Immunity, Vol. 18, 489–498, April, 2003, Copyright ©2003 by Cell Press

Asparagine Endopeptidase Can Initiate the Removal of the MHC Class II Invariant Chain Chaperone

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Summary

The invariant chain (Ii) chaperone for MHC class II molecules is crucial for their effective function. Equally important is its removal. Cathepsins S or L are known to be required for the final stages of Ii removal in different APCs, but the enzymes which initiate Ii processing have not been identified. Here we show that this step can be performed in B lymphocytes by asparagine endopeptidase (AEP), which targets different asparagine residues in the lumenal domain of human and mouse invariant chain. Inhibition of AEP activity slows invariant chain processing and hinders the expression of an antigenic peptide engineered to replace the groove binding region of Ii (CLIP). However, the initiation of Ii removal can also be performed by other proteases, reflecting the importance of this step.

Introduction

During biosynthesis, major histocompatibility complex (MHC) class II molecules associate with the invariant chain (Kvist et al., 1982), a type II membrane protein, which performs three main chaperone functions (reviewed in Sant and Miller, 1994; Cresswell, 1994; Stumptner-Cuvelette and Benaroch, 2002). First, the invariant chain (Ii) associates as a homotrimer with three $\alpha\beta$ heterodimers of MHC class II to form a nonameric structure (Roche et al., 1991; Lamb and Cresswell, 1992). Second, by interacting tightly with the MHC class II groove, it inhibits the loading of peptides generated in the ER (Roche and Cresswell, 1991; Bijlmakers et al., 1994a; Busch et al., 1996) and at the same time stabilizes the nonameric complex (Zhong et al., 1996). This interaction occurs via residues 81–104 of its lumenal domain (Romagnoli and Germain, 1994; Bijlmakers et al., 1994b; Ghosh et al., 1995) designated class II-associated invariant chain peptide or CLIP. Third, the Ii contains targeting signals in its cytoplasmic tail (Lotteau et al., 1990; Bakke and Dobberstein, 1990; Pond et al., 1995) which allow the delivery of the nonameric complexes to late endosome/lysosome compartments (Amigorena et al., 1994; Tulp et al., 1994; Arneson and Miller, 1995) which also receive endocytosed antigen (West et al., 1994).

A prerequisite for peptide loading of class II $\alpha\beta$ dimers is the proteolytic destruction of Ii. Ii appears to be processed in sequential steps, first giving rise to large class-II associated Ii fragments and finally the CLIP peptide whose removal is effected by another chaperone molecule, DM. The use of specific protease inhibitors and murine protease gene knockouts have helped to identify the key enzymes involved in the terminal stages of Ii processing (Villadangos and Ploegh, 2000; Riese and Chapman, 2000). Experiments performed with human B cells treated with a cathepsin S (catS) inhibitor (Riese et al., 1996) or with catS deficient APCs (Nakagawa et al., 1999; Shi et al., 1999) indicate that catS is critically involved in generating CLIP in bone marrow-derived professional APCs. In contrast, cathepsin L (catL) mediates the same late stage of Ii degradation in cortical thymic epithelial cells (Nakagawa et al., 1998). In the absence of these activities an N-terminal fragment of approximately 10 kDa, referred to as p10, accumulates on MHC class II. An additional protease, cathepsin F, expressed in macrophages can substitute for the function of catS and catL in CLIP formation (Shi et al., 2000). The enzyme(s) that generate the p10 intermediate is not known but a similarly sized intermediate accumulates in the presence of the broad spectrum cysteine protease inhibitor leupeptin (Amigorena et al., 1995). In human B lymphoblastoid cells, leupeptin induces the accumulation of a larger intermediate of 22 kDa (Blum and Cresswell, 1988; Nguyen et al., 1989) known as LIP (leupeptin-induced peptide). This blockage of Ii processing leads to a decrease in class II expression at the cell surface (Neefjes and Ploegh, 1992; Brachet et al., 1997). The fact that processing of Ii still occurs in the presence of broad specificity cysteine protease inhibitors shows that another as yet unidentified enzyme or enzymes can initiate Ii processing. It has generally been assumed that this step was performed by a noncysteine protease.

Asparagine endopeptidase (AEP) or mammalian legumain is an asparagine-specific cysteine protease that has been recently implicated in the MHC class II pathway. AEP has been shown to initiate processing of the tetanus toxin antigen in B cells (Manoury et al., 1998; Antoniou et al., 2000; Watts, 2001) and has recently been shown to be capable of destroying an immunodominant peptide of myelin basic protein (MBP, 85-99), an autoantigen implicated in the inflammatory demyelinating disease multiple sclerosis (Manoury et al., 2002). Thus, AEP can have both positive and negative effects on the outcome of antigen processing. AEP is unrelated to the papain-like cysteine proteases such as cathepsins S and L and instead is grouped together with the cas-

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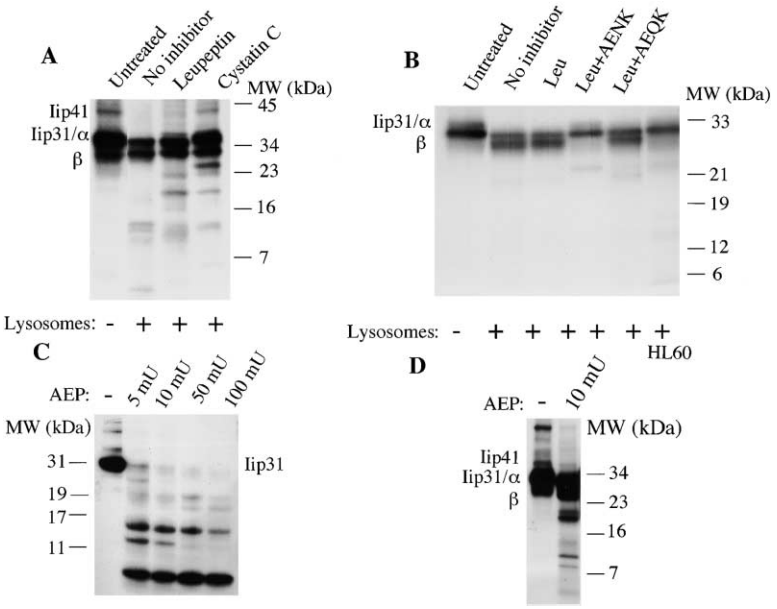

Figure 1. Digestion of ^{35}S-Labeled Class II/Ii Complexes with Disrupted Lysosomes or Purified AEP

(A and B) ^{35}S-Met/Cys labeled class II/Ii complexes were immunoprecipitated (EDR cells) with DA6.231 mAb and digested with EBV-B cell lysosomal fractions (5 μg) in the absence or presence of the indicated inhibitors. The reactions were separated on a 12% Bis-Tris Nupage gel. Track 6 shows inefficient degradation of class II/Ii complexes with disrupted lysosomes from the HL-60 monocytic cell line.

(C) ^{35}S-Met/Cys-labeled murine Ii chain (LB27.4 cells) was immunoprecipitated with the IN1 mAb and digested with increasing amounts of purified pig kidney AEP for 1 hr. Digestion products were analyzed on a 12% Bis-Tris Nupage gel.

(D) As in (C), but human class II/Ii complexes were immunoprecipitated with DA6.231 mAb. 10 mU of AEP was used to digest the complexes in vitro.

pases, separase, and some bacterial proteases in clan CD (Chen et al., 1998). It is unique among lysosomal cysteine proteases in that it is insensitive to E-64 and leupeptin (Chen et al., 1997; Manoury et al., 1998). Because the initial step of degradation of Ii has been shown to be leupeptin insensitive, we investigated the possible role of AEP in this process.

Here we show that intact Ii is a substrate for AEP both in vitro and in living cells. Blocking AEP activity either by mutating the AEP cleavage sites in the Ii chain or by use of a specific AEP inhibitor delays the appearance of the p10 and p22 intermediate fragments in murine and human B cells, respectively. Finally, when the CLIP region of the Ii chain is replaced by flu HA 306-318, loss of AEP processing sites adversely affects presentation of HA to T cells. Our data suggest that AEP plays an important but not exclusive role in initiating Ii processing in living cells.

Results

Lysosomal Proteases Involved in Ii Chain Processing In Vitro

To analyze the processing of the Ii chaperone molecule in vitro, we incubated radiolabeled MHC class II /Ii chain complexes with lysosomes isolated from the human Epstein-Barr virus (EBV)-transformed B cell lines EDR or PALA. The main p31 isoform and the splicing variant p41 form of the Ii chain were digested to produce a discrete pattern of bands at mildly acidic pH (e.g., Figure 1A, track 2) whereas the α and β chains of MHC class II were comparatively resistant. We then used various protease inhibitors to identify the enzymes involved in Ii degradation in vitro. As shown in Figures 1A and 1B, leupeptin had relatively little effect on p31 degradation consistent with in vivo data showing that Ii processing still proceeds in its presence (Blum and Cresswell, 1988). In contrast, addition of cystatin C which, like leupeptin, blocks the cysteine proteases from the papain family but which also blocks AEP (Alvarez-Fernandez et al., 1999), was a much more effective inhibitor of Ii degradation (Figure 1A, track 4). To investigate this further, we used the F-moc-Ala-Glu-Asn-Lys-NH2 (AENK) tetrapeptide, previously described to specifically inhibit AEP (Manoury et al., 1998). AENK together with leupeptin showed a clear inhibition of Ii chain degradation (Figure 1B, track 4) whereas, in contrast, no inhibition was observed with the control peptide AEQK (Figure 1B, track 5). Furthermore, lysosomes purified from the primary monocytic cell line, HL-60, which lacks AEP activity (B.M, unpublished data) but expresses other cysteine proteases, digested the Ii chain poorly (Figure 1B, track 6). These results suggested that, in vitro, AEP makes a significant contribution to Ii processing by B cell lysosomes.

AEP Processing Sites in the Ii Chain

To identify asparagine residues targeted by AEP on the Ii chain, Ii molecules from mouse B cells were labeled with ^{35}S-Cys/Met, immunoprecipitated, and incubated with purified AEP. Several cleavage products were generated depending on the amount of protease added during the digestion (Figure 1C). Similar results were obtained when MHC class II-Ii chain complexes from human B cells were used as substrates for AEP digestion (Figure1D). AEP cleavage products from human and mouse Ii chains were radiosequenced by Edman degradation. We could identify a predominant cleavage product from the mouse Ii chain consistent with a cleavage after Asn105 localized just downstream of the CLIP peptide. In human Ii, radiosequencing indicated two predominant cleavages after Asn76 and 155. We also observed a cleavage at Asn11, but this is in the cytoplasmic tail and so cannot be relevant to Ii processing in living cells (data not shown). These results demonstrate that Asn105 for mouse Ii and Asn155 for human Ii as well as Asn76 for human Ii can be recognized by AEP in vitro.

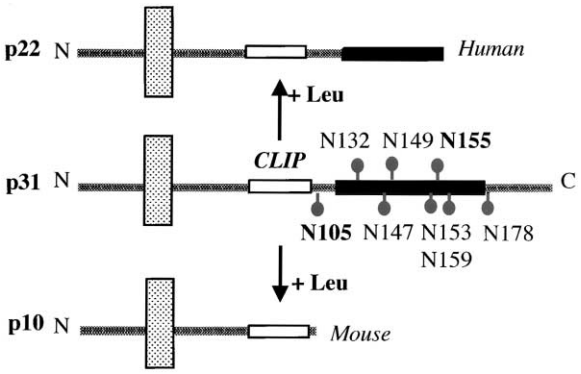

Figure 2. Schematic Representation of Potential AEP Processing Sites in the Mouse and Human Ii Chain Protein

The vertical box represents the lipid bilayer, and the black bar shows the trimerization domain. Lollipop symbols indicate where Asn sites are located in the human (top) and mouse (bottom) invariant chain. Asn residues upstream (N-terminal) of CLIP are not shown. Residues targeted by AEP which could potentially generate mouse p10 and human p22 fragments are shown in bold. Distances are not to scale.

Other Asn residues may well be targeted by AEP but may not be easily identified by radiosequencing techniques.

Mutating AEP Cleavage Sites Delays Murine Ii Processing in Living Cells

The two AEP processing sites downstream of CLIP could potentially generate the p10 and p22 Ii products induced by leupeptin in mouse and human Ii chain, respectively, as shown in Figure 2. To establish whether AEP could generate the p10 intermediate product in living cells, site-directed mutagenesis was performed on AEP cleavage sites in the mouse Ii. Asparagine residue 105 was mutated to Gln along with three potential downstream cleavage sites (153, 159, and 178) some of which we had identified as AEP targets in the human Ii. Wild-type (N) or mutated (Q) Ii cDNA was cotransfected together with cDNA for the α and β chains of the MHC class-II H-2^b haplotype into COS cells. Forty-eight hours later, the COS cells were labeled with ^{35}S-Cys/Met and chased for different times in the presence of leupeptin. MHC class II/Ii chain complexes were then immunoprecipitated with the Y3P antibody. As expected, the p10 fragment of wild-type Ii chain (N) appeared after 1 hr of chase in the presence of leupeptin and accumulated progressively thereafter (Figure 3A). (Note that the signal increases between 2 and 3 hr of chase because theY3P antibody preferentially recognizes mature forms of MHC class II). In contrast, we observed a clear delay in p10 formation when the mutated Ii chain (N105, 153, 159, 178Q) was expressed in COS cells (Figures 3A and 3B). Low levels of p10 were made from the mutant Ii after 1 hr of chase in the presence of leupeptin and still remained lower than wild-type even at 4 hr. Interestingly, we observed instead a small but distinct accumulation of higher molecular weight products (* in Figure 3A). Similar results were obtained when processing of mutated and wild-type Ii was examined in Chinese hamster ovary cells (CHO) transfected with α and β chains of the H-2^d class II haplotype (data not shown). Mutating N105 alone also delayed the appearance of p10, but the effect was less dramatic (data not shown and see Discussion).

We then performed similar experiments in the absence of leupeptin to assess the rate of processing of the wild-type or mutated Ii p31 when all proteases were active. Radiolabeled MHC class II/Ii chain complexes were first immunoprecipitated with M5/114 antibody and then reimmunoprecipitated using the IN1 antibody. Figure 3C shows that at early times of chase, the mutated Ii (Q) was less susceptible to degradation compared to the WT Ii (N). Quantification of these results showed after 2 hr of chase a significant difference in the amount of the full-length p31 Ii chain remaining when AEP sites were missing (wild-type Ii, 7.7%; mutated Ii chain, 40%, Figure 3D). Taken together, these results demonstrate that mutating AEP cleavage sites delays Ii processing in living cells.

AEP Overexpression Boosts the Appearance of the p22 Intermediate Ii Processing Product

The above results suggested that increasing expression of AEP might accelerate Ii processing. To test this, we established MelJuSo cell lines in which overexpression of AEP is under the control of tetracycline or doxycycline

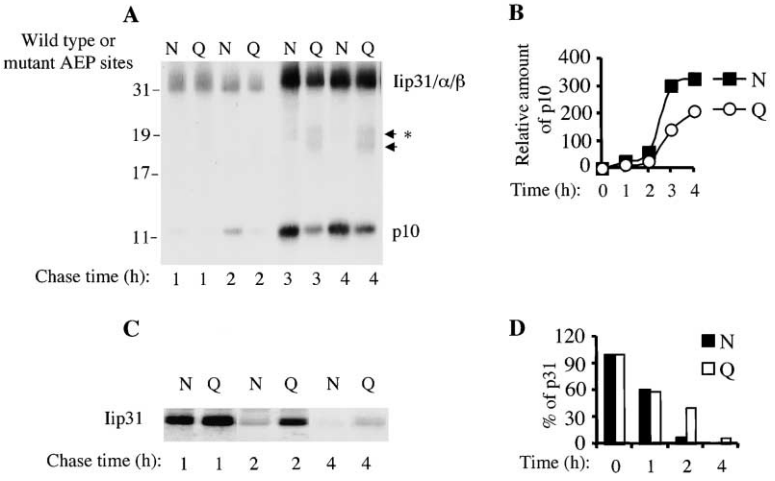

Figure 3. Mutating AEP Cleavage Sites on Murine Ii Chain Slow Down Its Processing

(A) Wild-type (N) or mutated (N105, 153, 159, 178Q) murine Ii chain cDNAs were transiently transfected into COS cells together with A^b α and A^d β cDNAs. Forty-eight hours after transfection, the cells were labeled with ^{35}S-Met/Cys and chased for different times in the presence of leupeptin (1 mM). Class II/Ii complexes were immunoprecipitated with mAb Y3P and analyzed by 12% SDS-PAGE.

(B) Quantification of the experiment shown in (A).

(C) As in (A), but the chase was in the absence of leupeptin, and Ii molecules were reimmunoprecipitated with the IN1 mAb from M5/114-classII/Ii precipitates.

(D) Quantification of the experiment in (C).

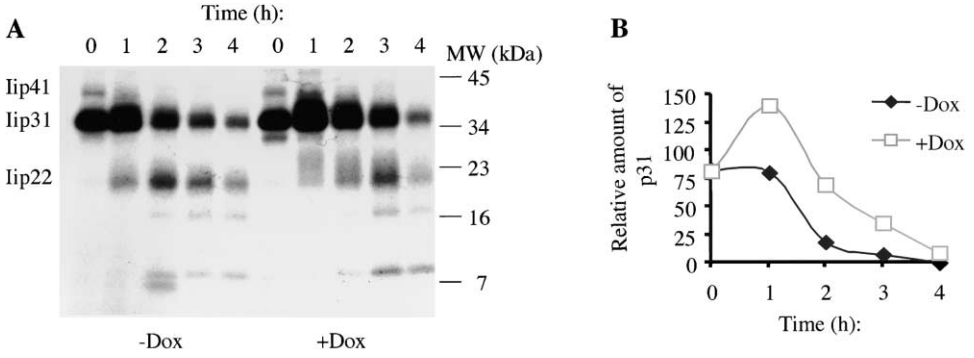

Figure 4. Overexpression of AEP Accelerates Ii Processing

(A) Control (+Dox) and AEP overexpressing (−Dox) MelJuSo cells were labeled with ^{35}S Met/Cys and chased for different times. Ii molecules were reimmunoprecipitated with VIC-Y1 mAb from DA6.231-class II/Ii precipitates. Samples were analyzed by 14% SDS-PAGE.

(B) Quantification of the experiment in (A). Note that the additional product around 7 kDa generated under conditions of AEP overexpression was not consistently seen in all experiments.

using the Tet-Off system. A clone of the cell line MelJuSo was generated that expresses normal amounts of AEP in the presence of doxycycline, but upon removal of the drug an approximately 10-fold increase in AEP activity is induced (Manoury et al., 2002). To assess the kinetics of Ii degradation in the overexpressing and control MelJuSo cell lines, the cells were pulsed with ^{35}S-Cys/Met for 30 min and chased for 1, 2, 3 and 4 hr. Class II-Ii complexes were isolated by immunoprecipitation with the HLA class II-specific mAb, DA6.231, and Ii was then reimmunoprecipitated using the VICY1 mAb. As shown in Figure 4A, in the presence of doxycycline a rather diffuse set of Ii products appeared during the chase migrating between 22–25 kDa (Pieters et al., 1991). In contrast, when AEP was overexpressed, a more distinct 22 kDa intermediate was generated (Figure 4A) which was more pronounced when leupeptin was included to isolate the initial stages of Ii processing (data not shown). In addition, faster p31 processing was observed in cells overexpressing AEP (Figure 4B and compare the p31/p22 ratio at the 2 and 3 hr time points). Note that the increase in p31 precipitation seen after 1 hr of chase is due to an excess of p31 made during the pulse associating with unlabeled α and β chains during the chase. Faster p31 processing in AEP overexpressing cells diminishes this increase. Thus, overexpression of AEP accelerates p31 to p22 processing in MelJuSo cells.

Specific Inhibition of AEP Slows Down Ii Processing in Professional APC

We next tested the effect of inhibiting AEP in human B cell lines and dendritic cells. We took advantage of a newly generated acyloxymethylketone inhibitor of AEP 2,6-dimethyl-benzoic acid 3-benzyloxycarbonylamino-4-carbamoyl-2-oxo-butyl ester (MV026630) that will be more fully described elsewhere (K.L. et al., unpublished data). We first confirmed that in living cells MV026630 blocked AEP but not other proteases. EDR cells were incubated with MV026630, and lysates were tested for their ability to hydrolyse different fluorometric substrates. As shown in Figure 5A, MV026630 treatment inhibited hydrolysis of the AEP substrate Z-Ala-Ala-Asn-NHMec but had no effect on hydrolysis of Z-Val-Val-Arg-NHmec or Z-Phe-Arg-NHmec preferred by cathepsin S and cathepsins B/L, respectively (Figure 5A). Conversely, leupeptin and LHVS treatment blocked hydrolysis of the catB/L and catS substrates but not the AEP substrate (Figure 5A). To provide independent confirmation that MV026630 did not react with other cysteine proteases, we incubated cells with the iodinated active-site-directed probes ^{125}I-MuTyrAlaCH$_2$F and ^{125}I-JPM-565-OEt (Morton et al.,1995; Bogyo et al., 2000) known to react selectively with most papain-related lysosomal cysteine proteases. As previously described (Bogyo et al., 2000) and shown in Figure 5B, ^{125}I-JPM-565-OEt (top panel) labeled a number of bands presumed to be lysosomal cysteine proteases. For example, cathepsin S can be identified due to the fact that preincubation of the cells with LHVS led to a selective loss of labeling (Riese et al., 1996, and Figure 5B, track 5). Leupeptin pretreatment also resulted in loss of cathepsin S labeling and almost complete loss of a band migrating immediately above it which is assumed to be cathepsin B (track 4). ^{125}I-MuTyrAlaCH$_2$F only labeled cathepsin S and B, and this was abolished by leupeptin treatment and, in the case of cathepsin S, by LHVS treatment. In contrast, exposure of cells to MV026630 did not block the labeling of cathepsins by either of the two active-site-directed probes (Figure 5B, compare track 1 with tracks 2 and 3). We conclude that MV026630 specifically blocks the AEP protease but not other lysosomal cysteine proteases.

We then tested the effect of the AEP inhibitor on Ii degradation. PALA cells were preincubated with MV026630 for 2 hr, pulsed for 30 min with ^{35}S-Met/Cys, and then chased for different times in the presence of leupeptin with or without MV026630. In the control cells the LIP fragment of 22 kDa appeared after 1 hr and accumulated thereafter (Figure 5C). In cells preincubated with MV026630, there was a pronounced delay in the appearance of the LIP fragment which was only just detectable after 3 hr of chase (Figure 5C). A similar striking block in p22 production was obtained (quantitated by phosphoimaging) when a second EBV cell line, EDR, was pulse labeled and chased in the presence of MV026630 over a 4 hr period (Figure 5D). We also tested

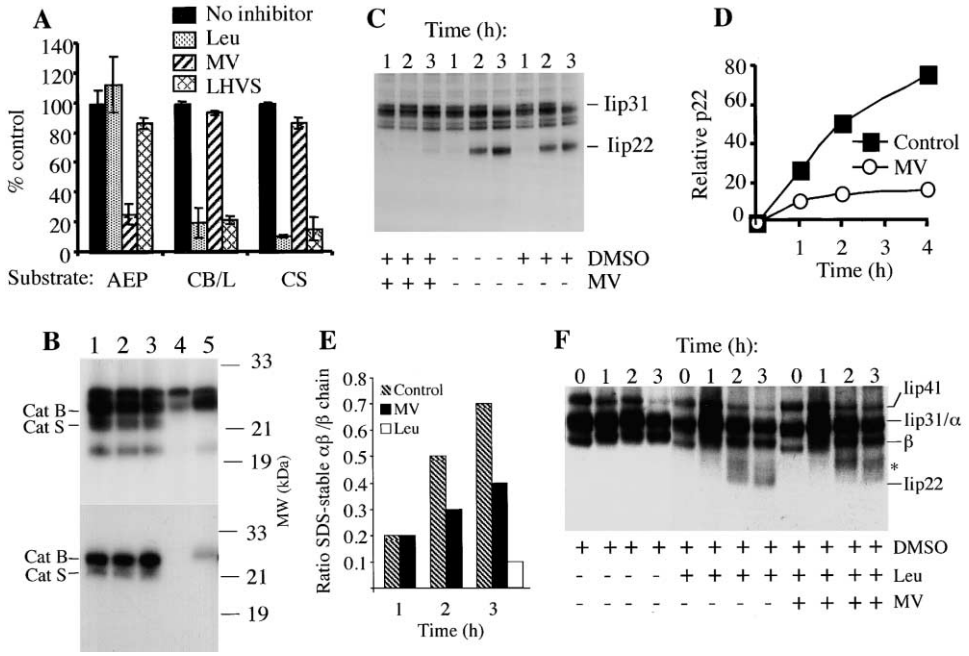

Figure 5. Inhibition of AEP Activity Interferes with Ii Processing and SDS-Stable Dimer Formation in Human Antigen-Presenting Cells

(A) Lysates from EDR cells preincubated with leupeptin (1 mM), MV206630 (20 μM), or LHVS (200 nM) were prepared and used to measure hydrolytic activity remaining for the substrates indicated. MV026630 is abbreviated to MV in this figure.

(B) EDR cells were preincubated in the absence (track 1) or presence of MV026630 (20 or 50 μM; tracks 2 and 3), leupeptin (track 4), or LHVS (track 5). The active-site-directed probes ^{125}I-JPM-565-OEt (top gel) or ^{125}I- Mu-Tyr-Ala-CH_2F (bottom gel) were added for 3 hr, and samples were analyzed using SDS-PAGE.

(C) PALA cells were labeled with ^{35}S-Met/Cys and chased for the indicated times in the presence of leupeptin (1 mM) with or without MV026630 (20 μM). Lysates were then immunoprecipitated with DA6.231 mAb and analyzed by SDS-PAGE.

(D) Quantification of a similar experiment to that shown in (C) but performed in EDR cells with chase for up to 4 hr.

(E) EDR cells were labeled and chased in the absence or presence of leupeptin or MV026630 for the indicated times. Class II/Ii complexes were immunoprecipitated with mAb DA6.231 and eluted for 30 min in SDS sample buffer at room temperature. SDS-resistant αβ-peptide dimers and β chain were quantitated and expressed as a ratio.

(F) Human monocyte-derived dendritic cells were labeled with ^{35}S-Met/Cys and chased for different times in the absence or presence of leupeptin (1 mM) with or without MV026630 (20 μM) or vehicle (DMSO). Lysates were immunoprecipitated with DA6.231 mAb and separated on 12% Bis-Tris Nupage gel.

the effect of inhibition of AEP on the formation of SDS-stable αβ dimers, a frequently used measure of normal MHC class II-peptide loading and maturation (Davidson et al., 1991; Germain and Hendrix, 1991). As shown in Figure 5E, SDS-stable dimers accumulated during a chase following ^{35}S-pulse labeling of EDR cells. MV026630 clearly had an inhibitory effect on dimer formation and/or stability though not as great as leupeptin. Taken together, these results show that AEP is the enzyme acting on the intact Ii substrate to generate the p22 LIP product and that inhibition of AEP causes a measurable reduction in the appearance of fully mature SDS-stable αβ dimers.

We also tested the effect of inhibiting AEP on Ii processing in human dendritic cells (DC). Immature monocyte-derived DC were preincubated with MV026630, and newly synthesized MHC class II molecules were pulse labeled as before followed by a chase in the absence or presence of leupeptin with or without MV026630. The monocyte-derived DC showed essentially the same kinetics of appearance in Ii processing products although the size range of the leupeptin-induced products was clearly broader compared with EBV-B cells (Figure 5F). In the presence of the AEP inhibitor, Ii products were still generated but were of higher molecular weight (* in Figure 5F), and the 22 kDa size product was essentially absent (compare 2 and 3 hr time points in Figure 5F). Overall, our data demonstrate that AEP is the enzyme that generates the 22 kDa Ii processing product seen in the presence of leupeptin. However, other processing activities continue to degrade Ii, and this is particularly evident in DC.

Presentation of flu HA epitope (306-318) to Specific T Cells Is Decreased When Ii Processing Is Impaired

We performed a further series of experiments in Mel-JuSo cells to test the possibility that inhibition of AEP processing of Ii would slow down the arrival of newly synthesized MHC class II molecules on the cell surface. To do this, we took advantage of an Ii construct where the CLIP peptide was replaced (Figure 6A) with the flu HA epitope 306-318 (Stumptner and Benaroch, 1997). This HA/Ii construct is processed like the Ii chain, and the HA peptide which remains associated with HLA-DR1 molecules can be presented to the HA-specific T cell line THA1.7 (Vincent-Schneider et al., 2001). We mutated Asn residues 132 and 155 to Gln and first established that the mutated HA/Ii associated with HLA-DR1 class

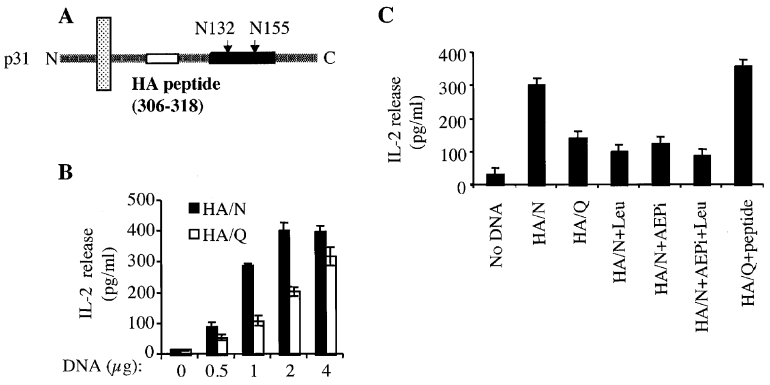

Figure 6. Mutating AEP Cleavage Sites on a flu HA/Ii Construct Delays the Appearance of MHC Class II/HA Complexes at the Cell Surface

(A) Schematic of the HA/Ii construct used. Arrows indicate Asn sites that were mutated.

(B) MelJuSo cells transiently transfected with DR1β cDNA and increasing concentrations of cDNAs coding for HA/Ii wild-type (HA/N, black bars) or mutated HA/Ii (HA/N132, 155Q, white bars) were incubated with HA-specific T cells for 24 hr. Supernatants were assayed for IL-2.

(C) MelJuSo cells transfected with DR1β and mutant or wild-type HA/Ii as in (B) were preincubated with the following inhibitors: leupeptin (1 mM), AEPi (MV026630, 20 μM), or leupeptin + AEPi and cocultured with HA-specific T cells. MelJuSo cells transfected with mutant HA/Ii (HA/Q) were pulsed with 0.5 mM of HA peptide (306-318) and incubated with HA-specific T cells for 24 hr.

II molecules as well as wild-type HA/Ii (data not shown). To test whether mutating AEP sites on HA/Ii chain would affect the presentation of the HA peptide, MelJuSo cells were cotransfected with increasing amounts of cDNA of DR1β*0101 plus HA/Ii wild-type (HA/N) or HA/Ii mutant (HA/Q) cDNA. Presentation of the HA epitope was monitored by IL-2 release from the HA-specific T cell hybridoma THA1.7. As shown in Figure 6B, there was a clear delay in the presentation of the HA peptide when the HA/Q construct was used compared with the HA/N construct. This decrease in antigen presentation was dependent on the amount of DNA transfected and, as expected, was partially overcome when high concentrations were used. Furthermore, if MelJuSo cell lines transfected with HA/N were incubated with MV026630, presentation was diminished to a comparable extent providing independent confirmation that AEP processing releases the class II/HA peptide complexes to the cell surface (Figure 6C). As expected, leupeptin alone or in combination with MV026630 also decreased presentation to T cells (Figure 6C). Inhibition of HA presentation by cells transfected with the HA/Q construct could be restored when the HA peptide (306-318) was added in the antigen presentation assay (Figure 6C). Thus, mutating AEP cleavage sites or blocking AEP activity can slow down the rate of expression of MHC class II molecules as measured with the HA/Ii probe.

Discussion

Proteolytic processing of Ii is a critical determinant of the timing and location of MHC class II peptide loading. While recent work has elucidated the terminal stages of Ii processing, little information exists on how Ii processing is initiated. A key early study showed that in the presence of the broad spectrum cysteine protease inhibitor leupeptin, a 22 kDa intermediate of human Ii accumulated (Blum and Cresswell, 1988). In murine B cells expressing MHC class II molecules of the d or b haplotypes, leupeptin induces instead the accumulation of a 10 kDa product (Amigorena et al., 1995; Brachet et al., 1997). A similar leupeptin-induced product (~10–12 kDa) can also be seen in some human cell lines (Maric et al., 1994). It has always been assumed that these leupeptin-induced products are generated by noncysteine proteases. Their identity, however, has never been established.

Maric et al. provided evidence that specific aspartic protease inhibitors blocked Ii degradation and MHC class II maturation in human B cells (Maric et al., 1994). However, Riese et al., using similar cells, found that specific inhibition of cathepsin D with a different compound did not result in accumulation of Ii fragments nor did it produce a decrease in SDS-stable MHC class II complexes (Riese et al., 1996). Furthermore, spleen cells from cathepsin D knockout mice did not show any alteration in the degradation of the Ii chain (Villadangos et al., 1997). Altogether, these results suggest that cathepsin D is not essential for Ii processing but do not exclude a possible role for a related aspartic protease.

We identify here a protease that initiates invariant chain processing. AEP is a cysteine protease but is insensitive to leupeptin (Chen et al., 1997). Using lysosomal fractions purified from EBV B cells as a source of proteases to digest Ii, we show that AEP is the major activity able to cleave Ii. Specific inhibition of AEP, overexpression of its activity, or mutation of AEP cleavage sites on Ii shows that in living cells AEP is involved in initiating Ii processing. Moreover, the sites targeted by AEP in vitro would generate fragments of 10 and 22 kDa in the mouse and human Ii, respectively, if they are recognized in vivo. In other words, the intermediates induced by leupeptin treatment could be generated by AEP. Consistent with this, the appearance of the LIP p22 Ii product was almost completely abolished by inhibition of AEP activity in human B cells. Furthermore, mutating AEP cleavage sites on a form of the invariant chain where the CLIP peptide was replaced with the flu HA epitope (306-318) induced a delay in the appearance of MHC class II/HA complexes at the cell surface.

The strict specificity of AEP has allowed us to identify and mutate protease cleavage sites in the invariant chain for the first time. Although a complete 3D structure for Ii is not known, structural information is available for the domain that mediates the trimerization of human Ii (residues 118–192; Bijlmakers et al., 1994b; Jasanoff et al., 1998). Of the four Asn residues found on each protomer of the trimer, one (N120) is glycosylated (Machamer and Cresswell., 1982) and cannot be a target for AEP. The remaining three are found on helices A and B which surround the central core of C helices in the

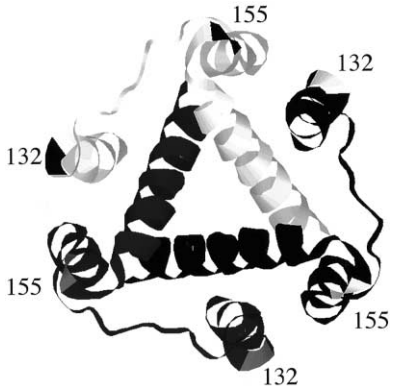

Figure 7. AEP Processing Sites on the Human Ii Trimer

Figure generated from coordinates deposited by Jasanoff et al. (1998) for Ii residues 118–192. Only residues 123–177 are shown for clarity. Each copy of the Ii trimerization region contributes an inner (C) helix and outer (A and B) helices. The AEP processing site at Asn155 is shown by contrasting shading on each ribbon as is the presumptive site at Asn132.

structure of Jasanoff et al., which is also shown in Figure 7 (residues 123–177). However, N149 faces inward at the N-terminal end of helix B and is unlikely to be accessible to AEP in the intact trimer. N155, located at the C-terminal end of helix B, faces outward (Figure 7), and indeed we find that the N155/T156 bond is readily cleaved. We cannot be certain whether or not N132, located at the C-terminal end of helix A, is also cleaved. It is located on the outside of this helix as shown in Figure 7, but radiosequencing would not easily have identified this cleavage product since the nearest labeled residue is Met157, 25 residues downstream and past the 155 cleavage site. Assuming that N132 is also an AEP target, it is clear that there are Asn residues placed at precisely the right positions to attack the outer helices of the Ii trimer (Figure 7). AEP cleavage at either N132 or N155 would separate the p22/$\alpha\beta$ products from the central core of C helices which appear crucial for trimer stability (Jasanoff et al., 1998). In spite of this, both p22/$\alpha\beta$ and p10/$\alpha\beta$ complexes retain the nonameric configurations found in intact Ii/$\alpha\beta$ (Newcomb and Cresswell., 1993; Amigorena et al., 1995). This may be explained by the fact that the transmembrane domain of Ii also plays an important role in trimerization (Ashman and Miller., 1999).

In the mouse, N149 and N155 are conserved. There is an additional Asn in murine Ii at position 178, which in the human trimer structure is at the end of the central C helix, i.e., in the core of the trimer. It seems unlikely that this is a good AEP target, at least in the intact trimer. Blockade of Ii processing with leupeptin is well known to induce the accumulation of a smaller fragment of about 10 kDa in size (Amigorena et al., 1995) in several mouse MHC class II haplotypes, although a p22 fragment can also sometimes be seen (Villadangos et al., 1997). The predominance of the murine p10 Ii intermediate can be explained by the presence of an additional Asn residue in the murine Ii chain at position 105 (Ala106 in human Ii). This residue is located between the CLIP region and the two N-glycosylation sites at N113 and N119. Both in vitro AEP digestion and mutagenesis studies confirm that this site is attacked by AEP. Although we observed slowing down of the appearance of p10 when N105 alone was mutated, the most efficient blockade of the appearance of this leupeptin-induced product was obtained when downstream Asn residues were also mutated, suggesting that efficient cleavage at Asn105 requires prior upstream AEP cleavage, probably at N153 in the trimerization region. Mutagenesis of the N105 and upstream sites did not completely block the appearance of the p10 fragment. This suggests that an alternative but less favored cleavage site for another enzyme is also located in this region. This is consistent with the fact that the human Ii, which does not have an Asn residue in this position, can sometimes be processed to give a similar sized intermediate, for example, in PALA cells (Riberdy et al., 1994; Pond and Watts., 1999). This may be the same as the small leupeptin-induced Ii product seen in some studies (Maric et al., 1994), suggesting that the enzyme responsible may be a leupeptin-insensitive enzyme other than AEP. Finally, although we found some evidence for an AEP cleavage at N76 (N-terminal to the CLIP region) in human Ii, the persistence of the p10 fragment containing this site in the presence of a catS inhibitor (Riese et al., 1996) indicates that it cannot be efficiently recognized by AEP in living cells. This site is in any case altered to Ser in mouse Ii.

Although AEP is involved in both human and murine Ii processing, our studies to date do not reveal an absolute requirement for this enzyme in initiating p31 processing and MHC class II maturation. Human B lymphoblastoid cells treated with the MV026630 inhibitor of AEP for 24 or 48 hr showed essentially the same steady-state level of surface MHC class II molecules (data not shown). This is similar to what was observed in cells from catS null mice where steady-state MHC class II levels were essentially normal (Shi et al., 1999; Nakagawa et al., 1999). Apparently, blockade, even of key proteolytic enzymes involved in Ii processing, can eventually be overcome by the action of other enzymes. Redundancy might be predicted to be greatest at the earliest stages of Ii processing. Once processing has reached the p10 stage, the target for protease action is much smaller and to a significant extent unavailable due to sequestration within either the peptide binding groove or the membrane barrier.

Interestingly, even in the combined presence of MV026630 and leupeptin some p31 processing still occurred, suggesting that in human B cells and dendritic cells other still unidentified enzymes can act on the intact invariant chain substrate. Removal of the invariant chain in the endocytic pathway of antigen-presenting cells is as crucial as its presence is in the secretory pathway perhaps dictating that intact Ii/MHC class II complexes may be sensitive to several proteases. We believe that the dominant p31-processing activities will vary depending on the APC in question. In B cells and thymic APCs (Manoury et al., 2002), AEP may play a major role since it is a prominent activity. In other cell types it may be less important. For example, we have found variable but generally lower levels of expression of AEP in human monocyte-derived dendritic cells compared with EBV B cells (C. Moss and B.M, unpublished data). This could explain why, in the presence of leupep-

tin, a relatively indistinct p22 product is seen compared with B cells. Instead, a rather broad set of Ii processing products was observed between 22 and 25 kDa. Inhibition of AEP under these conditions eliminates the appearance of the fragments in the 22 kDa region but not the larger ones. Combined use of leupeptin and the AEP inhibitor in B cells also led to the appearance of Ii fragments in the 25 kDa range. The situation is somewhat similar to that seen in MelJuSo cells in the absence of AEP overexpression (compare Figures 4A and 5F, and data not shown). Taken together, the data point to the existence of at least one additional leupeptin-insensitive enzyme acting at the earliest stages of Ii processing. It is possible that this is an aspartic protease as suggested by the data of Maric et al. (1994). Thus, multiple enzymes are capable of initiating Ii processing. This situation is similar to the terminal stages of Ii processing where different cathepsins perform this step in different cell types (Nakagawa et al., 1998, 1999; Shi et al., 1999, 2000). In addition, the protease requirements for Ii removal vary among different MHC class II haplotypes in the mouse (Villadangos et al., 1997; Nakagawa et al., 1999). Thus far, we have only explored the AEP requirement for Ii processing in cells transfected with the d and b haplotypes. Ii processing was compromised in both when AEP sites were mutated but ablation of AEP activity in professional APC of different murine haplotypes will be needed to fully assess this.

Several of the cysteine proteases shown originally to play a role in Ii processing, such as cathepsins S and L, have recently been shown to be required for generation of specific peptides during antigen processing (Hsieh et al., 2002; Pluger et al., 2002). In addition, a potential role for cathepsin L in the generation of peptides for positive selection of CD4 T cells by cortical thymic epithelial cells was recently described (Honey et al., 2002). In contrast, AEP, first described as a protease required for antigen processing, emerges as a key participant in Ii processing as well. The developing picture is one where most of the major enzymes in the endosome system of antigen-presenting cells participate in both of these crucial proteolytic events.

Experimental Procedures

Peptides, Oligonucleotides, and Antibodies

AENK and AEQK tetrapeptides were used as described (Manoury et al., 1998). AEP was purified from pig kidney essentially as described (Chen et al., 1997). Leupeptin was obtained from Sigma and cystatin C from Calbiochem. Morpholine urea-Tyr-Ala-CH_2 F (Mu-Tyr-Ala-CH_2F), JPM-565-OEt, and morpholineurea-leucinyl-homophenylalanine-vinylsulfone-phenyl (LHVS) were generous gifts from Drs. P. Morton, M. Bogyo, and H. Ploegh, respectively. MV026630 is an acyloxymethyl ketone inhibitor of AEP that will be more fully described elsewhere. Stock solutions of inhibitors were prepared in DMSO or water. The HA306-318 peptide (PKYVKQNTLKLAT) was synthesized by Synt:em (Nimes, France). cDNAs for mouse Ii chain and for α and β chains of I-A^b and I-A^d were gifts from Drs. P. Pierre and R. Germain, respectively. DR1β* was subcloned from the plasmid pSP72-DR1β (Biljmakers et al., 1994) into the EcoRV/XhoI sites of the pcDNA3 plasmid (Invitrogen). Oligonucleotides were custom synthesized by MWG Biotech. The following primers were used. For mutagenesis of mouse Ii chain: Asn105, sense primer CCA ATG TCC ATG GAT CAG ATG CTC CTT GGG C, antisense primer GCC CAA GGA GCA TCT GAT CCA TGG ACA TGG G; Asn153, sense primer G AAG CAT CTT AAG CAG TCC ATG GAT GGC GTG, antisense primer CAC GCC ATC TAC AGG CTG GTT AAG ATG CTT C; Asn159, sense primer CC ATG GAT GGC GTG CAG TGG AAG ATC TTC G, antisense primer C AAG GAT CTT CCA CTG CAG GCC ATC CAT GG; Asn178 sense primer GAG ATG AGC AAG CAG TCC CTG GAG GAG AAG, antisense primer CTT CTC CTC CAG GGA CTG CTT GCT CAT CTC. For human Ii chain: Asn132, sense primer G CAC CTG CTC CAG CAA GCT GAC CCC CTG AAG G, antisense primer C CTT CAG GGG GTC AGC TTG CTG GAG CAG GTG C; Asn155, sense primer G AGA CAC CTT AAG CAG ACC ATG GAG ACC ATA G, antisense primer C TCT GGT CTC CAT GGT CTG CTT AAG GTG TCT C.

Pulse-Chase, Immunoprecipitation, and Radiolabeling of Peptide Inhibitors

CHO, COS, LB27.4, MelJuSo, and human EBV-B cells were preincubated in Met/Cys-free RPMI 1640 medium for 2 hr. Cells (10^6) were then pulsed for 30 min with 0.5 mCi of ^{35}S-Met/Cys (ICN) and chased in unlabeled medium supplemented with 5 mM cold methionine in the presence or absence of different inhibitors for the indicated times. Cells were then lysed in a buffer containing 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 2 mM $MgCl_2$, 1% Triton X-100, and an inhibitor cocktail (Roche). Each sample was normalized either for the same number of cells or for equivalent protein in the case of COS and CHO transfectants. Lysates were precleared with human or mouse serum, and MHC class II/Ii complexes were recovered by immunoprecipitation with the following antibodies: M5/114, Y3P for mouse or DA6.231 for human. For reprecipitation of MHC class II-associated Ii molecules, washed DA6.231 or M5/114 immunoprecipitates were resuspended in 50 μl phosphate buffer saline/1% SDS and incubated at 95°C for 10 min. The eluted proteins were diluted into 1 ml of lysis buffer, and Ii molecules were immunoprecipitated using mAbs IN1 for mouse or VICY1 for human. Samples were boiled in sample buffer and separated by 12% SDS-PAGE (Novex). Quantification of the results was made using a phosphoimager (Fuji). Reactive cysteine protease inhibitors (MuTyrAlaCH_2F and JPM-565-OEt) were iodinated using the reported protocol (Xing et al., 1998; Bogyo et al., 2000). In brief, the reagants were labeled with 20 μg Iodogen dried onto the bottom of a glass tube in 10 μl of 0.25 M phosphate buffer (pH 7.5) to which was added 10 μM Mu-Tyr-Ala-CH_2F (1.25 μl) or JPM-565-OEt (2.5 μl) followed by 0.5 mCi (5μl) of ^{125}I-Na (Amersham). The reaction was incubated for 10 min on ice and stopped by the addition of 2 μl of 20 mM tyramine and 1μl of 1 M KI. The volume of the reaction was adjusted to 0.5 ml with 0.5 M phosphate buffer (pH 7.5), and the labeled reagent was used without further purification. The cells (5×10^5) were incubated with 50 nM of either probe, washed, lysed, and clarified by centrifugation (2200 rpm for 10 min) prior to analysis of supernatents by 14% SDS-PAGE.

Site-Directed Mutagenesis

The human and mouse invariant chain and the HA/Ii cDNAs (Stumptner and Benaroch, 1997) were cloned into and mutated in plasmid pcDNA3 using the QuikChange Mutagenesis Kit (Stratagene) according to the manufacturer's conditions. Positive colonies which contained the desired mutations were then grown in 150 ml of LB supplemented with ampicillin (100 μg/ml), and DNA was extracted using the endotoxin-free Maxiprep kit (Qiagen). Purified DNA was then used to transiently transfect CHO, COS, or MelJuSo cells using a standard calcium phosphate protocol (Knight et al., 1997).

Fluorometric Assays and Inhibitors

EBV-B cells were incubated with 1 mM leupeptin (Sigma) or 20 μM of the AEP inhibitor 2,6-dimethyl-benzoic acid 3-benzyloxycarbonylamino-4-carbamoyl-2-oxo-butyl ester (MV026630) for 24 or 48 hr and then lysed in 50 mM sodium citrate buffer (pH 5.5), 5 mM DTT, 0.1% CHAPS, and 0.5% Triton X-100. Protein content was determined using a BCA assay (Pierce). Postnuclear lysates were incubated with 40 μM of the following protease substrates (Z-Phe-Arg-NHmec, Z-Val-Val-Arg-NHmec, Z-Ala-Ala-Asn-NHmec; preferentially cleaved by catB/L, catS, and AEP, respectively) for 30 min. Release of 7-amido-4-methyl coumarin was measured on a Cytofluor 4000 Fluorimeter (Applied Biosystems).

In Vitro Digestion and Radio Sequencing

Lysosomal fractions from the human B cell line EDR or the monocytic cell line HL-60 were prepared on 27% Percoll density gradient as described (Davidson et al., 1990) using β-hexosaminidase activity to identify lysosomal fractions. Radiolabeled Ii chain was digested with lysosomal membranes (5 μg) or pig kidney AEP (mU as indicated) in 20 μl 50 mM sodium citrate buffer (pH 5.5), 5 mM DTT, 0.1% CHAPS in the presence or absence of the inhibitors: leupeptin (100 μg/ml), cystatin C (100 μg/ml), AENK, or AEQK peptide (1 mg/ml) for 1 hr. The reactions were separated on Nupage Bis-Tris gels (Novex). In some experiments, labeled digestion products were transferred to nitrocellulose and subjected to 20 cycles of Edman degradation. The PTH derivatives obtained from each cycle were analyzed by scintillation counting.

Cell Culture and Antigen Presentation Assay

The human EBV-B cell lines (EDR and PALA) and mouse B cell (LB27.4) were maintained in RPMI 1640 medium supplemented with 2 mM L-glutamine, 5×10^{-5} M β2-mercaptoethanol, 100 U/ml of penicillin, 100 μg/ml of streptomycin, 10% fetal calf serum (all from Life Technologies). DMEM medium was used to grow CHO, COS, or MelJuSo cells. Monocyte-derived dendritic cells were prepared essentially as described (Sallusto and Lanzavecchia, 1994) but using MACS $CD14^+$ beads (Miltenyi Biotec) to isolate monocyte precursors. MelJuSo cells were transiently transfected using a standard calcium phosphate protocol (Knight et al., 1997) with HLA-DR1β cDNA (1 μg/well) and increasing amounts of wild-type (HA/N) or mutated (HA/Q) cDNA. Forty hours later, the cells were detached with PBS/EDTA, counted, and plated in 96-well plates at a density of 10^5 cells per well. Inhibitors were added to the antigen-presenting cells 16 hr before they were harvested, and the cells were further incubated in the presence of inhibitors for 4 hr. The cells were then extensively washed and cultured with the HA-specific T cell line THA1.7 (Hewitt et al., 1992). 5×10^4 T cells were used in a 24 hr assay. Fresh or frozen supernatants were tested for the presence of IL-2 using an enzyme-linked immunosorbent assay kit (Pharmingen).

Acknowledgments

We thank Leslie Pond for collaboration on pilot experiments, P. Pierre and R. Germain for plasmids, S. Amigorena and W. Knapp for antibodies, P. Morton, H. Ploegh, and M. Bogyo for cysteine protease labeling reagents, and C. Moss and S. Matthews for comments on the manuscript. This work was funded by a Wellcome Trust Programme grant to C.W. and by Medivir UK Ltd.

Received: September 20, 2002
Revised: January 10, 2003
Accepted: January 23, 2003
Published: April 15, 2003

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