Major Histocompatibility Complex Class II-associated p41 Invariant Chain Fragment Is a Strong Inhibitor of Lysosomal Cathepsin L

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Summary

The invariant chain (Ii) is associated with major histocompatibility complex class II molecules during early stages of their intracellular transport. In an acidic endosomal/lysosomal compartment, it is proteolytically cleaved and removed from class II heterodimers. Participation of aspartic and cysteine proteases has been observed in in vitro degradation of Ii, but the specific enzymes responsible for its in vivo processing are as yet undefined. We have previously isolated a noncovalent complex of the lysosomal cysteine protease cathepsin L with a peptide fragment derived from the p41 form of Ii from human kidney. Here we show that this Ii fragment, which is identical to the alternatively spliced segment of p41, is a very potent competitive inhibitor of cathepsin L (equilibrium inhibition constant $K_i = 1.7 \times 10^{-12}$ M). It inhibits two other cysteine proteases, cathepsin H and papain, but to much lesser extent. Cysteine proteases cathepsins B, C, and S, as well as representatives of serine, aspartic, and metalloproteases, are not inhibited at all. These findings suggest a novel role for p41 in the regulation of various proteolytic activities during antigen processing and presentation. The Ii inhibitory fragment shows no sequence homology with the known cysteine protease inhibitors, and may, therefore, represent a new class.

MHC class II molecules are transmembrane glycoproteins, composed of polymorphic α and β chains, whose crucial function is to present bound antigenic peptides to CD4+ T lymphocytes (1). Shortly after their synthesis in the endoplasmic reticulum (ER)1, class II dimers associate with a nonpolymorphic type II transmembrane protein called the invariant chain (Ii) (2). The resulting class II–Ii complexes exit the ER, and are transported through the Golgi apparatus to the endocytic pathway (3, 4).

Ii contributes in a number of ways to the proper functioning of MHC class II molecules. These include promoting effective association and folding of newly synthesized α and β subunits (5), increasing transit of assembled heterodimers out of the ER (6), blocking of peptide binding to class II molecules in the early compartments of the biosynthetic pathway (7), and sorting class II molecules into appropriate endocytic organelles (8, 9).

After arrival in endosomes, the COOH-terminal lumenal domain of Ii is degraded in distinct steps from the COOH terminus of the molecule, resulting in the sequential formation of processing intermediates, each lacking increasingly larger portions from the COOH-terminal side (10, 11). This results in removal of Ii from class II dimers (3, 12). The specific proteases responsible for its processing remain unknown, but in vitro degradation of class II–Ii complexes with cathepsin B removes Ii and allows the binding of antigenic peptides to free class II molecules (12, 13). Treatment of APCs with aspartic and cysteine protease inhibitors prevents complete proteolysis and dissociation of Ii from class II molecules (3, 11, 14). In B cells, an aspartic protease initiates Ii processing, whereas a cysteine protease completes the final stages of Ii degradation, as established using leupeptin and the specific aspartic protease inhibitors (14).

After the dissociation of Ii, class II molecules assemble with peptides derived mainly from extracellular antigens that enter the endocytic pathway by binding to specific receptors, or through pinocytosis (15). Antigens are degraded...
Materials and Methods

Enzymes and Inhibitors. The complex of cathepsin L with the li fragment was isolated from human kidney (27). The li fragment was supplemented from the complex using reversed-phase HPLC (27), freeze-dried on a speed-vac concentrator, and redissolved in distilled water. Free cathepsin L was isolated from the same source (28). Cathepsins B, H, and S were purified from human spleen (29), cathepsin C from human kidney (30), and cathepsin D from human liver (31). Papain (papaya) and trypsin (porcine pancreas) were purchased from Sigma Chemical Co. (St. Louis, MO), and aminopeptidase M (porcine kidney) from Boehringer Mannheim (Mannheim, Germany). L-3-carboxy-trans-2,3-epoxypropyl-leucylamido-(3-guanidino) butane (Ep-475), a specific inhibitor of cysteine proteases used for active-site titrations, was a generous gift from Dr. Nobuhiko Katunuma (University of Tokushima, Tokushima, Japan).

Affinity Chromatography on Con A-Sepharose. The li fragment was isolated from rabbit and purified by affinity chromatography on protein A-Sepharose (Pharmacia, Uppsala, Sweden).

C0OH-terminal Sequence Analysis. li fragment was reduced with 2-mercaptoethanol, alkylated with iodoadetic acid, and exposed to β-tryptic cleavage. The resulting peptides were identified by their amino acid composition using an amino acid analyzer (model 421; Applied Biosystems, Foster City, CA). The sequence of the COOH-terminal peptide was determined using a liquid phase sequenator (model 475A; Applied Biosystems).

Enzymes and Inhibitors. Inhibitory effects of the li fragment were studied by incubating it with an enzyme for 30 min at 25°C before the addition of a substrate.

Active-site Titrations. 20 µl of cathepsin L (0.1 µM final pro-
tein concentration) was preincubated for 5 min with 410 μl of 8 
mM DTE in 0.34 M sodium acetate buffer, pH 5.5, containing 
0.1% Brij-35 (polyoxyethylene-lauryl ether) and 1 mM EDTA, 
and then added to 20 μl of increasing concentrations of the cy-
steine protease inhibitor (EPI, Epi-475). After a 30-min incubation 
at 25°C, 450 μl of 200 μM Z-Phe-Arg-p-nitroanilide in the same 
buffer was added. Product formation was followed continu-
ously at 410 nm for 1 min with a Lambda 3 spectrofluorimeter (Perkin-
Elmer, Beaconsfield, UK). The velocity was calculated using lin-
ear regression analysis, and the active concentration of cathepsin L 
determined from the plot of velocity against molar ratio of initial 
inhibitor and enzyme concentrations ([I]/[E]). All the other en-
zymes were active-site titrated in the same way using the corre-
sponding substrates and inhibitors. The li fragment was titrated 
with active-site–titrated cathepsin L.

Inhibition Kinetics of Cathepsin L. All experiments were carried 
at 24°C under pseudo first order conditions, with inhibitor 
concentrations at least 10-fold higher than enzyme concentrations 
(32). The progress curves were monitored continuously at excita-
tion and emission wavelengths of 370 and 460 nm, respectively, 
using a spectrofluorimeter (model LS-50B; Perkin-Elmer). The 
buffer used was 0.34 M sodium acetate buffer, pH 5.5, containing 
1 mM EDTA and 0.1% Brij-35. The reaction was started by the 
addition of 50 μl of the enzyme (6.6 pM final concentration, pre-
activated with 8 mM DTE) to 2.450 ml of the buffer containing 
the substrate (Z-Phe-Arg-MCA) and the inhibitor (the li frag-
ment). Six different inhibitor concentrations and four different 
substrate concentrations were used. All progress curves were fit-
ted by nonlinear regression analysis to the integrated rate equation 
(33): $P = v_t + (v_i - v_t)(1 - e^{-kt})/k$, where $P$ represents the 
product concentration, $v_t$ and $v_i$ are the initial and steady-state ve-
locities, respectively, $k$ is the observed pseudo first order rate con-
tant, and $t$ is time. The second order rate constant for complex 
formation, $k_{on}$, was calculated from the slope of the plot $k$ versus 
$[I]_0$ (slope = $k_{on}/(1 + [S]/K_m)$), where $K_m$ is the Michaelis con-
stant, using $[S]_0 = K_m = 2$ μM. The dissociation rate constant, 
$k_{off}$, was obtained from individual measurements $k_{on} = k_{fr}/k_{fr}$, 
and the equilibrium inhibition constant, or $K_i$ value, was calcu-
lated from $K_i = k_{off}/k_{on}$.

Inhibition Kinetics of Cathepsin H and Papain. Activities of ca-
thepsin H and papain in the presence of the li fragment were 
measured under the same experimental conditions as described 
above for cathepsin L, using 0.1 M phosphate buffer, pH 6.8, 
containing 1 mM EDTA, and the substrate H-Arg-MCA (20 
μM, 40 μM) for cathepsin H (23 pM final concentration, preac-
tivated with 8 mM DTE), and 0.34 M sodium acetate buffer, pH 
5.5, containing 1 mM EDTA, 0.1% Brij-35, and the substrate 
Z-Phe-Arg-MCA (10 μM, 16 μM, 20 μM) for papain (30 pM 
final concentration, preactivated with 8 mM DTE). The inhibitor 
concentrations were 10–100-fold higher than the enzyme con-
ncentrations. Since $v_t$ could not be determined due to very fast re-
actions, only $v_i$ was recorded and $K_i$ values were determined from 
Dixon plots in the form $1/v_i$ versus $[I]$ at different substrate con-
centrations (34).

Results

The li Fragment–Cathepsin L Complex. The NH$_2$- and 
COOH-terminal sequences of the peptide fragment, sepa-
rated from the complex, as well as its total amino acid com-
position, were determined, showing it to originate from the 
p41 form of li (27, and Fig. 1). It is identical to AS en-
coded by an alternatively spliced exon (20), which is found 
only in p41, and not in the major form of li, p31 (Fig. 1). 
The fragment consists of 65 amino acid residues, starting at 
residue 194 and ending at residue 258 (p41 numbering). 
The li fragment was eluted from the complex as a mixture of 
either two or three forms (number and proportions de-
pended on details in purification procedure) that differed 
slightly in their molecular masses as revealed by immuno-
blotting (Fig. 2 B). Amino acid compositions and the NH$_2$-
terminal sequences of the two main bands were shown to 
be identical. Only the higher molecular mass band was 
bound to Con A–Sepharose, and was therefore concluded to 
be glycosylated.

The complex was analyzed by SDS-PAGE and stained 
either with Coomassie blue (Fig. 2 A) or with anti-li frag-
ment Ab (Fig. 2 B). In the presence of SDS (Fig. 2, A and 
B, lane f) the li fragment remained associated with the en-
zyme, as revealed by anti-li immunostaining (Fig. 2 B, lane 
f). The complex migrated as a series of bands, a con-
sequence of the different forms of li fragment, with apparent 
molecular mass of ~32 kD. After incubating with SDS at 
100°C, the complex dissociated, giving rise to new protein

Figure 1. NH$_2$- (27) and 
COOH-terminal amino acid se-
cquences of the fragment that 
binds to cathepsin L, a schematic 
representation of p31 and p41 
(20), and the position of the in-
hibitory fragment within p41 
(horizontal bar). (AS) Additional 
segment derived from alterna-
tive splicing, unique to p41. 
Numbering is according to p41.
bands (Fig. 2, A and B, lane 2), the one with apparent molecular mass of 31 kD being cathepsin L, and the two with apparent molecular masses of ~14 kD being liberated Ii fragments. The surprisingly small difference in apparent molecular masses of free and complexed cathepsin L reflects the stability of the complex towards SDS. Immunostaining, which is more sensitive than Coomassie staining, also showed the third form of the Ii fragment. After incubating the complex with SDS at 100°C under reducing conditions (Fig. 2, A and B, lane 3), cathepsin L migrated as two bands with molecular masses of 25 kD (heavy chain) and <14 kD (light chain), but the position of the Ii fragment did not change. In lane 2, traces of reduced cathepsin L can be seen.

Inhibitory Activity of the Ii Fragment. During the purification procedure it was observed that complexed cathepsin L appeared much less active than its free counterpart (27). To investigate its inhibitory role, free Ii fragment was obtained by separating the complex components using HPLC (27), and a wide range of human lysosomal cysteine proteases, cathepsins B, C, H, L, and S, the plant cysteine protease, papain, as well as representatives of the other three classes of proteases, cathepsin D (aspartic protease), trypsin (serine protease), and aminopeptidase M (metalloprotease), were screened for inhibition (Fig. 3 A). None of the enzymes tested, except cathepsin L, cathepsin H, and papain, was inhibited, even at inhibitor concentrations of 10^{-7} M.

Inhibition Kinetics of Cathepsin L. Since cathepsin L, after dissociation of the Ii fragment from the complex by HPLC, was irreversibly denatured, it was isolated in a free form from human kidney (28). Complex formation in vitro with the Ii fragment was confirmed by native PAGE (Fig. 4). The inhibition kinetics of the Ii fragment with cathepsin L were characterized under pseudo first order conditions. All progress curves showed an exponential approach to a final, steady-state rate, and were analyzed by the least-squares fitting of the appropriate integrated rate equation (33) to the experimental data (Fig. 3 B). In a control experiment with the inhibitor omitted, fluorescence increased linearly, verifying that the enzyme was stable during the experiment. Dependence of the observed \( k_s \) on substrate concentration demonstrated a competitive inhibition mechanism (Fig. 3 C), and its linear dependence on inhibitor concentration allowed us to calculate \( k_{in} \) (Fig. 3 D). \( k_{in} \) was measured directly, and \( K_i \) was calculated from \( K_i = k_{in} / k_{cat} \). It is evident that the Ii fragment binds very tightly (\( K_i = 1.7 \times 10^{-12} \) M) and rapidly (\( k_{cat} = 4.6 \times 10^7 \) M^{-1}s^{-1}) to the active site of cathepsin L (competitive inhibition, Fig. 3 C) in contrast to the weakly inhibitory activity of intact p31 reported elsewhere (35). To confirm the accuracy of the value of \( K_i \), the slope of the plot \((v/v_s - 1)\) versus \([I]_0\) led to \( K_i = 1.1 \times 10^{-12} \) M, in good agreement with the value calculated from \( k_{in} \) and \( k_{cat} \).

The low activity of the complex as isolated is accounted for by the concentration of free enzyme expected to exist at equilibrium using the value of \( K_i \) determined from the above experiments. This confirms the close similarity and probable identity between the complex as isolated, and that generated in vitro.

Discussion

We have isolated from human kidney and characterized a noncovalent complex between the lysosomal cysteine protease, cathepsin L and a peptide derived from the p41 form of Ii. Although the complex was purified from a tissue homogenate, there are good reasons to believe that the complex must be formed in vivo in the endocytic vesicles of Ii-positive cells. First, Ii and cathepsin L are colocalized in the cell: class II–Ii complexes are transported from the ER to endocytic compartments (10, 36–38), and the lysosomal hydrodases are present all along this endocytic path-
way (39). Second, the value of $K_i$ is sufficiently low ($K_i \sim 10^{-12}$ M) to ensure the formation of the complex at the likely physiological concentrations of enzyme and peptide.

The $K_i$ value obtained for this interaction is comparable to the values reported for the inhibition of cathepsin L with the endogenous CPIs from the cystatin superfamily, which are potent cytosolic regulators of cysteine protease activity (40). The inhibition of cathepsin H by the li fragment is significantly weaker ($K_i \sim 10^{-9}$ M), indicating that it is less likely to be physiologically relevant. Since cathepsin L is by far the most potent cysteine protease of endocytic compartments, its inhibition must be very important in the regulation of its various functions during antigen processing and presentation. The li fragment shows no sequence homology to any of the known CPI (40), suggesting that it may represent a new class of inhibitors.

It is very interesting that the inhibition is a property of AS, the unique sequence that occurs in p41, but not in p31 (Fig. 1). Few studies have been made on p41 (24, 25, 37, 38), and its biological significance, as well as that of AS, is still unclear. It has been shown that p41 is able to impart the same folding and peptide-blocking functions as does p31 (25), but is superior in its ability to enhance antigen presentation for a subset of antigens (24). It has been suggested that p41 might contain a transport signal that, in contrast to p31, directs class II not only to early but to late

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**Figure 3.** Inhibition kinetics of the p41 fragment. (A) Inhibition of various proteases by the p41 fragment. The activities of cathepsin L ($[E_0] = 7$ pM, filled circles), papain ($[E_0] = 30$ pM, triangles), cathepsin H ($[E_0] = 23$ pM, squares) and cathepsin B ($[E_0] = 100$ pM, open circles) were measured in the presence of varying amounts of the fragment. The activities of cathepsins C, S, and D, trypsin, and aminopeptidase M were also measured ($[E_0] = 100$ pM) and were the same as that of cathepsin B. (B) Progress curves for the inhibition of cathepsin L ($[E_0] = 6.6$ pM, $[S_0] = 2$ nM) with different inhibitor concentrations: (i) $[I_0] = 0.066$ nM, (ii) $[I_0] = 0.132$ nM, and (iii) $[I_0] = 0.264$ nM. (+) Experimental points; (solid lines) theoretical curves. (C) Dependence of $k$ for the interaction between cathepsin L and the li fragment, on substrate concentration ($[E_0] = 0.064$ nM, $[I_0] = 0.64$ nM), and (D), on inhibitor concentration ($[E_0] = 6.6$ pM, $[S_0] = 2$ nM). The linearity of the plot in C indicates the competitive nature of the inhibition.

**Figure 4.** Formation of the li fragment–cathepsin L complex in vitro. Electrophoresis of proteins on a 12% polyacrylamide gel at pH 4.5 under native conditions. Samples: (lane 1) the complex as isolated; (lane 2) cathepsin L and the li fragment mixed together in the presence of 8 mM DTE 20 min before electrophoresis; (lane 3) the li fragment; and (lane 4) cathepsin L. The complex formed in vitro and excess li are seen as separate bands in lane 2.
endosomal compartments (24). p41 would, therefore, enhance the presentation of immunogenic peptides that are generated only after extensive proteolysis of the native antigen. This would be possible only in highly acidified late endosomal vesicles, rich in proteases. The putative transport signal would presumably be encoded in AS. From another observation that the half-life of p41 in the endosomal compartments is longer than that of p31, it has been proposed that p41 might be more resistant to proteolysis relative to p31 (38). This would enable p41 to penetrate more deeply into the endosomal system, and to survive in these more proteolytically active compartments. In addition, it has been reported that in vitro digestion of ovalbumin by cathepsins B and L destroyed its immunogenic epitope, which is recognized in vivo by specific CD4+ T cells (41). It has been proposed that in APCs, mechanisms must exist for the protection of epitopes from destructive proteolysis in processing compartments.

Our finding, that AS exhibits strong inhibitory activity against cathepsin L, illuminates these observations from an unexpected point of view. Since the class II–binding region of Ii presumably involves only sequences shared by both p31 and p41 forms of Ii, it is highly likely that the AS region is not involved in this interaction. This leads to the possibility that the inhibitory site for cathepsin L is available while p41 is still associated with the class II molecule. AS, the additional segment of 64 amino acids, unique to p41, would therefore encode the domain capable of protecting class II–Ii complexes and antigenic epitopes from undesired proteolytic action of one of the most powerful lysosomal proteases, cathepsin L, enabling them to survive in a harmful digestive milieu.

It is interesting that AS shares significant homology with 10 repetitive type 1 sequences of thyroglobulin, which is also targeted to lysosomes where, under conditions of limited proteolysis, it is processed to two thyroid hormones (21). For some other proteins, such as nidogen (42) and saxiphilin (43), insertions of homologous domains have been reported, showing that versions of this cysteine-rich structural motif occur in a variety of proteins, differing in function.

The hypothesis of simultaneous association of class II, p41, and cathepsin L is the most persuasive, but not the only possible explanation of our finding. It is possible that p41 is first proteolytically degraded, yielding a product that binds to cathepsin L and inhibits its activity. In this case, p41 would still regulate endosomal proteolytic activity during antigen processing and presentation. The level of regulator would depend on the relative proportions of the two splicing pathways that result in the two forms of Ii. It is worth noting that all studies on Ii degradation have been carried out either on p31 or on the mixture p31/p41 (3, 10–14). No data are available solely for p41 processing, which would be helpful in evaluating both aforementioned hypotheses.

It is also interesting that cathepsin B, which is thought to play a major role in antigen and Ii processing (11–14, 44), is not inhibited by the isolated Ii fragment. It has been reported that in rat macrophages under certain conditions, cathepsins B and L were found in different endosomal/lysosomal subpopulations (45), which would allow different roles for these two proteases. But even if they are not separately located, the specificity of inhibition shown in this paper would still strongly suggest that these two enzymes play different roles in class II–restricted antigen presentation.

The study presented here indicates that the biological role of Ii is not limited to the intensively studied functions connected with its association with MHC class II dimers. The novel and unexpected fact that the p41 form of Ii yields a peptide that is a very potent inhibitor of cathepsin L is worthy of further investigation. The future task is to define the precise site and mechanism of complex formation, as well as its genuine function within the cell.

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