather than remaining intracellular. In contrast, p70 (Ii-p10–αβ complexes) migrated as a major peak coincident with the major β-hexosaminidase-positive endosome/lysosome peak (Fig. 3, *B* and *C*, fractions 7–9). A second peak was found in the nonshifted regions (fractions 3–4) typical of plasma membranes, which also contained a minor peak of β-hexosaminidase (Fig. 3 *C*). Since p70 was not detected by biotinylation at the cell surface, we suspect that this second peak represented p70 found in residual lysosomes that did not shift during the electrophoresis step. p70 was also found trailing into the most anodally shifted fractions characteristic of CIIV. However, unlike Ii-p10–I-A^d class II complexes, most Ii-p10–I-A^b molecules were not restricted to CIIV, but were found in fractions containing endosomes and lysosomes.

I-A^b αβ–Ii-p10 Complexes Accumulate in Lysosomes

Using confocal microscopy, we next asked if the p70-containing vesicles were of endosomal or lysosomal origin. As expected, most of the I-A^b class II molecules detected using mAb Y3P in control cells were observed at the cell surface (Fig. 4). Some intracellular staining was observed in ~40% of the class II–expressing cells, however. Intracellular class II was observed in poorly resolved vesicles in the perinuclear region. These structures were negative for Ii chain (not shown) and also negative for the late endosome/lysosome membrane marker lgp-B/lamp-2 (Fig. 4, *lower left*).

After 3 h in leupeptin, the staining patterns observed were quite different. Abundant intracellular vesicles, positive for I-A^b class II, were now observed (Fig. 4, *upper right*). These class II–positive structures were also positive for lgp-B/lamp-2 (Fig. 4, *lower right*). Since the anti–I-A^b mAb used (Y3P) does not bind to class II molecules associated with intact Ii chain, the vesicles seen by confocal microscopy are likely to represent sites of I-A^b αβ–Ii-p10 accumulation in the presence of leupeptin; indeed, these structures were also positive for Ii chain, using the mAb to the Ii chain cytoplasmic tail IN-1 (not shown). The leupeptin-induced relocalization of I-A^b molecules to lgp-B/lamp-2–positive structures was selective, since transferrin (Tfn) receptor was not comparably redistributed as determined by confocal microscopy (not shown) or cell fractionation (see below).

To determine if the lgp/lamp-positive vesicles were lysosomes or late endosomes, we analyzed the distribution of I-A^b class II molecules by centrifugation in 27% Percoll gradients, which separate low density early and late endosomes from heavy density lysosomes. Metabolically labeled cells were chased for 1–4 h in the presence or absence of leupeptin, homogenized, and centrifuged in Percoll, and then individual fractions were monitored for marker enzymes and for MHC class II. As shown in Fig. 5 *A*, leupeptin had no effect on the sedimentation of lysosomes (β-hexosaminidase) that were recovered from the same heavy density fractions in control and treated cells (*left*). Similarly, conjugates of Tfn-HRP, a marker of the receptor recycling pathway (internalized for 30 min before cell homogenization), sedimented in the low density regions of the gradient both in the presence and absence of leupeptin (Fig. 5 *A*, *right*). Similar results were obtained for the early and late endosomal markers rab5 and rab7, respectively (not shown). Thus, leupeptin did not cause an overall shift of endosomal markers to heavy density lysosomes or an alteration in the density properties of lysosomes themselves.

In the absence of leupeptin, I-A^b molecules were barely detected in Percoll heavy fractions at 1–2 h of chase and were completely absent after 4 h (Fig. 5 *B*, *left*). Thus, under normal conditions, the majority of newly synthesized class II molecules does not appear to enter lysosomes at either short or long periods of chase. Leupeptin treatment did, however, result in the dramatic redistribution of I-A^b αβ dimers from low density fractions (containing plasma membranes, CIIVs, and endosomes) to the heavy density lysosomal fractions, with >80% of the total MHC class II being recovered from lysosomal fractions by 2–4 h of chase (Fig. 5 *B*, *right*). More than 90% of the Ii-p10 in leupeptin-treated cells was also recovered by immunoprecipitation from lysosomal fractions (Fig. 5 *B*, *bottom left*). No Ii-p10 was observed in Percoll gradients of control cells.

Taken together, these results show that leupeptin causes a progressive accumulation of newly synthesized I-A^b class II molecules in lysosomes. The redistribution of class II is also selective in that it is not accompanied by the lysosomal accumulation of an endosomal/plasma membrane marker (Tfn) or a second class II molecule (I-A^d; Amigorena et al., 1995). Given that I-A^b but not I-A^d αβ dimers formed stable complexes with Ii-p10 in the presence of leupeptin, it seems likely that the redistribution of I-A^b molecules reflected the accumulation of the Ii-p10–αβ complexes. Conceivably, the stable association with Ii-p10 might prevent egress of class II from lysosomes and/or cause the missorting to lysosomes of newly synthesized class II complexes after their arrival in endosomes. Since, under normal conditions, the bulk of new class II does not appear to enter lysosomes before reaching the cell surface, it seems likely that accumulation of class II into lysosomes after leupeptin reflects missorting in endosomes.

Characterization of Class II–positive Lysosomal Compartments in Leupeptin-treated Cells by Immunoelectron Microscopy

To further define the compartments where αβ–Ii-p10 complexes accumulated, leupeptin-treated and control cells were analyzed by immunoelectron microscopy on ultrathin cryosections. In nontreated cells, MHC class II molecules, detected by the anti–mouse MHC class II mAb M5.114, were found predominantly at the plasma membrane and very occasionally in clathrin-coated pits. A fraction of the class II molecules was also visualized intracellularly in tubules and vesicles that often displayed intraluminal membranes (Fig. 6 *A*). Since these vesicles were negative for lysosomal membrane markers, they are likely to be CIIV or early endosomes (see below). They were, however, strongly reminiscent of CIIV structures isolated from A20 cells by FFE (Amigorena et al., 1994).

In leupeptin-treated cells, the CIIV-like structures were also observed, but MHC class II was more often found in compartments whose content appeared more electron dense and replete with internal vesicles and membranes

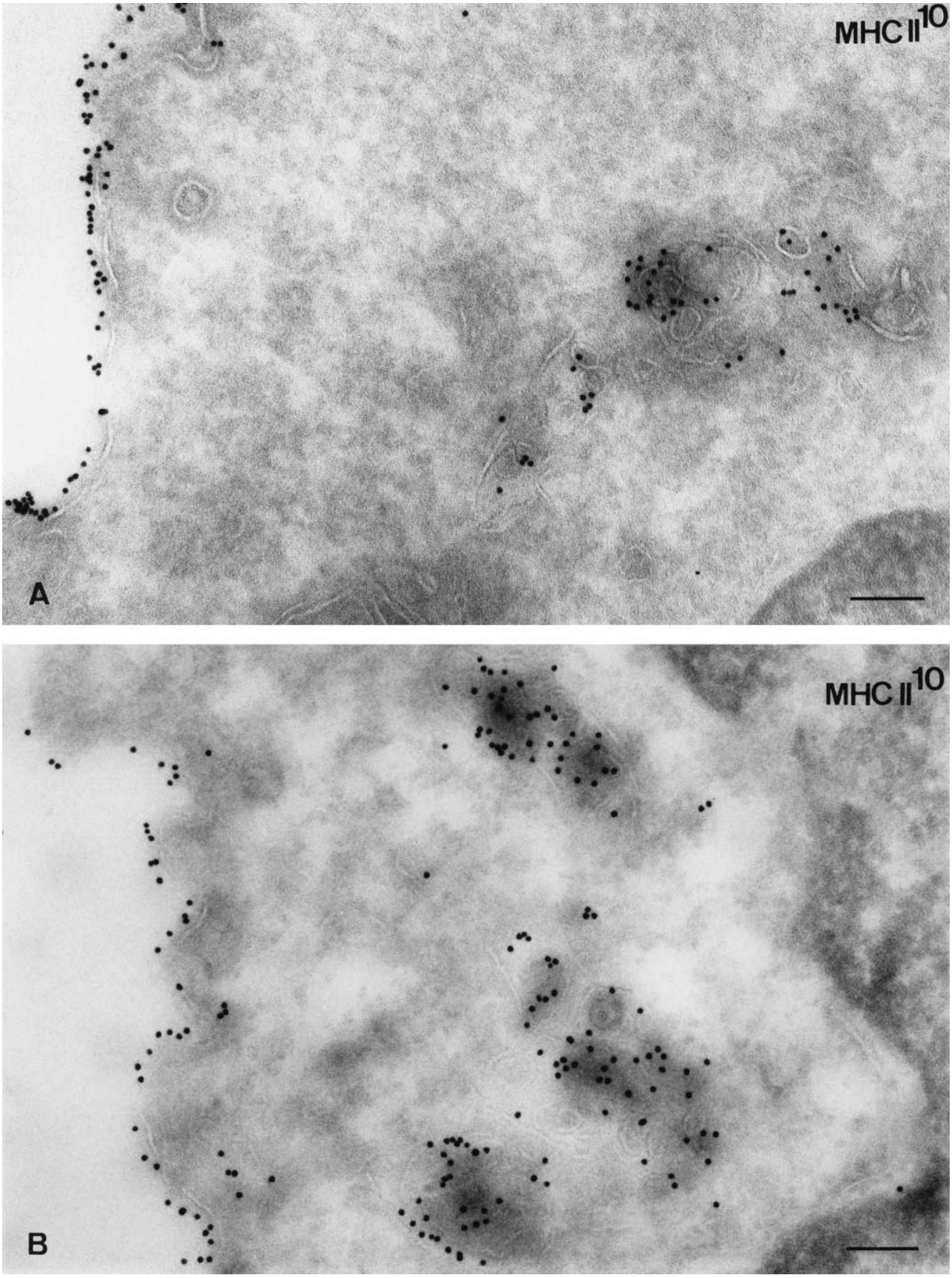


Figure 6. Immunogold localization of MHC class II molecules in leupeptin-treated cells. Ultrathin cryosections were immunogold labeled with the anti–class II mAb M5.114 and protein A–gold (PAG-10). (*A*) In control cells, MHC class II molecules are found on the plasma membrane, in intracellular compartments characterized by the presence of internal membranes. (*B*) In leupeptin-treated cells (18-h treatment), MHC class II molecules were detected on the plasma membrane and accumulate in electron-dense compartments displaying internal membranes. Bars, 120 nm.

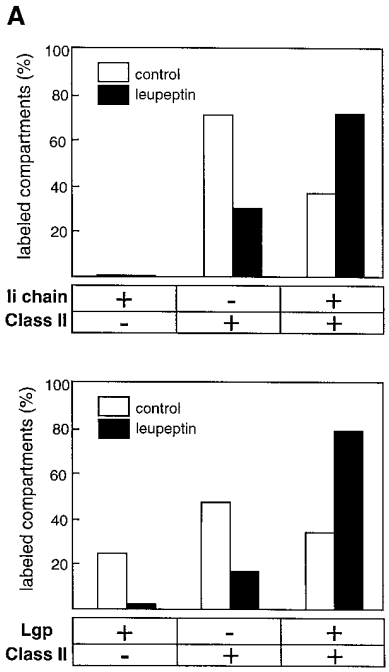
(Fig. 6 *B*). Although these denser structures were present in control cells, they were devoid of class II. Quantitation of the immunolabeling revealed that, in control cells, 13% of total class II was intracellular as compared with 43% in leupeptin-treated cells.

We next performed double labelings for MHC class II vs the cytoplasmic portion of Ii chain (using the IN-1 mAb), as well as for class II vs lgp-B/lamp-2 (GLA27 mAb). Since both antibodies were rat monoclonal IgGs and not strongly reactive with protein A, we used a rabbit polyclonal serum directed against the cytoplasmic domain of I-A β chain to visualize MHC class II. The labeling of MHC class II observed with the polyclonal antibody was less intense than that obtained using M5.114. As established previously for isolated CIIVs (Amigorena et al., 1995), CIIVs in control cells labeled poorly with IN-1: only 23% of class II–containing compartments also stained for Ii chain cytoplasmic tail (Fig. 7 *A*). Similarly, the majority of these class II–containing vesicles showed no or little lgp staining under normal conditions (Fig. 7 *A*). In contrast, after leupeptin treatment, the number of structures containing both class II and Ii chain, or class II and lgp-B, was greatly increased, accounting for >70% of the total intracellular class II–positive structures (Fig. 7, *A* and *B*). These results confirmed that leupeptin induced the intracellular accumulation of Ii-αβ complexes and illustrated the redistribution of MHC class II molecules to lysosomes. Thus, leupeptin-induced alterations in Ii chain cleavage would appear to induce class II transport to lysosomes and appearance of MIICs in cells that do not normally accumulate class II in lysosomal compartments.

Transport of Peptide-loaded Class II Molecules from Lysosomes to the Cell Surface

Lysosomes are classically defined as representing the terminal degradative compartment on the endocytic pathway (Kornfeld and Mellman, 1989). This feature raises the question of whether class II molecules present in lysosomes are degraded or can still be recruited to the cell surface. Transport to the plasma membrane may be possible given that MHC class II molecules are generally resistant to degradation and that lysosomal αβ dimers can be loaded by antigenic peptide (Castellino and Germain, 1995; Qiu et al., 1994; Rudensky et al., 1994; West et al., 1994). It has thus far been difficult to directly monitor the degree to which class II molecules in lysosomes survive to reach the cell surface. Therefore, we next determined whether the I-A^b molecules in lysosomes of leupeptin-treated cells could reach the plasma membrane after leupeptin removal.

Cells were metabolically labeled and chased in the presence of leupeptin for 4 h to accumulate αβ–Ii-p10 complexes in lysosomes. The cells were then washed and cultured for various periods of time in the absence of leupeptin. At each time point, molecules reaching the cell surface were detected by biotinylation and analyzed by SDS-PAGE with or without boiling.

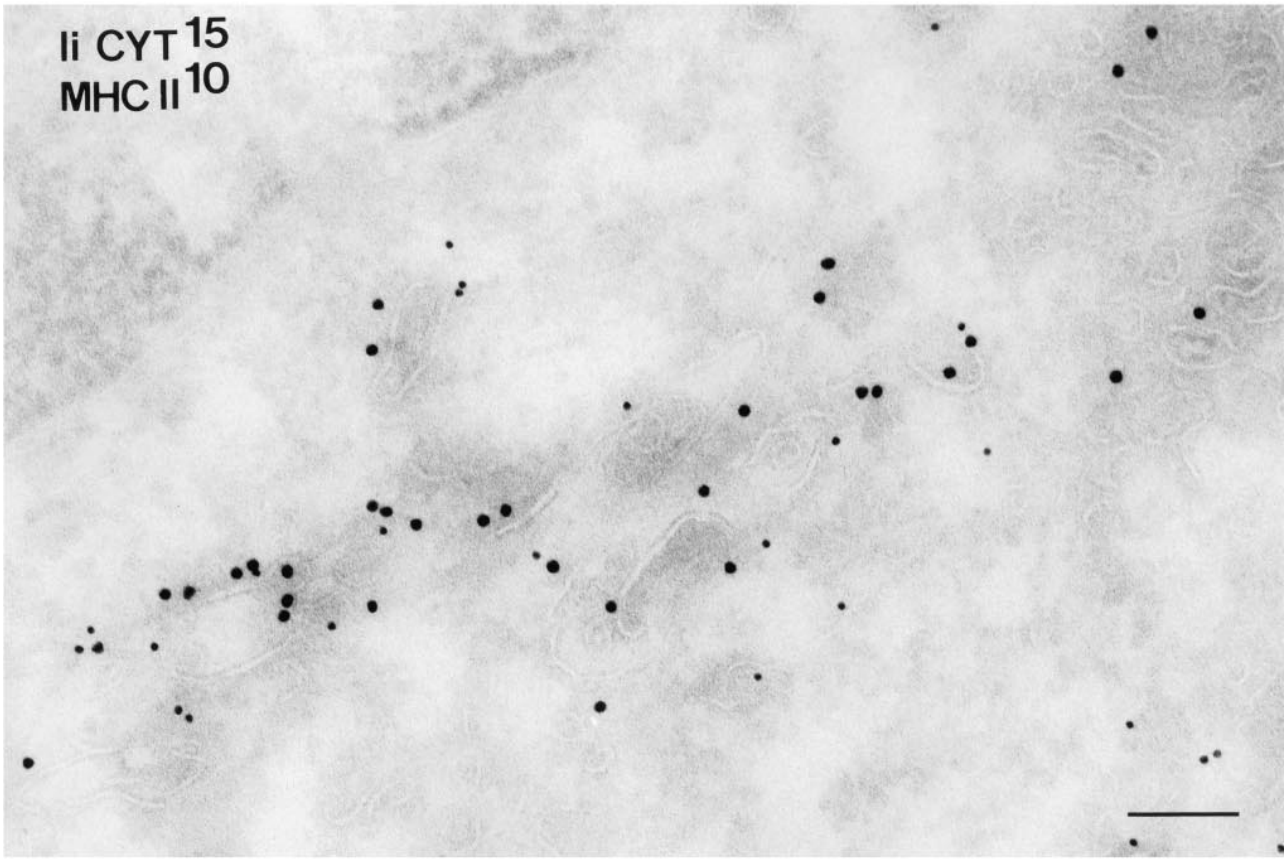


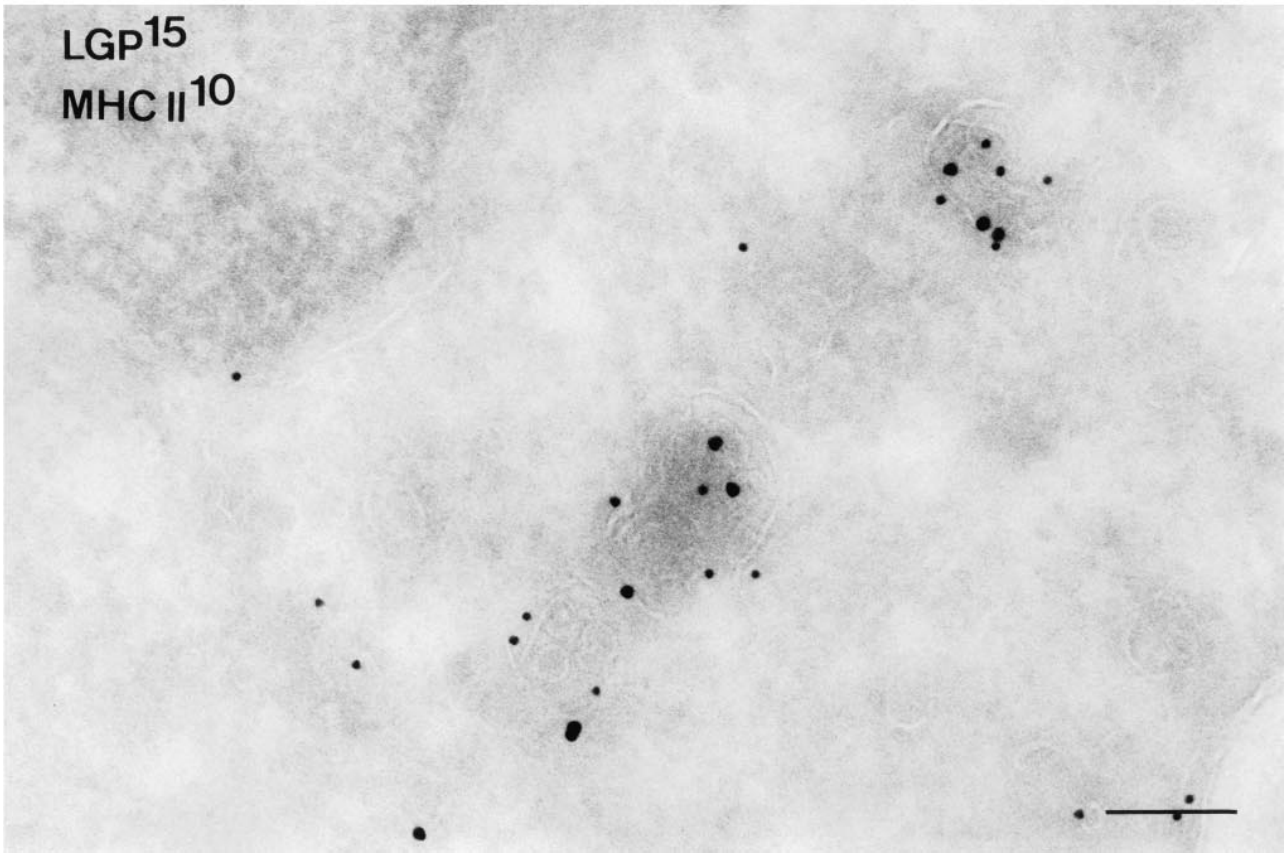
After 4 h in leupeptin, most of the I-A^b molecules were, as expected, present as αβ–Ii-p10 complexes that migrated as 70-kD complexes (p70) when not boiled, but as monomeric α, β, and Ii-p10 chains after boiling (Fig. 8 *A*, time *0*; *NB* and *B*, respectively). Some of the class II was also present as 60-kD peptide-loaded compact dimers ("*C*") not associated with Ii-p10. Relatively little class II was transported to the cell surface (Fig. 8 *A*, time *0*, *righthand panels*).

After culture in the absence of leupeptin, p70 was converted into compact dimers and transferred to the plasma membrane. Surface appearance was exceedingly slow, however, requiring up to 24 h for completion. Interestingly, over this time course, relatively little (~40%) class II was lost to degradation, a percentage not too different from the normal rate of degradation of MHC class II in control cells (Fig. 8 *A*, *TOTAL*, *right panel*). Thus, transit to lysosomes did not accelerate the rate of class II degradation but did dramatically retard its rate of surface appearance.

Figure 7. Distribution of MHC class II, Ii chain, and lgp-B in leupeptin-treated cells. (*A*) Quantification of MHC class II, Ii, and lgp-B in control and leupeptin-treated cells. The quantifications were carried out on ultrathin cryosections immunogold labeled for Ii chain (anti–Ii cytosolic tail mAb IN1) and MHC class II (rabbit anti–I-A cytoplasmic domain serum) or immunogold labeled for lgp-B (mAb GL2A7) and MHC class II (rabbit anti–I-A cytoplasmic domain serum) or immunogold labeled for lgp-B (mAb GL2A7) and MHC class II (rabbit anti–I-A cytoplasmic domain serum). In each case, 40 cell profiles were analyzed. (*B*) Immunogold localization of Ii chain, lgp-B, and I-A in leupeptin-treated cells. (*Upper panel*) Ultrathin cryosections were double immunogold labeled with the Ii chain antibody IN-1 (anti–Ii cytosolic domain; *Ii CYT*) and rabbit anti–I-A cytosolic tail polyclonal antibody. Ii chain accumulates in I-A–positive compartments displaying internal vesicles and electron-dense content. (*Lower panel*) Ultrathin cryosections were double immunogold labeled with the anti–lgp-B mAb GL2A7 and rabbit anti–I-A cytosolic tail polyclonal antibody. Class II molecules were visualized in electron-dense, lgp-B–positive compartments. The size of the gold particles is indicated. Bars, 120 nm.

B





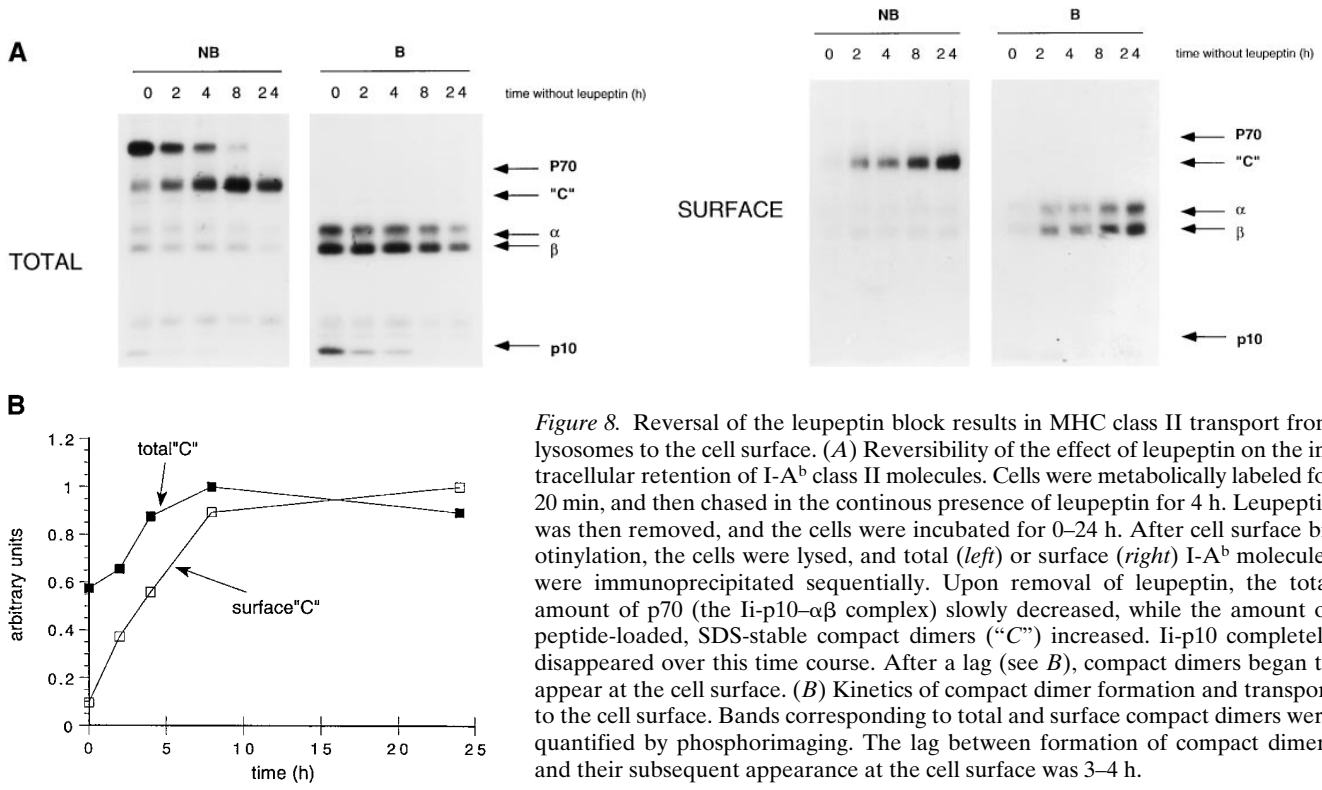


Figure 8. Reversal of the leupeptin block results in MHC class II transport from lysosomes to the cell surface. (*A*) Reversibility of the effect of leupeptin on the intracellular retention of I-A^b class II molecules. Cells were metabolically labeled for 20 min, and then chased in the continous presence of leupeptin for 4 h. Leupeptin was then removed, and the cells were incubated for 0–24 h. After cell surface biotinylation, the cells were lysed, and total (*left*) or surface (*right*) I-A^b molecules were immunoprecipitated sequentially. Upon removal of leupeptin, the total amount of p70 (the Ii-p10–αβ complex) slowly decreased, while the amount of peptide-loaded, SDS-stable compact dimers ("*C*") increased. Ii-p10 completely disappeared over this time course. After a lag (see *B*), compact dimers began to appear at the cell surface. (*B*) Kinetics of compact dimer formation and transport to the cell surface. Bands corresponding to total and surface compact dimers were quantified by phosphorimaging. The lag between formation of compact dimers and their subsequent appearance at the cell surface was 3–4 h.

Quantification of these results revealed a lag between the appearance of peptide-loaded compact dimers and their transfer to the plasma membrane (Fig. 8 *B*). Although most of the compact dimers that formed intracellularly eventually reached the cell surface, compact dimer formation was more rapid and efficient than plasma membrane appearance. Even at the start of the chase period, significant amounts of compact dimers were already present, none of which had reached the surface. If the time required to reverse the effect of leupeptin was taken into account (indicated by the rate of disappearance of p70 and appearance of compact dimers in total cell lysates), a 3–4-h delay was observed before newly formed compact dimers reached the surface (Fig. 8 *B*). This is slow when compared with the kinetics of transport of class II to the surface of A20 cells in the absence of leupeptin (1–2-h lag), under which conditions little if any newly synthesized class II ever reaches lysosomes (Amigorena et al., 1994). However, the rate was similar to the kinetics of class II transport to the surface of cells (e.g., human lymphoblasts) that accumulate significant amounts of class II in lysosomal compartments even in the absence of leupeptin (Neefjes et al., 1990).

Discussion

Ii Chain Processing Controls Class II Accumulation in Lysosomes

The Ii chain cytoplasmic domain has a well-characterized targeting determinant that specifies the transport of MHC class II molecules to endocytic organelles (Arneson and Miller, 1995; Bakke and Dobberstein, 1990; Lotteau et al., 1990; Nijenhuis et al., 1994; Odorizzi et al., 1994). The bulk of this transport reflects direct targeting of newly synthesized complexes from the TGN to endosomes, although a fraction may reach endosomes via the plasma membrane (Benaroch et al., 1995; Roche et al., 1993). Shortly thereafter, Ii chain is processed in a stepwise fashion, principally by the cysteine proteases cathepsins S and B (Cresswell, 1992; Morton et al., 1995; Riese et al., 1996). In both murine and human cells, a common processing intermediate is the 10-kD fragment Ii-p10 (Cresswell, 1996). This fragment exists as a trimer complexed with three αβ dimers; it also contains the Ii chain cytoplasmic tail, membrane anchor, and a segment of the luminal domain probably including the CLIP sequence thought to occupy the peptide binding groove of αβ dimers (Amigorena et al., 1995). Although a normal intermediate, Ii-p10–αβ complexes accumulate to high levels in cells treated with leupeptin (Amigorena et al., 1995). Leupeptin thus inhibits processing of Ii-p10 to yield shorter Ii chain fragments that can dissociate from the αβ dimer to permit binding of antigenic peptide.

Our most striking observation was that the inhibition of Ii chain processing by leupeptin alters the postendosomal transport of class II molecules resulting in their accumulation in lysosomes, even in cells that normally transport little class II to late endocytic compartments. We studied the behavior of two different class II haplotypes in A20 cells, the endogenously expressed I-A^d αβ dimers as well as stably transfected cells coexpressing dimers of the I-A^b haplotype. In the absence of leupeptin, both types of Ii-αβ complexes were transported through the Golgi complex and from the TGN (presumably via endosomes) to the plasma membrane with a lag of just 1–2 h. Cell fraction-

ation and immunocytochemistry both indicated that little of the class II remained intracellularly and, of this, none was detected in heavy density lysosomes. Leupeptin treatment changed this distribution dramatically but selectively. In the case of I-A^b, Ii-p10–αβ complexes accumulated to high levels, and this correlated with an almost complete inhibition of transport to the plasma membrane and accumulation of class II in lysosomes.

The redistribution of I-A^b αβ dimers to lysosomes did not reflect a generalized effect of leupeptin treatment on the endocytic pathway, since the inhibitor did not alter the distribution of the early endosome marker Tfn or the distribution of lysosomal enzymes. Nor did leupeptin dramatically affect the distribution of a second MHC class II haplotype, I-A^d. We previously found that leupeptin caused only a modest and transient increase in the appearance of Ii-p10–αβ complexes in cells expressing only I-A^d molecules (Amigorena et al., 1995). Moreover, there was only a minor increase in the fraction of I-A^d that reached lysosomes. Instead, the short-lived I-A^d Ii-p10–αβ complexes were restricted to CIIV, a distinct population of endosome-related vesicles enriched in newly synthesized MHC class II that appear to be positioned between endosomes and the plasma membrane (Amigorena et al., 1994; Mellman et al., 1995).

The different behaviors of the two haplotypes probably reflected inherent differences in their abilities to form stable complexes with Ii chain intermediates. I-A^b and I-A^d αβ dimers have different affinities for CLIP, an Ii domain thought to play a role in stabilizing the interaction between the Ii chain trimer and αβ dimers (Sette et al., 1995; Cresswell, 1996). Additionally, different class II haplotypes can alter the efficiency of processing of bound Ii chain (Ploegh, H., personal communication).

Although we have not established the mechanism whereby stable association with Ii chain or Ii-p10 blocks surface transport and triggers lysosomal accumulation, based on our understanding of intracellular class II transport and Ii chain processing, it is possible to suggest a plausible mechanism. After the delivery of new Ii chain–class II complexes to endosomes, Ii chain processing would ordinarily be expected to occur rapidly, yielding first Ii-p10 and then CLIP. Since αβ dimers bound to intact Ii chain or Ii-p10 rarely reach the plasma membrane in A20 cells, the inhibition of Ii chain processing combined with the stable association of Ii chain (or Ii-p10) with class II would result in the intracellular retention of the complex in endosomes. As would be the case for almost any membrane protein prevented from recycling out of endosomes, the Ii-αβ or Ii-p10–αβ complexes would then be delivered to late endosomes and lysosomes (Mellman, 1997). Alternatively, if newly synthesized Ii–class II complexes are transported to the plasma membrane via lysosomes, the inhibition of Ii chain processing induced by leupeptin might act at the level of preventing egress from lysosomes. We were unable to obtain evidence in favor of this latter pathway. Regardless of the actual mechanism, the most important point is that an alteration in Ii chain cleavage results in a dramatic relocalization of class II molecules to lysosomes. This finding not only provides a rationale for understanding postendosomal transport of class II, but also for understanding why different cell types exhibit significant differences in class II distribution.

Variations in class II transport may reflect important functional differences among different types or different developmental stages of antigen presenting cells. For example, we have found that immature bone marrow–derived dendritic cells target virtually all of their newly synthesized class II molecules to lysosomes where they accumulate as Ii-p10–αβ complexes. When the cells are induced to mature, however, Ii-p10 is found only transiently, and new class II molecules are efficiently delivered to the plasma membrane (Pierre, P., S. Turley, E. Gatti, P. Webster, M. Hull, J. Meltzer, A. Mirza, K. Inaba, R. Steinman, and I. Mellman, manuscript submitted for publication). Conceivably, such alterations in transport might reflect the regulated expression of proteases highly efficient at Ii chain proteolysis, such as cathepsin S (Riese et al., 1996). Low protease expression in immature cells, for example, might result in inefficient Ii chain processing and a leupeptin-like phenotype of class II transport to lysosomes; high expression, perhaps induced by cell differentiation, would more efficiently cleave Ii chain, resulting in efficient cell surface transport.

What's in a Name? CIIVs, MIICs, Endosomes, and Lysosomes

The nature of the endocytic compartments in which class II molecules bind peptides has been a topic of considerable interest since the initial description of a heterogeneous but largely lysosome-like compartment containing MHC class II, originally designated MIIC (Peters et al., 1991). The heterogeneity of class II–containing structures is clearly illustrated by the fact that in many murine B cell lines, such as the A20 cell line used here, little MHC class II is normally detected in lysosome-like structures. Instead, much of the intracellular class II in such cells were found associated with a population of endosome-like structures, designated CIIV. It has been of interest to determine the structural and functional relationship between CIIV and MIIC, and between both these structures and conventional endosomes or lysosomes. In other words, to what extent (if at all) do antigen presenting cells possess specializations of the endocytic pathway? CIIV have been physically separated from endosomes in B cells and dendritic cells (Amigorena et al., 1994; Pierre et al., manuscript submitted for publication), suggesting that they represent a discrete population of vesicles. On the other hand, MIIC have been defined as a population of class II–positive lysosomes (Peters et al., 1991, Pierre et al., 1996). Our present results support this possibility. By treating A20 cells with leupeptin, we could "generate" a population of vesicles morphologically and immunocytochemically similar to MIIC. Importantly, the same type of lysosomal structures rich in lgp/lamp (and HLA-DM) were also visible in control cells before leupeptin treatment. Under these conditions, however, they were largely negative for class II molecules and thus were identified as lysosomes or late endosomes.

MHC Class II Transport to the Plasma Membrane

Given the functional importance of HLA-DM in peptide loading, the facts that DM is highly enriched in lysosomal MIIC, as well as being present to a lesser extent in CIIV,

support the probability that immunogenic peptide–class II complexes can probably form at multiple sites intracellularly, even within lysosomes (Castellino and Germain, 1995; Pierre et al., 1996; Sanderson et al., 1994). It has remained unclear, however, whether class II complexes formed in lysosomes could be efficiently recruited to the plasma membrane, since transport from lysosomes to the plasma membrane is thought to be a relatively rare event (Kornfeld and Mellman, 1989). Thus, another important observation in this paper was the direct demonstration that lysosomal class II can reach the cell surface efficiently, if at a slow rate.

After 4 h in leupeptin, we determined that >85% of new I-A^b αβ dimers had accumulated in lysosomes. After an 8-h chase without leupeptin, ~70% of the newly synthesized cohort was detected at the cell surface by biotinylation. Less than 40% of the αβ dimers was lost to degradation of this time course. Thus, most of the new class II molecules that reached the surface during the 8-h chase must have been derived from the lysosomal pool. Despite being relatively efficient, this pathway was slow compared with the normal kinetics of transport of class II molecules to the cell surface in A20 cells, normally complete within 2 h of chase. We cannot completely eliminate the possibility that, for nonspecific reasons, leupeptin slowed a normally rapid rate of class II transport from lysosomes to the surface. However, this is unlikely in that (*a*) leupeptin's inhibition of SDS-stable dimer formation was reversed within 2 h of leupeptin removal; (*b*) the slow rate of transport is in accord with the expectedly slow rate of lysosome to plasma membrane transport; and (*c*) our pulse-chase experiments did not reveal lysosomes as being a normal intermediate in the more rapid pathway of class II transport seen in control cells.

Little is known about the final steps in class II transport to the plasma membrane, and a role for CIIV in this process is still unclear. Recent evidence has shown that, in cells that normally localize considerable amounts of class II to MIIC, some of the lysosomal class II reaches the surface by direct fusion of lysosomes with the plasma membrane (Raposo et al., 1996; Wubbolts et al., 1996). However, a major consequence of this fusion event is the release of class II–containing vesicles, called exosomes (Raposo et al., 1996). In addition, some lysosomal class II may be transported in a retrograde fashion after the normal fusion of lysosomes with incoming early or late endosomes (Mellman, 1997). Such retrograde delivery to endosomes might support the "recycling" of class II molecules that had been released from Ii chain or Ii-p10, which initially prevented transport to the cell surface when the complexes were first delivered to endosomes. Alternatively, lysosomes or late endosomes may selectively bud nascent transport vesicles containing peptide-loaded class II dimers that directly fuse with the plasma membrane. Entry into such lysosome-derived vesicles may be dependent on the normal processing of class II–associated Ii chain.

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