Role for Cathepsin F in Invariant Chain Processing and Major Histocompatibility Complex Class II Peptide Loading by Macrophages

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Abstract

The major histocompatibility complex (MHC) class II–associated invariant chain (Ii) regulates intracellular trafficking and peptide loading of MHC class II molecules. Such loading occurs after endosomal degradation of the invariant chain to a $\sim$3-kD peptide termed CLIP (class II–associated invariant chain peptide). Cathepsins L and S have both been implicated in degradation of Ii to CLIP in thymus and peripheral lymphoid organs, respectively. However, macrophages from mice deficient in both cathepsins S and L can process Ii and load peptides onto MHC class II dimers normally. Both processes are blocked by a cysteine protease inhibitor, indicating the involvement of an additional Ii-processing enzyme(s). Comparison of cysteine proteases expressed by macrophages with those found in splenocytes and dendritic cells revealed two enzymes expressed exclusively in macrophages, cathepsins Z and F. Recombinant cathepsin Z did not generate CLIP from Ii–MHC class II complexes, whereas cathepsin F was as efficient as cathepsin S in CLIP generation. Inhibition of cathepsin F activity and MHC class II peptide loading by macrophages exhibited similar specificity and activity profiles. These experiments show that cathepsin F, in a subset of antigen presenting cells (APCs), can efficiently degrade Ii. Different APCs can thus use distinct proteases to mediate MHC class II maturation and peptide loading.

Key words: cysteine protease • antigen presentation • protease inhibitor • proteolysis • antigen presenting cell

Introduction

The MHC class II–associated invariant chain (Ii)[1] is a type II transmembrane protein that binds to the peptide binding groove of newly synthesized MHC class II α/β heterodimers, thus preventing their premature association with endogenous polypeptides. Ii also regulates intracellular trafficking of MHC class II dimers. The Ii NH_2-terminal cytoplasmic domain contains an endosomal targeting signal that promotes Ii/MHC class II trafficking through the endosomal compartments of APCs (1–4). Within endosomal/lysosomal compartments, the Ii lumenal domain undergoes stepwise proteolytic degradation to yield progressively smaller fragments. The smallest fragment that contains both the NH_2-terminal endosomal targeting sequence and a COOH-terminal extension through the MHC class II peptide–binding groove has been termed Iip10. The Iip10 fragment is converted subsequently to CLIP (class II–associated invariant chain peptide), a $\sim$3-kD peptide that is ultimately exchanged for antigenic peptide in the final step of peptide loading (5–9). Once free from endosomal retention, peptide-loaded MHC class II dimers move to the cell surface. Thus, the timing and pattern of proteolysis of Ii dictate the route of maturation of MHC class II molecules and likely the antigenic peptides that are ultimately presented.

Degradation of Iip10 to CLIP is an important regulatory step in the maturation of MHC class II dimers. First, only two of the known acidic cathepsins (Cats) have been demonstrated to degrade Iip10 to CLIP, Cats S and L (10–12). Cat S is the major Ii-degrading enzyme of B cells and den-

dritic cells. B cells and dendritic cells from mice deficient in Cat S do not convert Ii to CLIP and also accumulate MHC class II–Iip10 complexes within lysosomes (13). Mice deficient in Cat L show Iip10 accumulation to some extent, but only in the thymic cortex, suggesting that Cat L is important for generation of CLIP at this site (14). The ubiquitous and abundant endosomal proteases Cats B and D do not degrade Iip10 (10). Second, immature dendritic cells express MHC class II molecules but accumulate these dimers largely within lysosomes because Ii is not degraded beyond Iip10. This accumulation has been attributed to inhibition of Cat S by intracellular cystatins (15). Maturation of dendritic cells is accompanied by a reduction in cystatin levels and rapid Cat S–dependent degradation of Iip10, peptide loading, and surface expression of peptide–MHC class II complexes. Thus, conversion of Iip10 to CLIP is a key step in the proteolytic processing of Ii and is regulated both by the pattern of cysteine protease expression and expression of their endogenous inhibitors.

Splenocytes from Cat S–deficient mice have defective MHC class II peptide loading, and Cat S$^{-/-}$ mice exhibit defective Th1-type antibody responses. However, IgE and pulmonary eosinophilic responses to antigen challenge in these mice are normal (11). This dichotomy raises the possibility that additional mechanisms exist to promote peptide loading and MHC class II antigen presentation in these mice. Here we explore this possibility by examining MHC class II maturation in mice deficient in both Cat S and L, currently the only known proteases capable of efficiently degrading Ii beyond Iip10 in vivo. Because lung and peritoneal macrophages of Cat S$^{-/-}$L$^{-/-}$ mice show normal peptide loading, we searched for additional cysteine proteases expressed preferentially in macrophages. We describe the identification and Ii-degrading functions of two such enzymes, Cats Z and F.

Materials and Methods

Materials and Antibodies. N-morpholinurea-leucine-homophenylalanine-vinylsulfone-phenyl (LHVS) and the cysteine protease active site probe ^{125}I–JPM565 were synthesized as described (16, 17) and further purified by HPLC. Recombinant human Cats S and F were prepared as described (18, 19). Recombinant human Cat B was purchased from Calbiochem Corp. N22 is a hamster mAb against mouse MHC class II molecules (20) and was a gift of Dr. R.M. Steinman (The Rockefeller University, New York, New York). All chemical reagents were purchased from Sigma Chemical Co. unless otherwise indicated. Cat L$^{-/-}$ mice (C57BL6/J129) were obtained from Zeneca Pharmaceuticals and phenotyped by hair loss (14). Correlation between hair loss and genotype was confirmed by Southern blot analysis using mouse Cat L cDNA. Cat S$^{-/-}$ mice (C57BL6/J129) were genotyped as described (11) and bred with Cat L$^{-/-}$ mice to generate mice deficient in both Cats. Such mice, other than hair loss, appeared healthy.

Mouse Immunization and IgE Measurement. Cat S$^{+/+}$ and Cat S$^{-/-}$ mice were immunized intraperitoneally with 8 μg of OVA admixed with 1 mg of aluminum hydroxide (Goldline Labs.), boosted with 8 μg of OVA 5 d later, and nebulized with 0.5%

OVA in PBS on day 12. On day 15, mice were killed for serum collection and bronchioalveolar lavage. Lavage fluid eosinophils were visualized by eosin and hematoxylin staining of cytospin preparations. Total IgE levels in serum were measured by ELISA as described previously (11). In some experiments, Cat S$^{-/-}$ mice were also injected intraperitoneally with LHVS (100 mg/kg body weight) every 48 h starting 2 d before the OVA immunization to the end of the protocol. Both lavage fluids and serum were again collected.

Metabolic Labeling of Mouse Peritoneal Macrophages. Cat S$^{+/+}$, Cat S$^{-/-}$, and Cat S$^{-/-}$L$^{-/-}$ mice were injected with 1 ml of 3% thioglycollate (Sigma Chemical Co.). After 72 h, peritoneal macrophages were prepared by three washes with PBS containing 6 mM EDTA. The macrophages were cultured in RPMI (MediaTech) with 10% FBS, 2 mM l-glutamine, 100 μg/ml penicillin/streptomycin, and 100–500 U/ml of mouse IFN-γ (Endogen). After overnight culture in the absence or presence of protease inhibitor LHVS (5–1,000 nM), cells were pulsed with ^{35}S protein labeling mix (New England Nuclear) for 1 h and chased with RPMI (10% FBS) overnight in the absence and presence of protease inhibitors. Cells were then lysed in NP-40 lysis buffer (5 mM MgCl$_2$, 0.5% NP-40, and 50 mM Tris-HCl, pH 7.4), and the protein concentration was normalized to radioactivity of trichloroacetic acid precipitates. Each cell lysate was immunoprecipitated with N22, boiled or nonboiled in nonreducing sample buffer, and separated on 12% SDS-PAGE as previously described (11). In comparison, mouse splenocytes were also isolated from Cat S$^{+/+}$, Cat S$^{-/-}$, and Cat S$^{-/-}$L$^{-/-}$ mice for pulse, chase, and N22 immunoprecipitation.

Mouse alveolar macrophages were harvested from lavage fluids as previously described (21). In brief, mouse lung was washed repeatedly with 1 ml of PBS with 6 mM EDTA. Macrophages were pelleted and cultured in RPMI with 10% FBS and mouse IFN-γ (100–500 U/ml). Cells were pulsed, chased, and immunoprecipitated with N22 as described above.

Cloning and Expression of Mouse Cat F cDNA. A probe based on 300 bp of human Cat F cDNA (19) was used to screen a mouse brain UNI-ZAP XR cDNA library (Stratagene Inc.). Two 1.9-kb full length Cat F cDNAs were isolated and fully sequenced with T7-Sequenase according to the manufacturer (Amersham). The 1.9-kb cDNA was subcloned into the XhoI and NotI restriction sites of the pcDNA-I expression vector (Invitrogen Corp.). The expression construct was purified and transfected into human embryonic kidney 293 cells with Lipofectamine as recommended by the manufacturer (GIBCO BRL). The expression of mouse Cat F was visualized by both Western blotting analysis with mouse Cat F polyclonal antibodies (discussed below) and reaction with the cysteine protease active site probe ^{125}I–JPM565 (22).

Preparation of Mouse Cat F Polyclonal Antibodies. Mouse Cat F cDNA was digested with EcoRI and fused in frame with maltose binding protein expression vector pMAL-c (New England Biolabs). The construct was transformed into DH5α, and fusion protein expression was induced with 0.3 mM isopropyl-b-d-thiogalactoside for 7 h to overnight. Fusion protein was then separated on 10% SDS-PAGE and purified as described previously (23). The purified fusion protein was used as antigen to immunize rabbits for generating polyclonal antisera (Charles River Labs.).

Cloning and Expression of Mouse Cat Z. Three partial EST-tag sequences (AA116604, AA870150, and AI304158) were used to design both 5′ end sense primer and 3′ end antisense primer. The full length cDNA was generated with reverse transcription PCR

using mouse lung total RNA and high fidelity Expander (Boehringer Mannheim) as previously described (22). The full length 1-kb mouse Cat Z cDNA was fully sequenced with ABI automated DNA Sequencer (model 377). PCR fragment was subcloned into PCR-Script™ Amp Cloning vector (Stratagene Inc.). The fragment was further released with XhoI and XbaI and subcloned into pcDNA-I expression vector (Invitrogen Corp.). The construct was also transfected into 293 cells as described above.

Northern Blot Analysis. Mouse splenocyte, peritoneal macrophage, and flt-3–stimulated dendritic cell total RNA was prepared using guanidine isothiocyanate lysate/cesium chloride gradient ultracentrifugation as described previously (22). 20 μg of total RNA from each sample was separated on 1.2% agarose gel. After blotting onto Zeta-Probe GT Genomic Tested Blotting Membrane (Bio-Rad Labs.), the membrane was probed with [^{32}P]dCTP-labeled full length mouse Cat F, Z, and S cDNA using express hybridization buffer (Clontech). After washings with 2× SSC, 2% SDS; 1× SSC, 1% SDS; and 0.1× SSC, 0.1% SDS, the membrane was exposed to Kodak BioMax MR-2 film.

Cysteine Protease Active Site Labeling and Mouse Cat F Immunoprecipitation. Mouse peritoneal macrophages were incubated overnight in the presence or absence of protease inhibitor LHVS (5 nM, 1 μM) or IFN-γ (100–500 U/ml). Cells were collected and lysed in lysis buffer (50 mM sodium acetate, 1 mM EDTA, 1% Triton X-100, pH 4.2) for 1 h on ice. Protein concentration was determined using a Bio-Rad Dc Protein Assay kit. Equal amounts of cell lysate from each treatment were labeled with HPLC-purified ^{125}I–JPM565 (17) for 1 h at 37°C and boiled for 5 min, followed by a 10-fold dilution with NP-40 lysis buffer. Mouse Cat F polyclonal antiserum–coated protein A–agarose beads were added, and the samples were incubated overnight at 4°C, followed by at least five washes with the washing buffer containing 50 mM Tris-HCl, pH 7.4, 0.5% NP-40, 150 mM NaCl, and 5 mM EDTA. Protein A–agarose beads were resuspended into 200 μl of 1× reducing protein sample buffer, boiled, and analyzed by 12% SDS-PAGE. One-tenth of the original labeled lysates were directly separated by 12% SDS-PAGE as a control for the immunoprecipitation. Dried gels were exposed to Kodak BioMax MR-2 film.

In Vitro Digestion of Ii with Recombinant Cats. Cat S gene–targeted mouse splenocytes were isolated as described (11). After incubation with 20 nM Con B (Ajinomoto Co.) and 0.5 mCi/ml of Express ^{35}S Protein Labeling Mix (New England Nuclear) in methionine/cysteine-free medium overnight, splenocytes were lysed in NP-40 lysis buffer for 1 h on ice. Samples were precleared and immunoprecipitated with N22-coated protein A–agarose beads, followed by another overnight incubation at 4°C as described above. The protein A–agarose beads were washed with washing buffer at least five times. The immunoprecipitated MHC class II αβ–Ii complexes were digested with recombinant Cats in 20 μl of cysteine protease assay buffer containing 50 mM sodium acetate, pH 4.2, 0.05% Triton X-100, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 5 μg/ml of pepstatin A, and 3 mM dithiothreitol for 1 h at 37°C, followed by separation on 10–20% tricine gel (Novex). The dried tricine gel was exposed to Kodak BioMax MR-2 film.

Kinetic Assays. Cat F enzyme assays were performed at a constant enzyme concentration (1 nM) in 100 mM potassium phosphate buffer, pH 6.5, containing 2.5 mM dithioerythreitol and 2.5 mM EDTA. *Z*-Leu-Arg-MCA (benzylcarbonyl-1-leucyl-1-arginine 4-methylcoumarinyl-7-amide) was used as the substrate, and its Cat F–catalyzed hydrolysis was measured at room temperature using a Perkin-Elmer fluorimeter (excitation and emission

wavelengths were 380 and 450 nm, respectively). Cystatins were dissolved in the reaction buffer and added to reaction mixtures. Cystatins A and B were provided by Dr. I. Bjork (Uppsala University, Uppsala, Sweden), and cystatin C was provided by Dr. M. Abrahamson (University of Lund, Lund, Sweden).

Determination of K_i Value. Steady-state rates were determined at eight different concentrations of inhibitor (0–5 μM) and three fixed concentrations of substrate (1, 2, and 5 μM). K_i values were determined by Dixon plots (plots of 1/v versus [I]), which gave three intersecting lines. All experiments were repeated twice, and the K_i values represent the mean values of the six intersecting points.

Results

Cat S–deficient Mice Show Defective IgE Responses in the Presence of LHVS. Cat S–deficient mice mount IgE responses and develop pulmonary eosinophilia normally after immunization and challenge with OVA (11). Cat S is not required for a normal Th2-like immune response. However, when Cat S$^{-/-}$ mice and wild-type mice were injected with the cysteine protease inhibitor LHVS (100 mg/kg) intraperitoneally every 48 h, the IgE response (Fig. 1) and perivascular eosinophilic infiltration (not shown) in lungs of OVA-immunized mice was totally abrogated. These results are similar to those observed with C57BL6 mice injected with LHVS during immunization with OVA (24) and raise the possibility that a cysteine protease(s) other than Cat S is required for IgE responses and pulmonary inflammation in this model. This finding led us to explore MHC class II processing and peptide loading in mice deficient in the two known cysteine proteases implicated in CLIP generation, Cats S and L (11, 12, 14).

MHC Class II Molecules from Macrophages of Cat S/L Double Knockouts Load Peptide Normally. To examine Ii processing and MHC class II peptide loading in the absence of known Ii-processing enzymes, mice deficient in Cats S and L were created by cross-breeding the respective single "knockouts." Processing of Ii–MHC class II complexes in Cat S$^{-/-}$L$^{-/-}$ mice was evaluated initially in splenocytes.
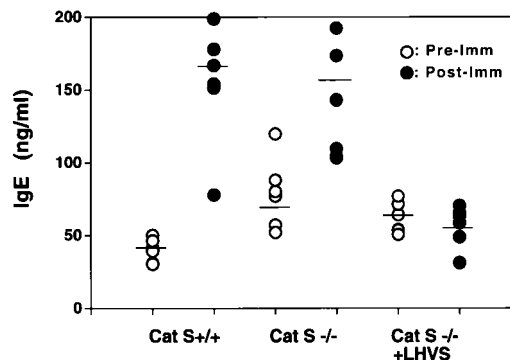
Figure 1. LHVS blocks IgE response in Cat S–deficient mice. Mouse sera from Cat S$^{-/-}$ mice, Cat S$^{-/-}$ mice injected with LHVS, and their wild-type siblings were used for total IgE ELISA. Cat S$^{-/-}$ and Cat S$^{+/+}$ mice have similar IgE responses to OVA immunization. However, LHVS (100 mg/kg/48 h) completely blocks the IgE response in Cat S$^{-/-}$ mice.

Cells were pulsed and then chased for 5 h to allow processing, and MHC class II heterodimers were immunoprecipitated. Splenocytes from Cat $S^{-/-}L^{-/-}$ and Cat $S^{-/-}$ mice showed identical accumulation of Iip10 and were equally defective in peptide loading (Fig. 2 A). This was expected, because mouse splenocytes express little or no Cat L. However, alveolar macrophages from Cat $S^{-/-}L^{-/-}$ mice degraded Ii and loaded peptide identically to macrophages from wild-type mice (Fig. 2 B), although a low amount of Iip10 accumulation was sometimes found in Cat $S^{-/-}$ or Cat $S^{-/-}L^{-/-}$ mouse macrophages. When the amounts of peptide–MHC complexes among the various samples were normalized by densitometry to MHC class II α chain in the boiled samples, instead of total radioactive protein, there was again no difference in formation of α/β peptide complexes among the various types of mice (not shown). Like alveolar macrophages, peritoneal macrophages from Cat $S^{-/-}L^{-/-}$ mice were found to process Ii and present antigen normally (Fig. 2 C). Thus, macrophages either have a mechanism of exchanging larger fragments of Ii for peptide that is distinct from splenocytes, or these cells express additional Ii processing enzyme(s) capable of generating CLIP.

We examined the importance of Ii processing for peptide loading in macrophages by exposing Cat $S^{-/-}L^{-/-}$ peritoneal macrophages to either low or high doses of the Cat S inhibitor LHVS. Prior studies showed that LHVS is selective for Cat S at low concentrations (<10 nM), whereas at higher concentrations additional cysteine proteases are inhibited (16, 25). In the presence of LHVS (5 nM), Cat $S^{-/-}L^{-/-}$ macrophages process MHC class II–Ii as effectively as controls (Fig. 2 C), consistent with the observation that macrophages from Cat S–deficient mice have unimpaired Ii processing and peptide loading. Macrophages cultured in 1 μM LHVS showed delayed Ii processing (accumulation of Iip10) and peptide loading remini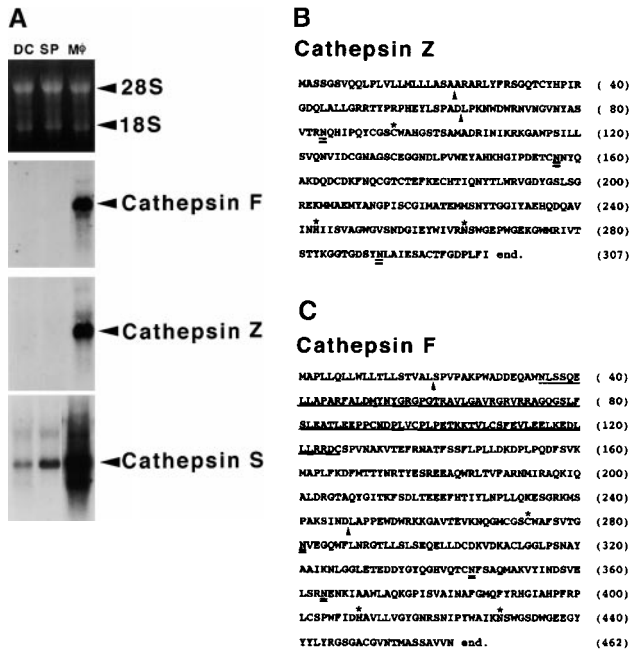scent of that seen in splenocytes from Cat $S^{-/-}$ or Cat $S^{-/-}L^{-/-}$ mice. Thus, an additional cysteine protease(s) in macrophages might substitute for Cat S in these cells.

Identification of Cysteine Proteases Expressed Preferentially in Macrophages. We next compared the set of cysteine proteases expressed by macrophages, B cells, and dendritic cells. EST databases were screened for murine cysteine protease cDNAs as a source of DNA probes. Sequences were obtained for murine Cats O, W, K, Z, and F in addition to the major Cats H, B, L, and S. PCRs of reverse-transcribed mRNA and Northern blot analyses were used to compare the expression of these enzymes in macrophages, B cells, and dendritic cells. Cat W and K mRNA was not seen in these cells, consistent with prior reports (26, 27). Low levels of Cat O were found in both B cells and macrophages. Because this enzyme was comparable in both cell types, Cat O was not further studied. Two enzymes, Cat Z and Cat F, were found to be expressed in macrophages but not splenocytes or dendritic cells by Northern blot analysis (Fig. 3 A). Comparison of mRNA levels of Cats Z and F with levels of Cat S in macrophages shows the relative level of Cat S to be considerably higher. Whereas Cat S mRNA is also evident in splenocytes and dendritic cells, longer exposure of the Northern blot shown in Fig. 3 A did not reveal a signal for either Cat F or Z mRNA in these cell types (not shown). Also unlike macrophages, PCR amplification of reverse-transcribed RNA failed to reveal Cats Z and F in splenocytes. Cats Z and F were therefore characterized in more detail.

Full length cDNAs encoding murine Cats Z and F were obtained as described in Materials and Methods. Comparison of the predicted amino acid sequences of human and murine Cats reveals extensive homologies (78% for Cat F and 79% for Cat Z). Cat Z has Cat B–like sequences with a predicted loop structure between the active site cysteine at amino acid residue 94 and the active site asparagine at amino acid residue 264 (reference 28; Fig. 3 B). Interest-
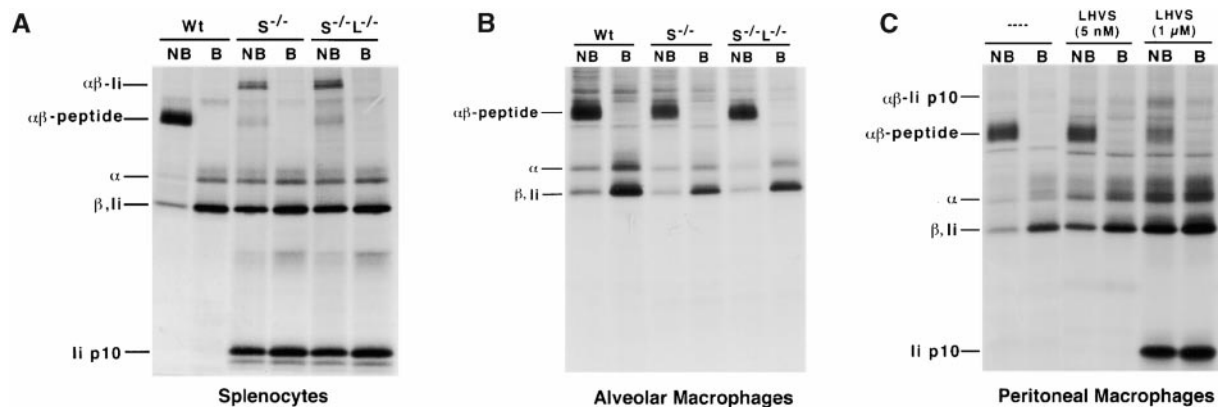
Figure 2. MHC class II from Cat $S^{-/-}L^{-/-}$ mice load peptide normally. (A) Mouse splenocytes from wild-type, Cat $S^{-/-}$, and Cat $S^{-/-}L^{-/-}$ mice were pulsed for 1 h and chased overnight. Macrophages were then lysed in NP-40 lysis buffer, followed by immunoprecipitation with N22. The precipitates were either boiled (B) or nonboiled (NB) before separation on 12% SDS-PAGE. (B) Mouse alveolar macrophages were metabolically labeled and immunoprecipitated with N22 and separated on 12% SDS-PAGE as splenocytes as in A. (C) Mouse peritoneal macrophages from Cat $S^{-/-}L^{-/-}$ were incubated in LHVS (0 or 5 nM or 1 μM) overnight, followed by 1 h of pulse and overnight chase. Cells were then lysed, immunoprecipitated with N22, and separated on 12% SDS-PAGE.

A

DC SP Mφ

◄ 28S
◄ 18S

◄ Cathepsin F

◄ Cathepsin Z

◄ Cathepsin S

B
Cathepsin Z

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MASSGSVQQLPLVLLMLLLASAARARLYPRSGQTCYHPIR   ( 40)
GDQLALLGRRTYPRPHEYLSPADLPKHWDWRHVNGVNYAS   ( 80)
VTRHQHIPQYCGSCWAHGSTSAMADRINIKRKGANPSILL   (120)
SVQNVIDCGNAGSCEGGNDLPVWEYAHKHGIPDETCHNYQ   (160)
AKDQDCDKFNQCGTCTEFKECHTIQNYTLMRVGDYGSLSG   (200)
REKHHAEMTANGPISCGIMATEMMSNYTGGIYAEHQDQAV   (240)
INHIISVAGWGVSNDGIEYWIVRNSWGEPWGEKGWHRIVT   (280)
STYKGGTGDSYNLAIESACTFGDPLFI end.           (307)
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C
Cathepsin F

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MAPLLQLLWLLTLLSTVALSPVPAKPWADDEQAWNLSSQE   ( 40)
LLAPARFALDMYNYGRGPQTRAVLGAVRGRVRRAAGQGSLF  ( 80)
SLEATLKKPPCNDPLVCPLPKTKKTVLCSFKVLEELKEDL   (120)
LLRRDCSPVNAKVTEFRNATFSSFLPLLDKDPLPQDFSVK   (160)
NAPLFKDFMTTYNRTYESREEAQWRLTVPARNMIRAQKIQ   (200)
ALDRGTAQYGITKFSDLTKEKFHTIYLNPLLQKESGRKMS   (240)
PAKSINDLAPPEWDWRKKGAVTEVKNQGWCGSCWAFSVTG   (280)
NVEGQWFLNRGTLLSLSEQELLDCDKVDKACLGGLPSNAY   (320)
AAIKNLGGLETEDDYGYQGHVQTCNFSAQMAKVYINDSVE   (360)
LSRNENKIAAWLAQKGPISVAINAFGMQFYRHGIAHPFRP   (400)
LCSPWFDHAVLLVGYGNRSNIPTWAIKNSWGSDWGEEGY    (440)
YYLYRGSGACGVNTMASSAVVN end.                (462)
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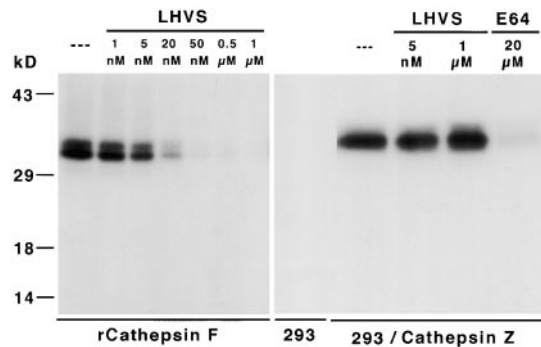
Figure 3. Isolation and expression of mouse Cats F and Z. (A) Northern blot analysis. Total RNA (20 μg/each) from mouse splenocytes, peritoneal macrophages, and flt-3–stimulated dendritic cells were separated on 1.2% agarose gel, blotted onto a nylon filter, and probed with full length mouse Cat F, Z, and S cDNAs. RNA loading control is shown by rRNAs (top panel). Both Cats F and Z can be detected from macrophages but not splenocytes or dendritic cells. In contrast, Cat S transcripts can be detected in all three cell types. (B) Amino acid sequence of mouse Cat Z. The active site amino acids are indicated with asterisks (*), and the potential sites for glycosylation are double underlined. Arrowheads indicate the potential cleavage sites of signal peptide and pro region of Cat Z. (C) Amino acid sequence of mouse Cat F. Arrowheads indicate the signal peptide and pro region cleavage sites. Three active site amino acids (Cys, His, and Asn) are indicated by asterisks (*), and three potential glycosylation sites are double underlined. The underlined region is the potential cystatin-like domain (32).

LHVS

```
kD   ---  1    5    20   50   0.5  1
          nM   nM   nM   nM   μM   μM
43 ▶
29 ▶
18 ▶
14 ▶
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LHVS   E64

```
---  5    1    20
     nM   μM   μM
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rCathepsin F   293   293 / Cathepsin Z

Figure 4. Cat F, but not Cat Z, fits the LHVS-inhibitable protease profile of macrophages. Recombinant Cat F (5 nM) was labeled with ^{125}I–JPM565 in the presence or absence of LHVS at the indicated concentrations for 1 h at 37°C in pH 4.2 assay buffer with 3 mM dithiothreitol (DTT) and then separated on 12% SDS-PAGE (left). Cat Z–transfected 293 cells were lysed in lysis buffer, pH 4.2, and 100 μg of protein was incubated with ^{125}I–JPM565 in the presence of 3 mM DTT and inhibitors and separated on 12% SDS-PAGE (right).

ingly, the dihistidines found in the "occluding loop" of Cat B appear to be replaced by asparagines in Cat Z (Fig. 3 B). Whether there is a true occluding loop in Cat Z remains to be determined. Notably, the Cat Z sequence has also been reported as Cat X in separate reports (29, 30).

Murine Cat F is also highly homologous to the previously reported human enzyme (19, 31, 32). As recently reported for the full length human enzyme, murine Cat F has an extended pro region with a cystatin-like sequence near the NH2 terminus (Fig. 3 C). This characteristic appears unique to Cat F and is a feature conserved between the human and murine enzymes (31). Whether this sequence has cystatin-like protease inhibitor activity also remains to be determined.

Inhibitor Profiles of Recombinant Cat F and Z. An enzyme substituting for Cat S in APC Ii processing should be sensitive to inhibition by high concentrations of LHVS. Therefore, we tested the sensitivity of Cats F and Z to increasing concentrations of LHVS. To conduct these studies, we used a probe based on the epoxide E-64, a class-specific inhibitor of cysteine (17, 22). This inhibitor, JPM565, was iodinated, and the sensitivity of human Cats F and Z to LHVS was determined by competition of active-site labeling by ^{125}I–JPM565. Recombinant Cat F was sensitive to LHVS but less so than Cat S. Inhibition is evident at 10–20 nM and virtually complete at 50 nM, making the enzyme ∼10–50-fold less sensitive to LHVS than Cat S (Fig. 4, left). In contrast to Cat F, mouse Cat Z expressed in 293 cells can be inhibited by 20 μM of E64 but not 1 μM LHVS (Fig. 4, right). This observation indicates Cat Z is an unlikely candidate to account for LHVS-sensitive Ii processing observed in Cat S/L–deficient macrophages (Fig. 2). These findings are therefore consistent with Cat F being a target enzyme for these inhibitors.

In additional experiments using recombinant human Cat F and inhibitors of the cystatin family, hydrolysis of the colorimetric substrate, Z-Leu-Arg-MCA by Cat F was found to be markedly inhibited by cystatin C ($K_i = 0.532 \pm 0.1991$ nM). The purified enzyme was much less sensitive to cystatin A ($K_i = 6.645 \pm 0.862$ nM) and cystatin B ($K_i = 9.156 \pm 1.287$ nM). These findings are in agreement with the overall sequence similarity between Cats S and F and imply an "open" substrate-binding cleft for Cat F, in line with its marked endoproteolytic activity (19). In contrast, recent observations suggest that human Cat Z confers stricter carboxypeptidase activity than Cat B. No endopeptidase activity was found with this exopeptidase, and no inhibition by cystatin C was detected even up to a concentration of 4 μM of inhibitor (33). Because Cat F, and not Cat Z, displayed the correct inhibitory profile with LHVS, we focused on the expression of this enzyme in murine macrophages.

Expression of Cat F in Murine Macrophages. Polyclonal antibodies were raised against a fusion protein comprised of the maltose-binding protein and a fraction of the mouse Cat F coding sequence (from Glu221 to Asn462). These antibodies were used to explore expression of Cat F in
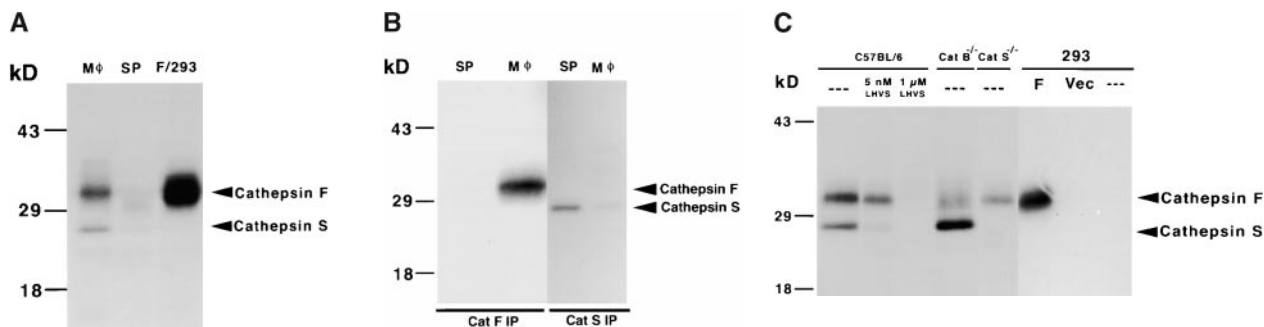
Figure 5. Cat F is expressed in macrophages and not splenocytes. (A) Equal amounts, normalized to protein content, of mouse peritoneal macrophages, splenocytes, and Cat F–transfected 293 cell lysates were labeled with ^{125}I–JPM565, preboiled for 5 min, and immunoprecipitated with mouse Cat F antibodies. The precipitates were separated on 12% SDS-PAGE. Cat F is not detected in splenocytes, whereas macrophages showed the 30–31-kD active Cat F that was also present in Cat F–transfected 293 cells. (B) Splenocyte lysates containing 10-fold greater protein content than macrophages were directly immunoprecipitated with Cat F antibodies without preboiling. Cat F can only be detected in macrophages and not splenocytes. The sequential lysates were then boiled and further immunoprecipitated with Cat S antibodies to reveal Cat S expression in both types of cells. (C) Peritoneal macrophages from wild-type, Cat B$^{-/-}$, and Cat S$^{-/-}$ mice were incubated with or without LHVS (5 nM, 1 μM) overnight. The cells were lysed and protein concentrations normalized. Equal amounts of protein from each sample were labeled with ^{125}I–JPM565 and precipitated with Cat F polyclonal antisera. Cat F activity was completely inhibited in cells incubated with 1 μM but not 5 nM LHVS. Immunoprecipitations of ^{125}I–JPM565-labeled lysates of macrophages from Cat S$^{-/-}$ and Cat B$^{-/-}$ mice indicate that the anti–Cat F antibodies cross-react with Cat S but not Cat B when boiled before immunoprecipitation. The size of mouse Cat F and the specificity of Cat F antibodies was also examined with Cat F–transfected 293 cell lysate and empty vector– or sham-transfected 293 cell lysate.

macrophages and splenocytes. The cysteine protease inhibitor ^{125}I-JPM565 was added to equal amounts of cell lysates, normalized for protein concentration, from macrophages and splenocytes. The lysates were then immunoprecipitated with anti–Cat F antibodies, and the precipitates were analyzed by SDS-PAGE and autoradiography to reveal active enzyme. Active site–labeled Cat F, migrating as a ∼31–33 kD protein, was recovered from lysates of macrophages but not splenocytes (Fig. 5 A). Notably, when cell lysates were boiled for 5 min before immunoprecipitation, the Cat F antibodies cross-reacted with and immunoprecipitated Cat S, seen as the lower band in the macrophage lane of Fig. 5 A. This was confirmed by performing immunoprecipitations with the Cat F antibody in Cat S–deficient mice (Fig. 5 C) as well as Cat S–transfected 293 cell lysates (data not shown).

To further determine whether low levels of Cat F are expressed in splenocytes, ^{125}I–JPM565-labeled Cat F was immunoprecipitated from both splenocyte and macrophage lysates under conditions in which the splenocyte lysate contained 10-fold greater protein content than the macrophage lysate. This experiment was conducted without preboiling of lysates, which clearly diminishes Cat S cross-reactivity. Again, no Cat F signal can be detected from splenocytes (Fig. 5 B). However, a sequential Cat S immunoprecipitation revealed Cat S expression in both splenocytes and macrophages (Fig. 5 B). This is consistent with the results of Northern blot analyses (Fig. 3).

As judged by [25]I–JPM565 labeling, activity of Cat F was blocked by 1 μM LHVS but not by a low dose of LHVS (5 nM; Fig. 5 C). Because Cat F migrates with a molecular mass similar to that of Cat B, we tested the possibility that Cat F polyclonal antibodies cross-react with Cat B. Cat B–deficient macrophages were labeled and immunoprecip-

itated with Cat F antibodies. Active Cat F was again observed (Fig. 5 C). In addition, the antibody specificity was also confirmed using Cat F–transfected 293 cell lysates. No active cysteine proteases were revealed by this antibody in sham-transfected cells nor in cells transfected with empty vector (pcDNA-I; Invitrogen Corp.; Fig. 5 C). We conclude that the 31–33-kD active Cat F is expressed in macrophages and not B cells.

Cat F Degrades Iip10 and Generates CLIP. Recombinant enzymes were used to test the ability of Cats Z or F to degrade Ii to CLIP. The amounts of active enzyme were determined by active site titration as described (10). Complexes of full length Ii and MHC class II dimers were obtained by immunoprecipitation of metabolically labeled splenocytes in the presence of Con B. Con B inhibits endosomal ATPase, which disrupts the normal pH of these compartments and results in a blockade of Ii processing. Immunoprecipitated complexes were resuspended at acidic pH (pH 4.2) and exposed to various amounts of recombinant Cats as indicated in Fig. 6 A. After 1 h at 37°C, the reactions were stopped in SDS sample buffer, and the reaction products were separated by SDS-PAGE and visualized by autoradiography. The ability of these cysteine proteases to degrade Iip10 was quantified by densitometry and normalized to that seen with 5 nM recombinant Cat S (Fig. 6 B). As reported previously, compared with Cat S, Cat B had no activity as an Ii-degrading protease (10). Murine Cat Z, expressed in 293 cells, did not degrade Iip10 at any concentration tested (Fig. 6 B). In contrast, human Cat F was found to be as or more active than Cat S in Iip10 degradation and CLIP formation (Fig. 6). Lysates of 293 cells expressing murine Cat F also degraded Iip10, resulting in CLIP formation (Fig. 6 B), whereas cells expressing murine Cat Z had no activity in this assay.
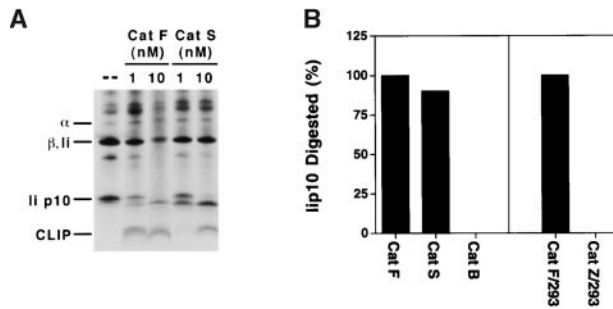
Figure 6. Cat F, but not Cat Z, degrades Iip10 from MHC class II–Iip10 complexes, generating MHC class II–CLIP. (A) N22 immunoprecipitates from metabolically radiolabeled Cat S$^{-/-}$ mouse splenocytes treated with Con B (20 nM) were digested with either purified recombinant human Cat F or purified recombinant human Cat S at the indicated concentrations in assay buffer, pH 4.2, for 1 h at 37°C. The digested products were separated on 10–20% Tricine gel (Novex). (B) N22 immunoprecipitates were digested with 5 nM (as determined by cysteine protease active site titration; reference 10) of purified recombinant human Cat F–, S–, and B– or mouse Cat F– and Z–transfected 293 cell lysates and analyzed on a 10–20% Tricine gel. The Cat F and Z activity from cell lysate was examined with ^{125}I–JPM565 active site labeling. Densitometry analysis was used to quantitate the Iip10 digestion products of each Cat. CLIP-forming activities of recombinant Cat S and B are shown relative to Cat F (as 100%). Also, Cat Z–transfected 293 cell lysate CLIP-forming activity is shown relative to Cat F–transfected 293 cell lysate (as 100%).

Discussion

Proteolytic processing of Ii is a critical determinant of the timing and location of MHC class II peptide loading. Although a number of proteases have been implicated in progressive COOH-terminal cleavages of Ii to produce Iip10, only a limited number of cysteine proteases, i.e., Cats S and L, appear capable of degrading Iip10 to generate CLIP. We have identified an additional protease capable of cleaving Iip10 to form CLIP: Cat F. Our data support the conclusion that Cat F is a relevant protease in APC Iip10 processing. First, purified Cat F is at least as potent as Cat S in Ii processing and CLIP formation (Fig. 6). Second, macrophages deficient in Cats S and L process Ii and load peptides onto MHC class II heterodimers normally (Fig. 2). Nonetheless, Iip10 processing and peptide loading in these cells remains sensitive to high concentrations of the cysteine protease inhibitor LHVS. Concentrations of LHVS that result in abrogation of MHC class II α/β–peptide SDS-stable complexes in macrophages (Fig. 2 B) also inhibit Cat F (Figs. 4 and 5 B), consistent with Cat F being the protease responsible for Ii processing in the absence of Cats S and L. Finally, macrophages express active Cat F. Nonetheless, we cannot exclude the possibility that as yet unknown additional cysteine proteases contribute to Iip10 processing in macrophages. Our data are consistent with the notion that in macrophages, Cat F substitutes for and potentially complements the function of Cat S in Iip10 processing. If so, manipulation of Iip10 processing and MHC class II maturation by APCs in vivo may require an inhibitor that attacks Cats F, L, and S. From the viewpoint

of Ii processing, these three enzymes appear to form a distinct subfamily of papain-type cysteine proteases.

Professional APCs are functionally heterogeneous but have been clarified on the basis of distinct surface markers and morphology into lymphoid- and myeloid-like dendritic cells, B cells, and macrophages. Recent evidence indicates that flt-3 ligand promotes development of lymphoid-type dendritic cells, whereas GM-CSF promotes development of predominantly myeloid-type dendritic cells (34). B cells are functionally distinct from dendritic cells in that they can use their surface Ig to capture and process low levels of extracellular antigens. Macrophages also present antigen efficiently in the context of inflammation (35–39). Although all types of APCs appear to express Cat S, only macrophages express Cat F, whereas this enzyme is not detected in B cells or flt-3–stimulated murine dendritic cells (Figs. 2 A and 5 A). The different pH profiles of Cats F and S allow the possibility that these enzymes may function in distinct antigen processing compartments. The pH optimum of Cat F favors its main site to be the lysosome (19), whereas Cat S activity is present throughout the endosomal/lysosomal pathways (13). The trafficking of endogenous Cat inhibitors such as cystatin C may also favor, at least under some conditions, one enzyme over the other in Iip10 processing. The true functional importance of the finding of a second Iip10 processing enzyme in APCs will require gene deletion and analysis of mice with selective loss of Cat F.

The use of cysteine protease inhibitors in vivo has recently emerged as a strategy to regulate immune responses and the life cycle of certain parasites (40, 41). For example, mice infected with *Leishmaniasis major* showed a shift of CD4$^+$ T cell differentiation from Th2 to Th1 when mice were treated with the Cat B inhibitor CA074 (42). IgE responses and pulmonary eosinophilia in mice immunized with OVA are blocked by high concentrations of LHVS (24). That this blockade cannot be explained by simple inhibition of Cat S is now clear given the fact that IgE responses in this OVA model are normal in Cat S–deficient mice (Fig. 1). Th2-type T cell responses are still impaired in LHVS-treated, Cat S–deficient mice. The identification of Cat F as an LHVS-inhibitable protease (Fig. 4) fully competent to process Ii-p10 and generate CLIP (Fig. 6) may explain the normal Th2-type immune responses in these mice. Stimulated lung macrophages could thus play a more prominent role in antigen presentation than is currently believed. Further experiments are required to establish whether other populations of APCs within lungs express Cat F or if indeed lung macrophages, at least in the setting of airway inflammation, become fully competent and important for antibody responses to antigens that access the immune system by this route.

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