Essential Role for Cathepsin S in MHC Class II–Associated Invariant Chain Processing and Peptide Loading

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Summary

Destruction of Ii by proteolysis is required for MHC class II molecules to bind antigenic peptides, and for transport of the resulting complexes to the cell surface. The cysteine protease cathepsin S is highly expressed in spleen, lymphocytes, monocytes, and other class II–positive cells, and is inducible with interferon-γ. Specific inhibition of cathepsin S in B lymphoblastoid cells prevented complete proteolysis of Ii, resulting in accumulation of a class II–associated 13 kDa Ii fragment in vivo. Consequently, the formation of SDS-stable complexes was markedly reduced. Purified cathepsin S, but not cathepsin B, H, or D, specifically digested Ii from αβIi trimers, generating αβ–CLIP complexes capable of binding exogenously added peptide in vitro. Thus, cathepsin S is essential in B cells for effective Ii proteolysis necessary to render class II molecules competent for binding peptides.

Introduction

Major histocompatibility complex (MHC) class II αβ heterodimers associate early during biosynthesis with a type II membrane protein, the invariant chain (Ii) (Roche et al., 1991; Lamb and Cresswell, 1992). The Ii contains a signal in its cytoplasmic tail (Lotteau et al., 1990; Bakke and Dobberstein, 1990) that delivers a nonameric complex of αβIi to intracellular compartments with late endosomal/lysosomal characteristics (Amigorena et al., 1994; Tulp et al., 1994; West et al., 1994). In these acidic compartments, class II molecules encounter and bind antigenic peptides, and are then deposited on the cell surface for recognition by CD4^{+} T helper cells. Ii associates with class II molecules via direct interaction of residues 81–104 of its lumenal domain (Bijlmakers et al., 1994; Ghosh et al., 1995), designated class II–associated invariant chain peptides (CLIP), with the antigen-binding groove of class II (Rudensky et al., 1991; Riberdy et al., 1992; Chicz et al., 1992). Most class II alleles require an additional class II–like molecule, HLA-DM, to liberate the peptide-binding groove of CLIP, and to facilitate

loading with antigenic peptide (Denzin and Cresswell, 1995; Sherman et al., 1995; Sloan et al., 1995).

A prerequisite for peptide loading of class II αβ dimers is the proteolytic destruction of Ii, as intact αβIi trimers themselves are unable to bind peptides (Roche and Cresswell, 1990). In vivo, inhibition of all cysteine class proteases impairs Ii breakdown and induces accumulation of class II–associated Ii fragments in B lymphoblastoid cells (Blum and Cresswell, 1988; Nguyen et al., 1988; Newcomb and Cresswell, 1993). Consequently, acquisition of antigenic peptide by class II αβ dimers is prevented (Neefjes and Ploegh, 1992), expression of class II molecules at the cell surface is decreased (Neefjes and Ploegh, 1992; Bénaroch et al., 1995), and antigen-stimulated T cell proliferation is attenuated (Buus and Werdelin, 1986; Diment, 1990). Despite the absolute requirement for Ii destruction to render class II molecules capable of binding peptide, the primary proteases that perform this task remain unidentified. Previous attempts at identifying these proteases are difficult to interpret, owing to the rather nonspecific action of the inhibitors used and possible contamination of commercially available protease preparations with other proteases. For example, leupeptin, a commonly utilized protease inhibitor (Nguyen et al., 1988), acts on many proteases of the cysteine and serine class. Lysosomotropic agents, such as quinidine (Humbert et al., 1993) and concanamycin B (Bénaroch et al., 1995), neutralize acidic compartments in a nonspecific manner. Also, analysis of some commercially available cathepsin B preparations in our laboratory have demonstrated the presence of additional cysteine proteases (H. A. C., unpublished data).

Cathepsin S, a cysteine protease originally cloned from human alveolar macrophages, is highly expressed in the spleen and professional antigen-presenting cells, including B lymphocytes, macrophages, and other class II–positive cells (Shi et al., 1992, 1994; Morton et al., 1995). Moreover, it is inducible with interferon-γ (IFNγ), it is a potent endoprotease, and it has a broad pH activity profile (Shi et al., 1992, 1994; H. A. C., unpublished data; Brömme et al., 1993). These characteristics distinguish cathepsin S from other cysteine proteases, and make cathepsin S an ideal candidate for Ii processing. To avoid the problems associated with the use of less specific inhibitors or impure protease preparations, we utilized a novel specific irreversible inhibitor of cathepsin S, and purified recombinant enzyme (Brömme and McGrath, 1996), to probe directly the relationship between cathepsin S activity, Ii proteolysis, and subsequent peptide binding by class II molecules. Cathepsin S alone, in marked contrast with other endosomal proteases, was required for efficient Ii processing to permit subsequent peptide binding by class II molecules. These findings suggest that cathepsin S, along with HLA-DM, may be new essential elements for MHC class II antigen presentation.

Results

Active Site Labeling of Cysteine Proteases
The role of cathepsin S in the proteolytic processing of Ii was investigated by exploiting the properties of a
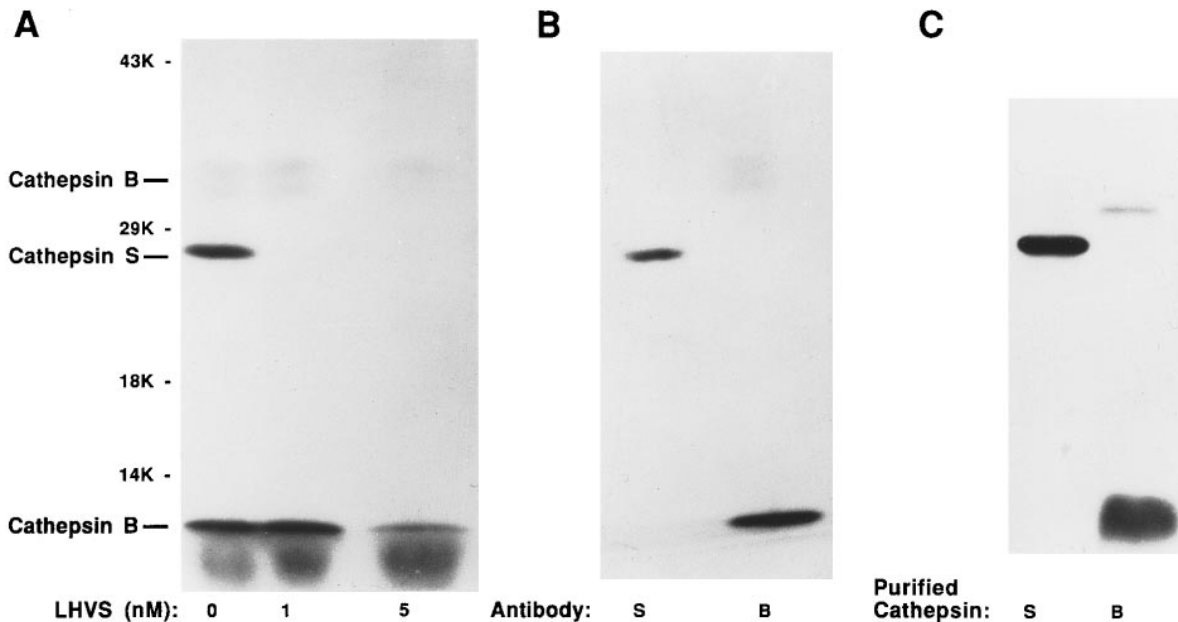
Figure 1. Cbz-[^{125}I]–Tyr–Ala–CN$_2$ Labeling of HOM2 Cells and Purified Human Cathepsins

(A) HOM2 cells were labeled with active site cysteine protease inhibitor Cbz-[^{125}I]–Tyr–Ala–CN$_2$ after incubation with 0, 1, and 5 nM specific cathepsin S inhibitor LHVS, lysed and analyzed by 12% SDS–PAGE.

(B) HOM2 cells were labeled as above without inhibitor, lysed and immunoprecipitated with an antibody against cathepsin S and an antibody against cathepsin B, and analyzed by 12% SDS–PAGE.

(C) Purified human cathepsins B and S were labeled as above and analyzed by 14% SDS–PAGE.

number of protease inhibitors, both novel and previously described. The cysteine protease inhibitor Cbz–Tyr–Ala–CN$_2$ irreversibly binds to the active site of cysteine proteases in proportion to their activity. A profile of the active cysteine proteases present within a given cell type can be directly established by incubating the cells with an iodinated form of this inhibitor, Cbz–[^{125}I]–Tyr–Ala–CN$_2$, and visualizing the labeled proteases on SDS–PAGE (Mason et al., 1989). Cysteine proteases in the cell that are first inactivated with other cysteine class inhibitors prior to incubation with Cbz–[^{125}I]–Tyr–Ala–CN$_2$ produce a corresponding decrease in labeling. Inhibition specific for a given protease will affect subsequent labeling with Cbz–[^{125}I]–Tyr–Ala–CN$_2$ of that particular protease, but not other enzymes present in the preparation. To examine the cysteine protease profile of professional antigen-presenting cells, and measure specifically the activity of cathepsin S, the B lymphoblastoid cell line HOM2 was labeled with Cbz–[^{125}I]–Tyr–Ala–CN$_2$ after incubation with varying concentrations of the specific cathepsin S inhibitor, morpholinurea–leucine–homophenylalanine–vinylsulfone–phenyl (LHVS). LHVS has a second order rate constant for inhibition of cathepsin S, which is 67 times greater than that for cathepsin L and 6000 times greater than that for cathepsin B, illustrating the high degree of specificity for inhibition of cathepsin S activity (Palmer et al., 1995). Lysates prepared from the labeled cells were analyzed either directly (Figure 1A), or subjected to immunoprecipitation with antibodies specific for cathepsins S and B (Figure 1B). In the absence of cathepsin S inhibitor, three polypeptides were labeled, migrating at 33 kDa, 28 kDa, and 6 kDa (running with the dye front in this 12% gel) (Figure 1A). The 33 kDa

and 6 kDa polypeptides were immunoprecipitated with an antiserum specific for cathepsin B (Figure 1B), and represent the single and light chain forms of the active enzyme, respectively. The more intense labeling of the 6 kDa polypeptide compared with the 33 kDa protein suggests that most of the cathepsin B present in HOM2 cells is in the light chain form. The 28 kDa polypeptide was identified as the active form of cathepsin S, as it was immunoprecipitated with an antibody specific for this enzyme (Figure 1B). The labeling of cathepsin S, but not cathepsin B, was effectively inhibited at an LHVS concentration of 1 nM (Figure 1A). At an inhibitor concentration of 5 nM, the labeling of cathepsin B was decreased, although some activity remained. Thus, LHVS can be utilized at 1–5 nM concentrations to inhibit specifically cathepsin S in HOM2 cells, leaving other cysteine proteases functionally active. The active site labeling of cysteine proteases with Cbz–[^{125}I]–Tyr–Ala–CN$_2$ was then used to determine that the purified human cathepsins B and S, used in subsequent experiments described in this manuscript, were not cross-contaminated with other proteases (Figure 1C).

Inhibition of Cathepsin S Prevents Ii Processing

To determine whether inhibition of cathepsin S interferes with processing of Ii and subsequent peptide binding by class II molecules, HOM2 cells were pulsed-labeled with [^{35}S]methionine/cysteine and chased for 5 hr in the presence or absence of LHVS, followed by immunoprecipitation of class II $\alpha\beta$ dimers and $\alpha\beta$Ii complexes with the monoclonal antibody (MAb) Tü36 (Figure 2). One half of the samples were analyzed under mildly denaturing
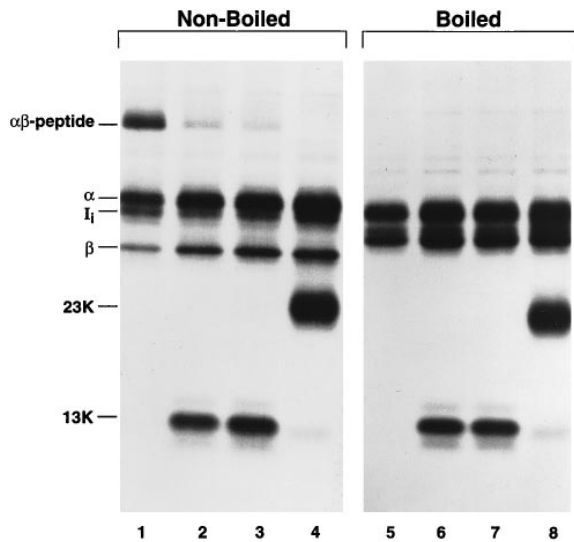
Figure 2. Inhibition of Cathepsin S and Its Effect on Ii Processing and Formation of SDS-Stable Complexes In Vivo

HOM2 cells were labeled with [^{35}S]methionine/cysteine and chased for 5 hr without inhibitor (lanes 1, 5), in the presence of 1 nM LHVS (lanes 2, 6), 5 nM LHVS (lanes 3, 7), and 20 μM E64D (lanes 4, 8). Class II–Ii complexes were immunoprecipitated from cell lysates with MAb Tü36 and analyzed by 14% SDS–PAGE under mildly denaturing conditions (nonboiled, nonreduced) (lanes 1–4) and denaturing conditions (lanes 5–8).

(nonboiled, nonreduced) conditions (Figure 2A) to visualize SDS-stable complexes that migrate at approximately 50 kDa. These SDS-stable complexes represent peptide-loaded $\alpha\beta$ dimers, which decrease upon inhibition of cysteine proteases with leupeptin (Neefjes and Ploegh, 1992). Specific inhibition of cathepsin S with 1 nM and 5 nM LHVS resulted in accumulation of a class II–associated 13 kDa Ii fragment, and a concomitant reduction in peptide loading as evidenced by a marked decrease in formation of SDS-stable complexes (Figure 2A, lanes 2, 3). Inhibition of all cysteine proteases with the cysteine-class inhibitor 2S, 3S-trans-epoxysuccinyl-L-leucylamido-3-methylbutane ethyl ester (E64D) resulted in a buildup of a class II–associated 23 kDa Ii fragment with a decrease in SDS-stable dimer formation

(Figure 2A, lane 4), similar to that seen with leupeptin. This suggests that cathepsin S acts on a relatively late Ii breakdown intermediate and is required for efficient proteolysis of Ii necessary for subsequent peptide loading.

Cathepsin S Selectively Digests Ii Participating in $\alpha\beta$Ii Complexes

To examine the effects of proteolysis by cathepsin S on class II molecules and Ii, individual α, β, and Ii polypeptides were translated in vitro both separately and together under conditions compatible with complex formation (Bijlmakers et al., 1994), followed by digestion with varying concentrations of purified cathepsin S at pH 5.5. Cathepsin S readily digested the α, β, and Ii chains when translated alone (Figure 3). However, when the α and β chains were translated together so that dimer formation occurred, they displayed resistance to proteolysis. When all three moieties were translated together and digested with cathepsin S, only Ii was degraded, illustrating the marked sensitivity of Ii to cathepsin S digestion (Figure 3). The formation of $\alpha\beta$ dimers and $\alpha\beta$Ii trimers were confirmed following each step by immunoprecipitation (data not shown). Thus, cathepsin S selectively degrades Ii molecules that are part of $\alpha\beta$Ii complexes, leaving the $\alpha\beta$ dimers intact.

Can cathepsin B digest $\alpha\beta$Ii complexes with similar efficiency? To compare directly the ability of cathepsins S and B to degrade Ii participating in $\alpha\beta$Ii trimers, the activity of the two proteases must be measured, independent of their differences in substrate specificity. Normalization to total protein content is misleading because only a portion of the total protein may be active. To overcome this difficulty, the molar activity of the purified cathepsin B and cathepsin S preparations were measured by using E64D as an active site titrant with the substrate Z–Phe–Arg–AMC, as previously described (Barrett and Kirschke, 1981). The activities of the enzymes measured in this manner are independent of the substrate used, because E64D irreversibly inhibits both cathepsins S and B on an equimolar basis.

Using the concentrations determined by the above method, the ability of cathepsins S and B to digest Ii from $\alpha\beta$Ii heterotrimers immunoprecipitated from HOM2
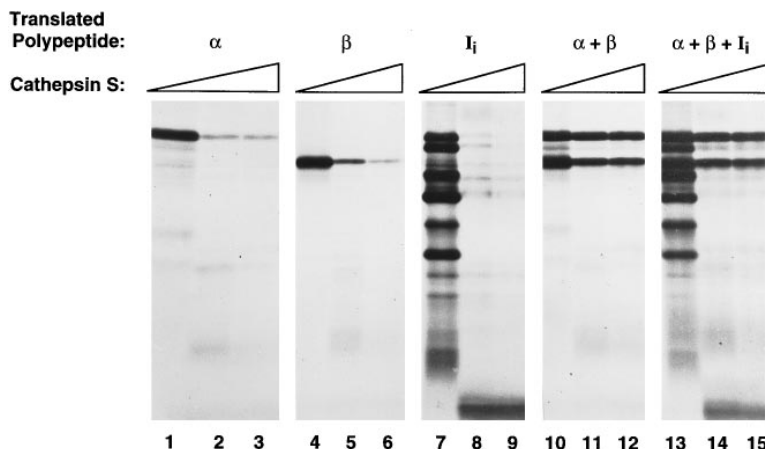

Figure 3. Digestion of In Vitro-Translated α, β, Ii Polypeptides with Purified Human Cathepsin S

MHC class II α chains, β chains, and Ii were translated alone (lanes 1–9), or together (α + β chains, lanes 10–12; α + β + Ii, lanes 13–15) followed by digestion with cathepsin S at pH 5.5. Translations were performed in conditions known to form $\alpha\beta$ dimers and $\alpha\beta$Ii complexes as previously reported (Bijlmakers et al., 1994). Cathepsin S concentrations were 0 μM (lanes 1, 4, 7, 10, 13), 0.19 μM (lanes 2, 5, 8, 11, 14), and 0.38 μM (lanes 3, 6, 9, 12, 15). Samples were analyzed by 15% SDS–PAGE under denaturing conditions.

A

B
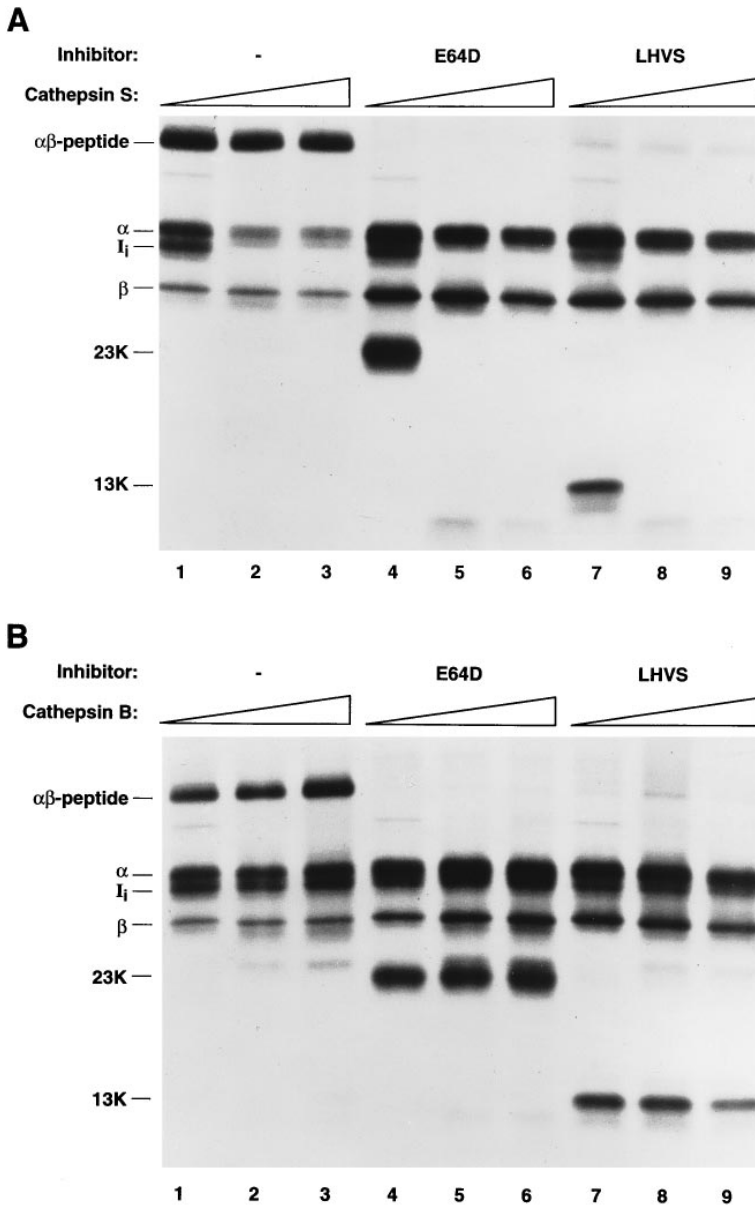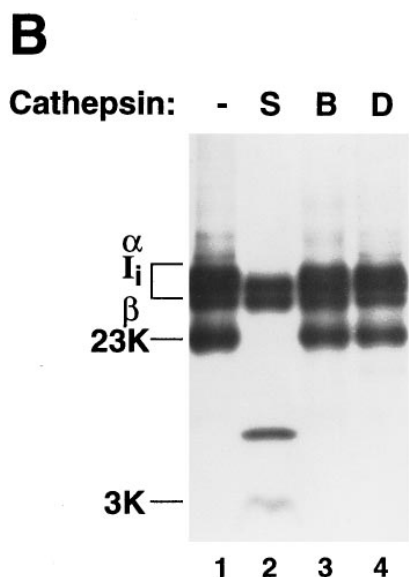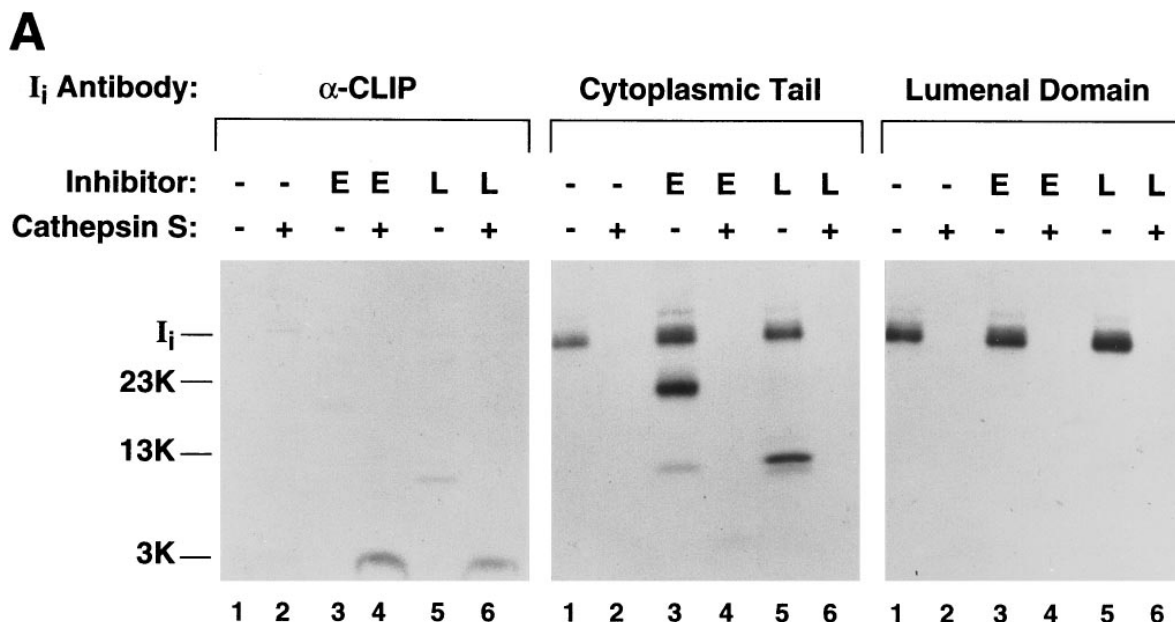

Figure 4. Digestion of Immunoprecipitated $\alpha\beta$Ii Trimers with Purified Human Cathepsins S and B

(A) HOM2 cells were labeled with [^{35}S]methionine/cysteine and chased for 5 hr in the absence and presence of inhibitors, lysed, and immunoprecipitated with Tü36. Immunoprecipitates were then digested with purified cathepsin S at 0 μM (lanes 1, 4, 7), 0.23 μM (lanes 2, 5, 8), and 0.76 μM (lanes 3, 6, 9), and analyzed by 14% SDS–PAGE under mildly denaturing conditions (nonboiled, nonreduced). (B) HOM2 cells were labeled and immunoprecipitated as in (A), and then were subjected to digestion with purified human cathepsin B at 0 μM (lanes 1, 4, 7), 6.6 μM (lanes 2, 5, 8), and 22 μM (lanes 3, 6, 9). Conditions for SDS–PAGE analysis were identical to those above.

cells was determined. HOM2 cells were incubated in the absence of inhibitor (Figure 4, lanes 1–3), or in the presence of inhibitors E64D (lanes 4–6) or LHVS (lanes 7–9), during a [^{35}S]methionine/cysteine pulse-chase. Cathepsin S specifically degraded intact Ii as well as the 23 kDa and 13 kDa Ii intermediates, while sparing the $\alpha\beta$ dimer and the $\alpha\beta$–peptide complexes (Figure 4A). In contrast, cathepsin B showed little proteolytic activity against immunoprecipitated $\alpha\beta$Ii trimers or $\alpha\beta$Ii fragment complexes, even at 100× the molar concentration of cathepsin S. The inability of cathepsin B to digest Ii was not merely a result of the enzyme not being active at the concentrations used, as evidenced by the slight change in migration of the class II β chain (Figure 4B, compare lane 3 with lane 4), suggesting activity of cathepsin B on the cytoplasmic portion of the β chain (Roche and Cresswell, 1991).

A defined intermediate in the maturation of class II molecules is a complex consisting of the $\alpha\beta$ heterodimer bound to the CLIP region of Ii (Avva and Cresswell, 1994). It is this intermediate that was proposed to be a substrate for HLA-DM. To determine whether cathepsin S could generate $\alpha\beta$–CLIP from $\alpha\beta$Ii, HOM2 cells were labeled in the absence and presence of inhibitors, immunoprecipitated with the MAb Tü36, digested with cathepsin S, and reimmunoprecipitated with antibodies directed against Ii (Figure 5A). Following digestion with cathepsin S, a 3 kDa polypeptide was immunoprecipitated with an anti-CLIP antibody (Figure 5A, lanes 4, 6), which was not found in the undigested samples (lanes 3, 5). An antibody directed against the N-terminal cytoplasmic tail, PIN-1, was able to immunoprecipitate intact Ii as well as the 23 kDa and 13 kDa Ii chain fragments (Figure 5A, lanes 1, 3, and 5), suggesting that both the 23

A

B

Figure 5. Generation of αβ–CLIP Complexes from Immunoprecipitated αβIi Trimers by Digestion with Cathepsin S
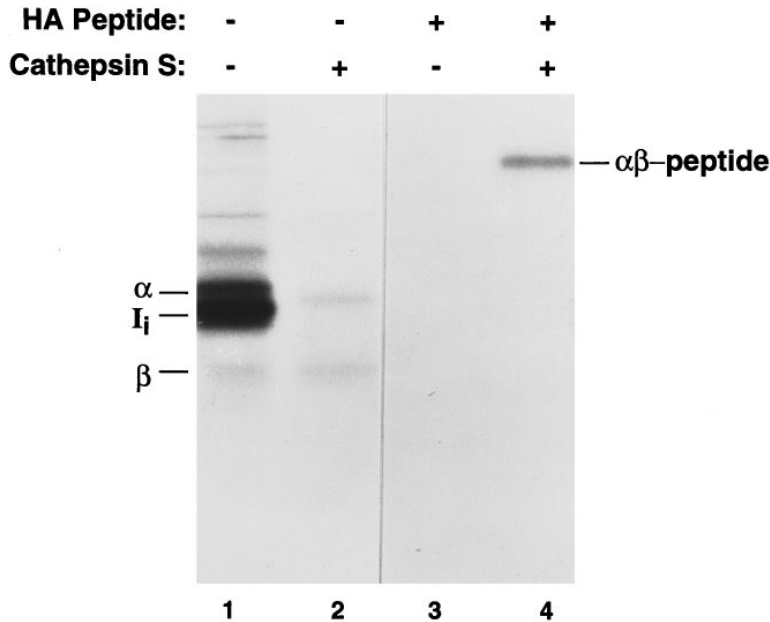
(A) HOM2 cells were labeled with [^{35}S]methionine/cysteine and chased for 5 hr in the absence and presence of inhibitors, immunoprecipitated with Tü36 MAb, and digested with 0.23 μM cathepsin S for 1 hr at 37°C. Immunoprecipitates were then boiled for 3 min in the presence of 1% SDS to unfold the αβIi complexes, diluted 10-fold, and reimmunoprecipitated sequentially with antibodies against the CLIP region (left), cytoplasmic tail (PIN-1 antibody, center), and lumenal domain (LN-2 antibody, right) of Ii. Samples were analyzed by 10%–20% gradient tricine SDS–PAGE under denaturing conditions.

(B) HOM2 cells were labeled with [^{35}S]methionine/cysteine and chased for 5 hr in the presence of 0.5 mM leupeptin, immunoprecipitated with Tü36, and incubated at pH 5.5 in the absence of enzyme (lane 1), 0.23 μM cathepsin S (lane 2), 66 μM cathepsin B (lane 3), and 2.4 μM cathepsin D (lane 4) for 1 hr at 37°C. The digested complexes were then analyzed by 10%–20% tricine gel under denaturing conditions.

Abbreviations: minus, no inhibitor; E, 20 μM E64D; L, 5 nM LHVS; S, cathepsin S; B, cathepsin B; D, cathepsin D.

kDa and 13 kDa intermediates are N-terminal fragments. The LN-2 antibody against the Ii lumenal domain precipitated only the full-length Ii (Figure 5A, lanes 1, 3, and 5). Thus, cathepsin S is able to produce αβ–CLIP, a known intermediate in αβIi proteolysis.

In contrast with cathepsin S, neither purified cathepsin B nor D could produce αβ–CLIP from αβIi (Figure 5B). HOM2 cells were pulse–chased in the presence of leupeptin and class II molecules were immunoprecipitated with Tü36. The isolated class II complexes were then digested with the different purified cathepsins. Digestion with cathepsin S alone resulted in the production of a 3 kDa polypeptide associated with αβ dimers (Figure 5B, lane 2). This 3 kDa fragment could also be reimmunoprecipitated with the anti-CLIP reagent used above (data

not shown). Both cathepsins B and D, when used at high concentrations, could produce large molecular weight Ii cleavage products, but not αβ–CLIP, illustrating the essential role of cathepsin S in complete and efficient Ii processing.

While inclusion of HLA-DM facilitates exchange of CLIP bound to the αβ–CLIP complex for antigenic peptide (Denzin and Cresswell, 1995; Sherman et al., 1995; Sloan et al., 1995), the reaction can also occur, albeit less efficiently, in the absence of added HLA-DM. The displacement of larger Ii remnants with antigenic peptides is even less efficient. Can proteolysis of αβIi heterotrimers with cathepsin S allow peptide loading onto the resulting class II αβ–CLIP complexes? HOM2 cells were treated with concanamycin B to accumulate a large

Figure 6. Digestion of αβIi Complexes with Cathepsin S Generates Class II Molecules Capable of Binding Peptide

HOM2 cells were labeled with [^{35}S]methionine/cysteine and chased for 5 hr in the presence of 20 nM concanamycin B. Class II–Ii complexes were immunoprecipitated from cell lysates with LN-2. These immunoprecipitates were then incubated at pH 5.5 in the absence (lane 1) and presence (lane 2) of 0.23 μM human cathepsin S and immediately placed in SDS–PAGE sample buffer to stop the digestion. In a parallel experiment, unlabeled concanamycin B–treated HOM2 cells were lysed and the class II–Ii complexes were immunoprecipitated with LN-2. These immunoprecipitates were incubated in the absence (lane 3) and presence (lane 4) of 0.23 μM cathepsin S. The digested precipitates were then exposed to ^{125}I–HA at pH 5.5 for 4 hr. Samples were diluted to 0.8 ml with lysis buffer, precleared of LN-2 antibody, and immunoprecipitated with Tü36. All samples were analyzed by 14% SDS–PAGE under mildly denaturing (nonboiled, nonreduced) conditions.

amount of αβIi trimers (Bénaroch et al., 1995). The hemagglutinin peptide (HA), containing amino acids 306–318, was used as a DR1-restricted peptide (Rothbard et al., 1988). The αβIi complexes from concanamycin B–treated cells were initially immunoprecipitated with an antibody against the Ii lumenal domain (LN-2) to precipitate only intact αβIi complexes (Figure 6). These complexes were incubated in the absence and presence of cathepsin S at pH 5.5, and then exposed to ^{125}I–HA at the same acidic pH. After removal of unbound ^{125}I–HA, complex formation was assessed by SDS–PAGE. Digestion of αβIi with cathepsin S and subsequent exposure to ^{125}I–HA resulted in the formation of labeled αβ–peptide complexes, although this conversion was incomplete as evidenced by the continued presence of SDS-labile class II molecules (data not shown). Cathepsin S is thus able to process Ii while leaving the class II molecules functionally intact, which shows that cathepsin S is sufficient, by itself, to effectively degrade Ii in a manner that renders αβ dimers capable of binding peptide.

Inhibition of Cathepsin D Does Not Effect Ii Processin

Maric et al. (1993) have implicated the aspartyl protease cathepsin D in an early step of Ii breakdown. To examine this question in our system, a potent aspartyl class protease inhibitor, CGP 53437, which inhibits cathepsin D in the nanomolar range, was utilized. CGP 53437 inhibits cathepsin D activity in human monocytes by 75% and 90% at concentrations of 5 μM and 50 μM, respectively (D. Simon et al., unpublished data). Inhibition of cathepsin D with CGP 53437 did not result in accumulation of Ii fragments nor did it produce a decrease in SDS-stable complexes (Figure 7), suggesting that cathepsin D is not essential for Ii processing.

Cathepsin H, a lysosomal cysteine protease with good aminopeptidase but weak endopeptidase activity, is upregulated by IFNγ in mouse peritoneal macrophages (Lafuse et al., 1995). Purified human cathepsin H was not inhibited by LHVS nor did it display any proteolytic activity against immunoprecipitated αβIi (data not

shown), implying that cathepsin H is not an essential protease for Ii degradation.

Discussion

A noteworthy feature of the various components of the class II–restricted antigen presentation pathway is that
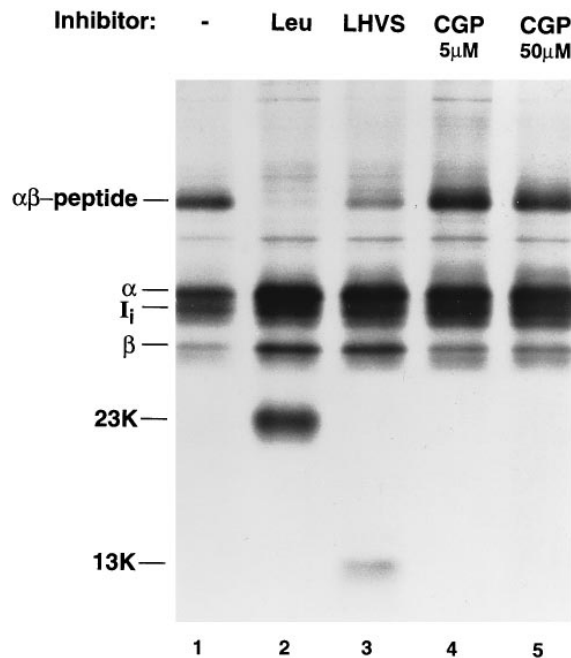

Figure 7. Inhibition of Aspartyl Proteases Does Not Effect Ii Processing

HOM2 cells were labeled with [^{35}S]methionine/cysteine and chased for 5 hr without inhibitor (lane 1), in the presence of 0.5 mM leupeptin (lane 2), 5 nM LHVS (lane 3), 5 μM CGP 53437 (lane 4), and 50 μM CGP 53437 (lane 5). Samples were analyzed by 14% SDS–PAGE under mildly denaturing conditions. Abbreviations: minus, no inhibitor; Leu, 0.5 mM leupeptin; L, 5 nM LHVS; CGP, CGP 53437.

they appear to be regulated in a coordinate fashion. Expression of the structural genes that encode class II α and β chains, Ii, and the accessory molecule HLA-DM, are all under the control of the MHC class II trans-activator, CIITA (Steimle et al., 1993, 1994; Siegrist et al., 1995). In professional antigen-presenting cells, CIITA regulates both constitutive as well as IFNγ-induced expression of these essential components of the class II processing and presentation pathway (Steimle et al., 1993, 1994). However, it would be naive to assume that all of the components involved in loading class II molecules with peptide are subject to control by a single transcription factor, and that all of the essential players have been identified. Indeed, induced expression of the class II subunits, Ii and HLA-DM in melanoma cells via transfection of CIITA alone, was not sufficient to achieve effective processing and presentation of protein antigen (Siegrist et al., 1995). In contrast, IFNγ stimulation by itself did confer the ability to present antigen in a class II–restricted fashion, and corrected the antigen-presentation defect of the CIITA transfectants. Thus, additional IFNγ-inducible factor(s), necessary to load class II molecules with antigenic peptide effectively, must exist (Siegrist et al., 1995).

We now provide evidence that the cysteine protease, cathepsin S, is one of these, as yet unrecognized, essential elements. A priori, cathepsin S is an excellent candidate for participation in the breakdown of Ii because it is a potent endopeptidase, has a broad pH activity range (Shi et al., 1994; Brömme et al., 1993), and is present in class II–positive antigen-presenting cells, including B lymphocytes (Morton et al., 1995), macrophages (Shi et al., 1994), and bone marrow–derived mouse dendritic cells (E. Schneeburger and R. J. R., unpublished data). We here show that cathepsin S, unlike cathepsins B or D, is required for complete Ii proteolysis, a step that is necessary for subsequent peptide loading in the B lymphoblastoid cell line HOM2. In addition, inhibition of cathepsin S attenuated tetanus toxoid–stimulated T cell proliferation from immune individuals in peripheral blood mononuclear cells, implicating participation of cathepsin S in presentation of peptide antigen by class II molecules (data not shown).

Specific inhibition of cathepsin S activity in HOM2 cells resulted in an accumulation of class II molecules associated with a 13 kDa Ii fragment, and a concomitant reduction in the appearance of SDS-stable complexes. Thus, in the absence of cathepsin S, class II–associated Ii molecules are susceptible to attack by other proteases, but the action of these proteases may not allow the production of the intermediates that favor peptide binding to class II molecules. Whether the activity we invoke for such additional proteases is essential in the course of normal breakdown of Ii is not clear. A 23 kDa Ii processing intermediate may be generated by noncysteine proteases, which is then further digested by cysteine proteases other than cathepsin S to generate the 13 kDa intermediate. This 13 kDa intermediate could serve as the final substrate for complete proteolysis by cathepsin S. However, given that in vitro, cathepsin S alone was capable of generating αβ–CLIP complexes capable of binding peptide, it appears more likely

that these additional proteases may provide a less efficient salvage pathway for Ii processing, used only when cathepsin S is absent.

Because cathepsin S is able to generate αβ–CLIP complexes from intact αβIi, it may thus be responsible for creating a suitable substrate for HLA-DM. In vitro experiments with both immunoprecipitated (Denzin and Cresswell, 1995; Sherman et al., 1995) and soluble DM (Sloan et al., 1995) have clearly shown that HLA-DM induces removal of CLIP from αβ–CLIP complexes, permitting, as well as facilitating, peptide loading. Inhibition of the N-terminal cleavage performed by cathepsin S may therefore inhibit DM-mediated dissociation of Ii-breakdown intermediates (i.e., CLIP). Just as the affinity of class II molecules for CLIP and the requirement for HLA-DM to remove CLIP appears to vary between class II alleles (reviewed by Wolf and Ploegh, 1995; Sette et al., 1995), allelic variation may dictate dependency on the proteolytic activity of cathepsin S to effectively remove Ii. In other class II–positive cells, we cannot rule out the participation of lysosomal enzymes with a substrate profile similar to that of cathepsin S (i.e., cathepsin L), absent from the B lymphoblastoid cell line utilized in the present study.

Although cathepsin S may be the primary protease involved in Ii breakdown, it is certainly not the only protease essential to class II–restricted antigen presentation. Proteases are not only necessary for Ii breakdown, but are required in the processing of protein antigen for presentation by class II molecules. Thus, other lysosomal enzymes, i.e., cathepsins B, H, D, and E, may primarily function in generating epitopes for capture by αβ dimers made competent to bind peptide by exposure to cathepsin S (Matsunaga et al., 1993; Rodriguez and Diment, 1992; Bennett et al., 1992). It is notable that cathepsins H and B are potent amino- and carboxypeptidases, respectively. The action of cathepsin D has been revealed in the generation of peptides suitable for binding to class II molecules (Van Noort and van der Drift, 1989; Van Noort et al., 1991).

What, then, is the specific substrate for the proteolytic activity of cathepsin S? The 13 kDa Ii fragment that accumulates when cathepsin S activity is inhibited can be coprecipitated with class II molecules by an antibody against the cytoplasmic tail of Ii, but not with an antibody directed against the lumenal domain. In a similar fashion, cells exposed to the cysteine protease inhibitor leupeptin accumulate a number of Ii breakdown products that remain associated with class II αβ chains (Neefjes and Ploegh, 1992), thus preventing peptide loading and intracellular transport of these molecules. One such N-terminal fragment, designated small leupeptin-induced peptide (SLIP) (Nguyen et al., 1988), is similar in molecular mass to the 13 kDa Ii fragment that accumulated when cathepsin S activity was inhibited. A similar N-terminal SLIP-like fragment is found associated with class II when the macrolide antibiotic concanamycin B is used to inactivate cellular vacuolar H+ ATPases, neutralizing the endocytic compartments where Ii breakdown and peptide binding occur (Bénaroch et al., 1995). Thus, the accumulation of class II molecules associated with SLIP-like fragments when the above inhibitors are

used may be a direct consequence of cathepsin S inhibition. The smallest N-terminal Ii fragment that can associate with αβ dimers and effectively inhibit peptide binding is comprised of amino acids 1–104 (Bijlmakers et al., 1994), which includes the CLIP region. Whereas other proteases may be able to digest the C terminus of Ii, cathepsin S appears essential for cleavage of the N terminus, a necessary step in generating αβ–CLIP complexes. Because the N-terminal cytoplasmic tail of Ii contains the signals for sorting and retention (Amigorena et al., 1994), removal of this segment by cathepsin S would also be necessary to free class II molecules from the retention mechanisms imposed by Ii and to allow transport to the cell surface.

Finally, based on the preferred substrate specificities of cathepsin S, we can hypothesize as to its likely cleavage sites near the N terminus of the CLIP region. Both Arg and Lys are good P1 residues for proteolysis by cysteine proteases (Mason et al., 1984, 1985), and cathepsin S prefers branched nonpolar residues such as Leu or Val in the P2 position (Brömme et al., 1989). This suggests that the Arg–Met bond between amino acids 78 and 79 with Leu in the P2 position, or the Lys–Leu bond between amino acids 80 and 81 with Met in the P2 position, may be good substrates for cathepsin S. CLIP fragments have been eluted from class II molecules exhibiting N termini beginning at amino acids 81–83, consistent with the putative cathepsin S cleavage sites listed above (Riberdy et al., 1992; Chicz et al., 1992). Further modification of the N terminus in vivo may occur following cathepsin S cleavage by endogenous aminopeptidases.

In summary, the finding that a single protease is crucial for Ii proteolysis is surprising indeed, and has several important implications both in the fields of lysosomal biology and immunology. This finding contrasts the prior view of lysosomes as pools of enzymes that mediate terminal degradation of endocytosed protein (Barrett and Kirschke, 1981). Cathepsin S expands the list of components essential to class II antigen presentation, and implies that the enzymes that mediate Ii proteolysis and antigen processing may be distinct. In turn, this makes targeting of cathepsin S a possibility for modulating class II–restricted immune responses.

Experimental Procedures

Materials
Cbz–Tyr–Ala–CN₂ was a gift from Dr. E. Shaw (Friedrich Miescher-Institut, Basel, Switzerland). E64D and leupeptin were obtained from Sigma Chemical Company (St. Louis, Missouri). LHVS, an irreversible specific cathepsin S inhibitor, was provided by Dr. J. T. Palmer (Arris Pharmaceuticals, South San Francisco, California) (Palmer et al., 1995). CGP 53437 was provided by Ciba–Geigy, Limited (Basel, Switzerland) (Alteri et al., 1993). Stock solutions of these inhibitors were made in dimethyl sulfoxide. Concanamycin B was obtained from Ajinomoto Corporation (Kanagawa, Japan). A 10 μM stock solution was prepared in ethanol. Purified human cathepsin S was obtained by expression in Sf9 cells using the Baculovirus expression system as described (Brömme and McGrath, 1996). Purified human cathepsins B, D, and H were obtained from Calbiochem–Novabiochem Corporation (San Diego, California). HA, a DR1-presentable peptide (amino acids 306–318), was synthesized (t-boc chemistry) on a Biosearch SAM 2 peptide synthesizer, dissolved in H₂O, and stored at −70°C.

[³⁵S]methionine/cysteine and ¹²⁵I were obtained from DuPont New England Nuclear (Boston, Massachusetts). Protein A–agarose was purchased from Boerhinger Mannheim (Indianapolis, Indiana). Methionine-free cysteine-free Dulbecco's modified Eagle's medium (DMEM), penicillin/streptomycin (100×), HEPES (100×), and glutamine (100×) were obtained from GIBCO BRL (Gaithersburg, Maryland). RPMI was purchased from Fisher (Pittsburgh, Pennsylvania) and fetal bovine serum (FBS) was obtained from Hyclone (Logan, Utah). Normal rabbit serum, normal mouse serum, and Histopaque were purchased from Sigma. Iodogen was obtained from Pierce (Rockford, Illinois) and the C18 Sep-pak column was purchased from Waters, Incorporated (Milford, Massachusetts).

Antibodies
Tü36 is a mouse MAb (Shaw et al., 1985) that recognizes HLA-DR1 αβ dimers alone or in association with Ii. Two Ii-reactive mouse MAbs were used: PIN-1, specific for the N terminus of Ii, provided by Dr. P. Cresswell (Yale University, New Haven, Connecticut), and the MAb LN-2, directed against the C terminus of Ii, provided by Dr. A. Epstein (University of Southern California Medical School, Los Angeles, California). The anti-CLIP reagent was generated by the laboratory of Dr. E. Weirtz (National Institute of Public Health and Environmental Protection, Netherlands) by injection of rabbits with two overlapping peptides, conjugated to KLH, spanning the region of residues 81–104 of intact human Ii. Antibody to human cathepsin S was prepared as described previously (Shi et al., 1994), and antibody to cathepsin B was purchased from Vital Products, Incorporated (St. Louis, Missouri).

Cell Culture
The B lymphoblastoid cell line HOM2 (homozygous for HLA-DR1) was maintained in RPMI with 10% FBS, 1/1000 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM glutamine.

Active Site Labeling of Cysteine Proteases
The cysteine protease inhibitor Cbz–Tyr–Ala–CN₂ was iodinated as previously reported (Mason et al., 1989). HOM2 cells (5 × 10⁶ cells/sample) were incubated with inhibitors E64D (20 μM), leupeptin (0.5 mM), concanamycin B (20 nM), or LHVS (1 or 5 nM) at 37°C for 1 hr prior to labeling. Cells were labeled by incubation with Cbz–[¹²⁵I]–Tyr–Ala–CN₂ for 1 hr at 37°C, washed twice with phosphate-buffered saline, and lysed in the SDS–PAGE sample buffer. The purified cathepsins B and S were labeled by addition of a 2 μl aliquot of purified enzyme stock to 50 μl of digestion buffer (50 mM Na acetate [pH 5.5], 1% Triton X-100, 3 mM cysteine, 1 mM EDTA) containing Cbz–[¹²⁵I]–Tyr–Ala–CN₂. Samples were incubated for 1 hr at 37°C and the labeling reaction was stopped by the addition of 50 μl of 2× SDS–PAGE sample buffer.

Immunoprecipitation of cathepsins B and S was performed by labeling 5 × 10⁶ HOM2 cells as above followed by cell lysis with 1 ml of 10 mM Tris–HCL (pH 7.5), 1 mM EDTA, 0.2% SDS, 1% Triton X-100 on ice. Lysates were collected, boiled for 5 min, and precleared with protein A–agarose (Boehringer Mannheim, Indianapolis, Indiana) plus normal rabbit serum (Sigma). The cysteine proteases were immunoprecipitated with anti-cathepsin S antibody and anti-cathepsin B antibody coupled to protein A–agarose. The pellets were washed and eluted with reducing SDS–PAGE sample buffer.

Metabolic Labeling, Class II Immunoprecipitation, and Proteolytic Digestion
HOM2 cells, 5 × 10⁶/sample, were preincubated in 1 ml methionine-free cysteine-free DMEM supplemented with protease inhibitors or appropriate solvent for 1 hr at 37°C prior to labeling with 0.25 mCi [³⁵S]methionine/cysteine for 1 hr at 37°C. The cells were centrifuged and resuspended at 1 × 10⁶/ml in RPMI, 10% FBS and chased for 5 hr in the presence of protease inhibitors or solvent. The HOM2 cells were then washed twice with cold phosphate-buffered saline and lysed in 1 ml of 50 mM Tris–HCL (pH 7.4), 0.5% NP-40, 5 mM MgCl₂. Lysates were precleared with protein A–agarose, 7 μl of normal rabbit serum, and 2 μl of normal mouse serum followed by immunoprecipitation with antisera coupled to protein A–agarose. Immunoprecipitates were washed 4–6 times with 1 ml of 50 mM Tris–HCL (pH 7.4), 0.5% NP-40, 5 mM EDTA, 150 mM NaCl. These

pellets were either eluted directly with nonreducing or reducing SDS–PAGE sample buffer, or used as starting material for proteolytic digestion and further immunoprecipitation.

Proteolytic digestion of immunoprecipitates was performed by incubation of precipitated pellets with purified proteases diluted in 50 μl of 50 mM Na acetate (pH 5.5), 1% Triton X-100, 3 mM cysteine, 1 mM EDTA at 37°C. Samples were eluted with SDS–PAGE sample buffer.

Peptide Loading
HA peptide was iodinated by incubation of HA (50 μl of 1 mM solution) with ^{125}I and 50 mM NaPO$_4$ (20 μl), pH 7.5, in a iodogen-coated glass tube on ice for 10 min. ^{125}I–HA was separated from free ^{125}I by passage over a C18 Sep-pack column, and eluted with acetonitrile. Aliquots of ^{125}I–HA were dried in a speed-vac and redissolved in digestion buffer for incubation with cathepsin S–treated and nontreated immunopecipitates. Following incubation with the peptide, samples were diluted to 0.8 ml with lysis buffer (pH 7.4), cleared of LN-2 antibody with protein A–agarose, and immunoprecipitated with Tü36. Samples were washed thoroughly prior to addition of SDS–PAGE sample buffer to remove unbound peptide.

In Vitro Translation of α, β, and Ii
cDNAs of HLA-DR1 α (Larhammar et al., 1982) and β chains (Bell et al., 1985), and the cDNA encoding the human p33 Ii (Claesson et al., 1983), were all cloned in pSP72 (Promega, Madison, Wisconsin) as described previously (Bijlmakers et al., 1994). The cDNAs were transcribed in vitro, either together or separately, using T7 RNA polymerase. RNA was stored in 70% ethanol at −80°C. The optimal amount of RNA utilized was determined empirically for each separate batch of RNA. The resulting RNAs were translated in vitro in rabbit reticulocyte lysate (Flexi, Promega), supplemented with canine microsomes prepared in the laboratory of Dr. H. Ploegh. Translations were performed for 90 min at 30°C, as previously described (Bijlmakers et al., 1994). Upon completion of translation the microsomes were pelleted by centrifugation for 4 min at 12,000 rpm, and resuspended in 20 μl of lysis/digestion buffer (50 mM Na acetate [pH 5.5], 1% Triton X-100, 3 mM cysteine, 1 mM EDTA) with or without cathepsin S at varying concentrations. Proteolytic digestion was performed by incubation of the above solubilized microsomes for 4 hr at 37°C. Digestion was stopped by the addition of reducing SDS-sample buffer, samples boiled, and analyzed directly by SDS–PAGE.

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