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

[1]*Abbreviations used in this paper:* AS, additional segment of p41; CPI, cysteine protease inhibitor; DTE, dithioerithritol; ER, endoplasmic reticulum; Ii, invariant chain; k, pseudo first order rate constant; k_{ass}, second order rate constant for complex formation; k_{diss}, dissociation rate constant; K_i, equilibrium inhibition constant; K_m, Michaelis constant; -MCA, 4-methyl-7-coumarylamide; v_s, steady-state velocity; v_z, initial velocity; Z-, benzyloxycarbonyl.

This paper is dedicated to Professor Hans Fritz on the occasion of his 60th birthday.

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by proteolytic enzymes in the endocytic pathway, and subsequent competition among the resulting peptides leads to class II selection of peptides with the highest affinities (16). Little is known about the specific proteases responsible for the generation of immunogenic peptides, but it is very likely that they are antigen and class II allele dependent (15).

Endosomes comprise structurally and functionally distinct compartments, and the actual vesicles involved in Ii cleavage and antigen degradation are as yet undefined. Recent studies (17) have indicated that both class II molecules and Ii are introduced into the endocytic pathway at the level of the early endosomes. Other reports (18, 19) have characterized endosome-related organelles, distinct from lysosomes and from both early and late endosomes, to which class II molecules are transported on their way to the plasma membrane. They arrive with an associated Ii, and leave the compartment devoid of it, but loaded with peptides (19).

Ii exists in humans in two alternatively spliced forms, p31 (comprises p31 and p33, generated by alternative initiation of translation) and p41 (comprises p41 and p43), the latter containing an additional 64-amino acid sequence in the lumenal domain (20). The additional segment (AS) is cysteine rich (20), and shares significant homology with the type 1 repetitive sequence of thyroglobulin (21). The p31 and p41 forms are naturally occurring and are expressed at relative levels dependent on cell type, the proportion of p41 in relation to p31 varying from 9% in B lymphocytes to 43% in epidermal Langerhans cells (22).

An interesting feature of Ii is that its various functions are associated with different functional regions contained in the Ii polypeptide. The endosomal localization signal can be attributed to the NH_2-terminal cytoplasmic and transmembrane domain (8, 9); class II association is mediated by amino acids encoded by exon 3 (23); antigen presentation can be enhanced by the p41 form of Ii (24, 25); and T cell interaction can be mediated by the chondroitin sulfate form of Ii (26).

We have previously shown that a noncovalent complex of cathepsin L and the fragment derived from the p41 form of Ii can be isolated from human kidney (27). We now report on the structural organization of the complex, and on the inhibitory effect of the Ii fragment on cathepsin L, which is the most potent lysosomal cysteine protease.

Materials and Methods

Enzymes and Inhibitors. The complex of cathepsin L with the Ii fragment was isolated from human kidney (27). The Ii fragment was separated 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).

Antibodies. Polyclonal antibodies against the Ii fragment were raised in rabbit and purified by affinity chromatography on protein A–Sepharose (Pharmacia, Uppsala, Sweden).

COOH-terminal Sequence Analysis. Ii fragment was reduced with 2-mercaptoethanol, alkylated with iodoacetic 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).

HPLC. The components of the complex were separated by HPLC (Milton Roy Co., Riviera Beach, FL) on a ChromSpher C8 column (Chrompack Int., Middelburg, The Netherlands) equilibrated with 0.1% TFA, and eluted with a linear gradient of acetonitrile (0–70%) in the starting solution.

Affinity Chromatography on Con A–Sepharose. The Ii fragment (a mixture of forms) was applied to Con A–Sepharose equilibrated with 0.1 M sodium acetate buffer, pH 6.0, containing 1 M NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, and 1 mM $MnCl_2$. Unbound proteins were washed off with the same buffer, and bound molecules were eluted with 0.2 M methyl-α-D-glucopyranoside in the buffer. Unbound and bound fractions were concentrated, dialyzed, and subjected to SDS-PAGE.

SDS-PAGE. Electrophoresis was performed using a PhastSystem (Pharmacia). Samples and molecular weight markers ranging from 14,400 to 94,000 M_r (Pharmacia) were run in the presence of 5% SDS on an 8–25%-gradient polyacrylamide gel (0.45 × 43 × 50 mm). For reduction, 10% 2-mercaptoethanol was added to samples. After electrophoresis, the gel was stained with Coomassie blue.

Native PAGE. Electrophoretic separation was carried out under native conditions in 8 × 8-cm slabs of 12% homogeneous polyacrylamide gel in a buffer, pH 4.5, containing 0.08 M β-alanine and 0.04 M acetic acid. The complex formed in vitro was made by mixing 10 μM of cathepsin L and 20 μM of the Ii fragment in 20 mM sodium acetate buffer, pH 5.5, in the presence of 8 mM dithioerithritol (DTE) 20 min before electrophoresis.

Immunoblotting. Proteins were separated by SDS-PAGE as described above, and transferred to Imobilon polyvinylidene difluoride transfer membrane (Millipore Corp., Bedford, MA) using passive diffusion. After immersion of the membrane in 0.1 M DTE for 5 min, nonspecific binding was blocked by preincubating the blot in PBS containing 4% BSA. After washing, the blot was first incubated with primary anti-Ii fragment antibody, washed again, and incubated with peroxidase-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch Labs., Inc., West Grove, PA). The blot was immunostained with 3-amino-9-ethylcarbazole (Sigma Chemical Co.) and hydrogen peroxide in 50 mM sodium acetate buffer, pH 5.0.

Enzyme Assays. Cathepsin L, cathepsin H, and papain activities were measured as described below. All the other enzymes were assayed following standard procedures using the substrate benzyloxycarbonyl (Z)-Phe-Arg-4-methyl-7-coumarylamide (-MCA) for cathepsins B and S, Gly-Phe-4-methoxy-β-naphthylamide for cathepsin C, hemoglobin for cathepsin D, Nα-benzoyl-DL-arginine-*p*-nitroanilide for trypsin, and L-Leu-β-naphthylamide for aminopeptidase M. Inhibitory effects of the Ii 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 cysteine protease inhibitor (CPI), Ep-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 continuously at 410 nm for 1 min with a Lambda 3 spectrofluorimeter (Perkin-Elmer, Beaconsfield, UK). The velocity was calculated using linear regression analysis, and the active concentration of cathepsin L determined from the plot of velocity against molar ratio of initial inhibitor and enzyme concentrations ($[I_o]/[E_o]$). All the other enzymes were active-site titrated in the same way using the corresponding substrates and inhibitors. The Ii fragment was titrated with active-site–titrated cathepsin L.

Inhibition Kinetics of Cathepsin L. All experiments were carried out 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 excitation 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, preactivated with 8 mM DTE) to 2.450 ml of the buffer containing the substrate (Z-Phe-Arg-MCA) and the inhibitor (the Ii fragment). Six different inhibitor concentrations and four different substrate concentrations were used. All progress curves were fitted by nonlinear regression analysis to the integrated rate equation (33): $P = v_s t + (v_z - v_s)(1 - e^{-kt})/k$, where P represents the product concentration, v_z and v_s are the initial and steady-state velocities, respectively, k is the observed pseudo first order rate constant, and t is time. The second order rate constant for complex formation, k_{ass}, was calculated from the slope of the plot k versus $[I_o]$ (slope $= k_{ass}/(1 + [S_o]/K_m)$), where K_m is the Michaelis constant, using $[S_o] = K_m = 2$ μM. The dissociation rate constant, k_{diss}, was obtained from individual measurements $k_{diss} = kv_s/v_z$, and the equilibrium inhibition constant, or K_i value, was calculated from $K_i = k_{diss}/k_{ass}$.

Inhibition Kinetics of Cathepsin H and Papain. Activities of cathepsin H and papain in the presence of the Ii 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, preactivated 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 concentrations. Since v_z could not be determined due to very fast reactions, only v_s was recorded and K_i values were determined from Dixon plots in the form $1/v_s$ versus $[I_o]$ at different substrate concentrations (34).

Results

The Ii Fragment–Cathepsin L Complex. The NH_2- and COOH-terminal sequences of the peptide fragment, separated from the complex, as well as its total amino acid composition, were determined, showing it to originate from the p41 form of Ii (27, and Fig. 1). It is identical to AS encoded by an alternatively spliced exon (20), which is found only in p41, and not in the major form of Ii, p31 (Fig. 1). The fragment consists of 65 amino acid residues, starting at residue 194 and ending at residue 258 (p41 numbering). The Ii fragment was eluted from the complex as a mixture of either two or three forms (number and proportions depended on details in purification procedure) that differed slightly in their molecular masses as revealed by immunoblotting (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-Ii fragment Ab (Fig. 2 *B*). In the presence of SDS (Fig. 2, *A* and *B*, lane *1*) the Ii fragment remained associated with the enzyme, as revealed by anti-Ii immunostaining (Fig. 2 *B*, lane *1*). The complex migrated as a series of bands, a consequence of the different forms of Ii fragment, with apparent molecular mass of ~32 kD. After incubating with SDS at 100°C, the complex dissociated, giving rise to new protein

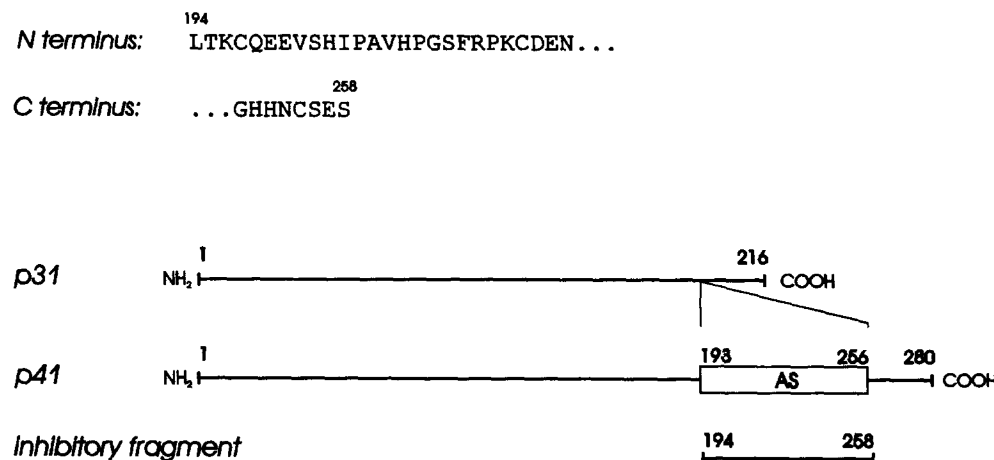

Figure 1. NH_2- (27) and COOH-terminal amino acid sequences of the fragment that binds to cathepsin L, a schematic representation of p31 and p41 (20), and the position of the inhibitory fragment within p41 (*horizontal bar*). (*AS*) Additional segment derived from alternative splicing, unique to p41. Numbering is according to p41.

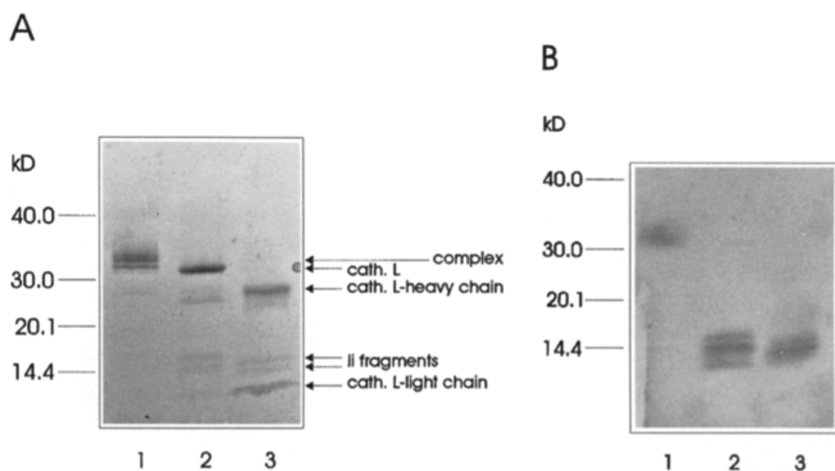

Figure 2. Structural organization of the Ii fragment–cathepsin L complex. (*A*) 8–25% SDS-PAGE of the complex stained with Coomassie blue. (*B*) Immunoblot of the equivalent gel stained with the anti-Ii fragment antibody. Samples: (lane *1*) nonreduced complex; (lane *2*) nonreduced complex exposed to 100°C in the presence of SDS for 5 min; and (lane *3*) reduced complex exposed to 100°C in the presence of SDS for 5 min.

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 the same source, 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, on substrate concentration demonstrated a competitive inhibition mechanism (Fig. 3 *C*), and its linear dependence on inhibitor concentration allowed us to calculate k_{ass} (Fig. 3 *D*). k_{diss}, was measured directly, and K_i was calculated from $K_i = k_{diss}/k_{ass}$. It is evident that the Ii fragment binds very tightly ($K_i = 1.7 \times 10^{-12}$ M) and rapidly ($k_{ass} = 4.6 \times 10^7\ M^{-1}s^{-1}$) 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 ($v_z/v_s - 1$) versus $[I_o]$ led to $K_i = 1.1 \times 10^{-12}$ M, in good agreement with the value calculated from k_{ass} and k_{diss}.

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.

Inhibition Kinetics of Cathepsin H and Papain. Under the experimental conditions used, cathepsin H and papain inhibition reactions were too fast to monitor the initial rates (v_z). Therefore, only v_s were recorded (data not shown), and the K_i values were calculated by the equilibrium Dixon method (34). Binding of the Ii fragment to cathepsin H ($K_i = 5.3 \times 10^{-9}$ M) and to papain ($K_i = 1.4 \times 10^{-9}$ M) was shown to be much weaker than binding to cathepsin L (Fig. 3 *A*).

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 hydrolases are present all along this endocytic path-

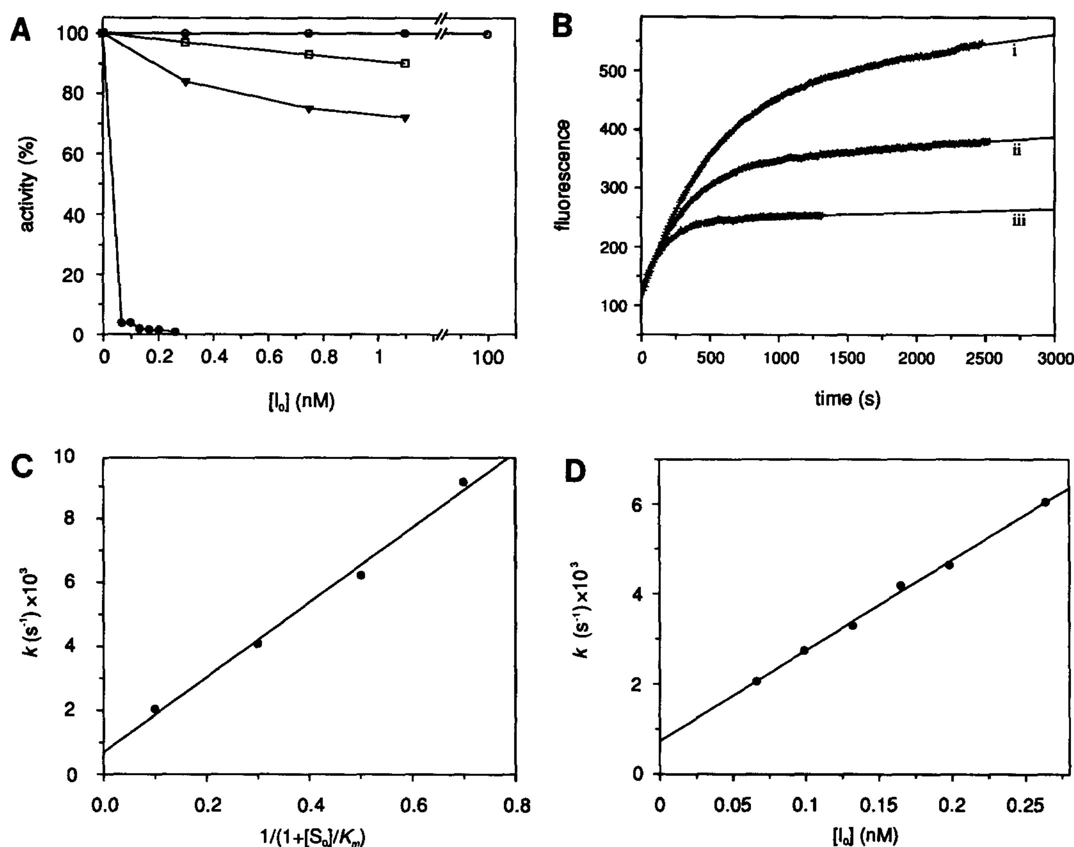

Figure 3. Inhibition kinetics of the p41 fragment. (*A*) Inhibition of various proteases by the p41 fragment. The activities of cathepsin L ($[E_o]$ = 7 pM, *filled circles*), papain ($[E_o]$ = 30 pM, *triangles*), cathepsin H ($[E_o]$ = 23 pM, *squares*) and cathepsin B ($[E_o]$ = 100 pM, *open circles*)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

way (39). Second, the value of K_i is sufficiently low (K_i ~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 Ii fragment is significantly weaker (K_i ~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 Ii 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

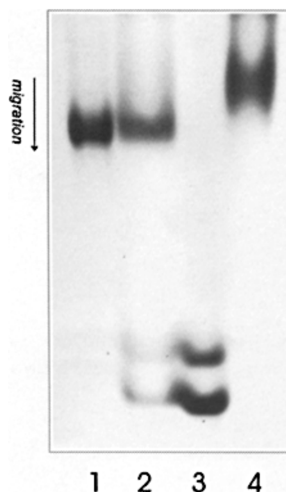

Figure 4. Formation of the Ii fragment–cathepsin L complex in vitro. Electrophoresis of proteins on a 12% polyacrylamide gel at pH 4.5 under native conditions. Samples: (lane *1*) the complex as isolated; (lane *2*) cathepsin L and the Ii fragment mixed together in the presence of 8 mM DTE 20 min before electrophoresis; (lane *3*) the Ii fragment; and (lane *4*) cathepsin L. The complex formed in vitro and excess Ii 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 it 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 hypothesis.

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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References

1. Germain, R.N., and D.H. Margulies. 1993. The biochemistry and cell biology of antigen processing and presentation. *Annu. Rev. Immunol.* 11:403–450.
2. Jones, P., D. Murphy, D. Hewgill, and H. McDevitt. 1979. Detection of a common polypeptide chain in I-A and I-E subregion immunoprecipitates. *Mol. Immunol.* 16:51–60.
3. Blum, J.S., and P. Cresswell. 1988. Role for intracellular proteases in the processing and transport of class II HLA antigens. *Proc. Natl. Acad. Sci. USA.* 85:3975–3979.
4. Neefjes, J., V. Stollorz, P. Peters, H. Geuze, and H. Ploegh. 1990. The biosynthetic pathway of MHC class II but not class I molecules intersects the endocytic route. *Cell.* 61:171–183.

5. Peterson, M., and J. Miller. 1990. Invariant chain influences the immunological recognition of MHC class II molecules. *Nature (Lond.).* 345:172–174.
6. Anderson, M.S., and J. Miller. 1992. Invariant chain can function as a chaperone protein for class II MHC molecules. *Proc. Natl. Acad. Sci. USA.* 89:2282–2286.
7. Roche, P., and P. Cresswell. 1990. Invariant chain association with HLA-DR molecules inhibits immunogenic peptide binding. *Nature (Lond.).* 345:615–618.
8. Bakke, O., and B. Dobberstein. 1990. MHC class II-associated invariant chain contains a sorting signal for endosomal compartments. *Cell.* 63:707–716.
9. Odorizzi, C.G., I.S. Trowbridge, L. Xue, C.R. Hopkins, C.D. Davis, and J.F. Collawn. 1994. Sorting signals in the MHC class II Ii cytoplasmic tail and transmembrane region determine trafficking to an endocytic processing compartment. *J. Cell Biol.* 126:317–330.
10. Pieters, J., H. Horstmann, O. Bakke, G. Griffiths, and J. Lipp. 1991. Intracellular transport and localization of MHC class II molecules and associated invariant chain. *J. Cell Biol.* 115: 1213–1223.
11. Morton, P.A., M.L. Zacheis, K.S. Giacoletto, J.A. Manning, and B.D. Schwartz. 1995. Delivery of nascent MHC class II–invariant chain complexes to lysosomal compartments and proteolysis of invariant chain by cysteine proteases precedes peptide binding in B-lymphoblastoid cells. *J. Immunol.* 154: 137–150.
12. Roche, P.A., and P. Cresswell. 1991. Proteolysis of the class II-associated invariant chain generates a peptide binding site in intracellular HLA-DR molecules. *Proc. Natl. Acad. Sci. USA.* 88:3150–3154.
13. Reyes, V.E., S. Lu, and R.E. Humphreys. 1991. Cathepsin B cleavage of Ii from class II MHC α- and β-chains. *J. Immunol.* 146:3877–3880.
14. Marić, M.A., M.D. Taylor, and J.S. Blum. 1994. Endosomal aspartic proteinases are required for invariant-chain processing. *Proc. Natl. Acad. Sci. USA.* 91:2171–2175.
15. Ojcius, D.M., L. Gapin, J.M. Kanellopoulos, and P. Kourilsky. 1994. Is antigen processing guided by MHC molecules? *FASEB (Fed. Am. Soc. Exp. Biol.) J.* 8:974–978.
16. Adorini, L., S. Muller, and F. Cardinaux. 1988. In vivo competition between self peptides and foreign antigens in T-cell activation. *Nature (Lond.).* 334:623–625.
17. Gorvel, J.P., J.M. Escola, E. Stang, and O. Bakke. 1995. Invariant chain induces a delayed transport from early to late endosomes. *J. Biol. Chem.* 270:2741–2746.
18. Peters, P.J., J.J. Neefjes, V. Oorschot, H.L. Ploegh, and H.J. Geuze. 1991. Segregation of MHC class II molecules from MHC class I molecules in the Golgi complex for transport to lysosomal compartments. *Nature (Lond.).* 349:669–676.
19. Tulp, A., D. Verwoerd, B. Dobberstein, H.L. Ploegh, and J. Pieters. 1994. Isolation and characterization of the intracellular MHC class II compartment. *Nature (Lond.).* 349:120–126.
20. Strubin, M., C. Berte, and B. Mach. 1986. Alternative splicing and alternative initiation of translation explain the four forms of the Ia antigen-associated invariant chain. *EMBO (Eur. Mol. Biol. Organ.) J.* 5:3483–3488.
21. Malthiéry, Y., and S. Lissitzky. 1987. Primary structure of human thyroglobulin deduced from the sequence of its 8448-base complementary DNA. *Eur. J. Biochem.* 165:491–498.
22. Kämpgen, E., N. Koch, F. Koch, P. Stöger, C. Heufler, G. Schuler, and N. Romani. 1991. Class II MHC molecules of murine dendritic cells: synthesis, sialylation of invariant chain, and antigen processing capacity are down-regulated upon culture. *Proc. Natl. Acad. Sci. USA.* 88:3014–3018.
23. Freisewinkel, I.M., K. Schneck, and N. Koch. 1993. The segment of invariant chain that is critical for association with MHC class II molecules contains the sequence of peptide eluted from class II polypeptides. *Proc. Natl. Acad. Sci. USA.* 90:9703–9706.
24. Peterson, M., and J. Miller. 1992. Antigen presentation enhanced by the alternatively spliced invariant chain gene product p41. *Nature (Lond.).* 357:596–598.
25. Anderson, M.S., K. Swier, L. Arneson, and J. Miller. 1993. Enhanced antigen presentation in the absence of the invariant chain endosomal localization signal. *J. Exp. Med.* 178:1959–1969.
26. Naujokas, M.F., M. Morin, M.S. Anderson, M. Peterson, and J. Miller. 1993. The chondroitin sulfate form of invariant chain can enhance stimulation of T cell responses through interaction with CD44. *Cell.* 74:257–268.
27. Ogrinc, T., I. Dolenc, A. Ritonja, and V. Turk. 1993. Purification of the complex of cathepsin L and the MHC class II-associated invariant chain fragment from human kidney. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 336:555–559.
28. Turk, B., I. Dolenc, V. Turk, and J.G. Bieth. 1993. Kinetics of the pH-induced inactivation of human cathepsin L. *Biochemistry.* 32:375–380.
29. Turk, V., and W. Bode. 1993. Lysosomal cysteine proteinases and their inhibitors cystatins. *In* Innovations in Proteases and Their Inhibitors. Walter de Gruyter, Berlin. 161–178.
30. Dolenc, I., B. Turk, G. Pungerčič, A. Ritonja, and V. Turk. 1995. Oligomeric structure and substrate induced inhibition of human cathepsin C. *J. Biol. Chem.* 270:21626–21631.
31. Smith, R., and V. Turk. 1974. Cathepsin D: rapid isolation by affinity chromatography on haemoglobin-agarose resin. *Eur. J. Biochem.* 48:245–254.
32. Morrison, J.F., and C.T. Walsh. 1988. The behavior and significance of slow-binding enzyme inhibitors. *Adv. Enzymol. Relat. Areas Mol. Biol.* 61:201–301.
33. Morrison, J.F. 1982. The slow-binding and slow, tight-binding inhibition of enzyme-catalysed reactions. *Trends Biochem. Sci.* 7:102–105.
34. Dixon, M., and E. Webb. 1979. Enzyme inhibition and activation. *In* The Enzymes. Academic Press, New York. 350–351.
35. Katunuma, N., H. Kakegawa, Y. Matsunaga, and T. Saibara. 1994. Immunological significances of invariant chain from the aspect of its structural homology with the cystatin superfamily. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 349:265–269.
36. Guagliardi, L.E., B. Koppelman, J.S. Blum, M.S. Marks, P. Cresswell, and F.M. Brodsky. 1990. Co-localization of molecules involved in antigen processing and presentation in an early endocytic compartment. *Nature (Lond.).* 343:133–139.
37. Romagnoli, P., C. Layet, J. Yewdell, O. Bakke, and R.N. Germain. 1993. Relationship between invariant chain expression and MHC class II transport into early and late endocytic compartments. *J. Exp. Med.* 177:583–596.
38. Arunachalam, B., C.A. Lamb, and P. Cresswell. 1994. Transport properties of free and MHC class II-associated oligomers containing different isoforms of human invariant chain. *Int. Immunol.* 6:439–451.
39. Ludwig, T., G. Griffiths, and B. Hoflack. 1991. Distribution of newly synthesized lysosomal enzymes in the endocytic pathway of normal rat kidney cells. *J. Cell Biol.* 115:1561–1572.

40. Turk, V., and W. Bode. 1991. The cystatins: protein inhibitors of cysteine proteinases. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 285:213–219.
41. Rodriguez, G.M., and S. Diment. 1995. Destructive proteolysis by cysteine proteases in antigen presentation of ovalbumin. *Eur. J. Immunol.* 25:1823–1827.
42. Nakae, H., M. Sugano, Y. Ishimori, T. Endo, and T. Obinata. 1993. Ascidian entactin/nidogen. Implication of evolution by shuffling two kinds of cysteine-rich motifs. *Eur. J. Biochem.* 213:11–19.
43. Morabito, M.A., and E. Moczydlowski. 1994. Molecular cloning of bullfrog saxiphilin: a unique relative of the transferrin family that binds saxitoxin. *Proc. Natl. Acad. Sci. USA.* 91:2478–2482.
44. Matsunaga, Y., T. Saibara, H. Kido, and N. Katunuma. 1993. Participation of cathepsin B in processing of antigen presentation to MHC class II. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 324:325–330.
45. Muno, D., N. Sutoh, T. Watanabe, Y. Uchiyama, and E. Kominami. 1990. Effect of metabolic alterations on the density and the contents of cathepsins B, H and L of lysosomes in rat macrophages. *Eur. J. Biochem.* 191:91–98.