Inhibitory Fragment from the p41 Form of Invariant Chain Can Regulate Activity of Cysteine Cathepsins in Antigen Presentation*

Marko Mihelčič, Andreja Dobersˇek, Gregor Guˇncar, and Duˇsan Turk
From the Department of Biochemistry and Molecular and Structural Biology, J. Stefan Institute, Ljubljana SI 1000, Slovenia

Cysteine cathepsins play an indispensable role in proteolytic processing of the major histocompatibility complex class II-associated invariant chain (Ii) and foreign antigens in a number of antigen presenting cells. Previously it was shown that a fragment of 64 residues present in the p41 form of the Ii (p41 fragment) selectively inhibits the endopeptidase cathepsin L, whereas the activity of cathepsin S remains unaffected. Comparison of structures indicated that the selectivity of interactions between cysteine cathepsins and the p41 fragment is far from being understood and requires further investigation. The p41 fragment has now been shown also to inhibit human cathepsins V, K, and F (also, presumably, O) and mouse cathepsin L with Ki values in the low nanomolar range. These Ki values are sufficiently low to ensure complex formation at physiological concentrations. In addition we have found that the p41 fragment can inhibit cathepsin S too. These findings suggest that regulation of the proteolytic activity of most of the cysteine cathepsins by the p41 fragment is an important and widespread control mechanism of antigen presentation.

In the processes of adaptive immunity, antigenic proteins are cleaved to peptides, some of which are loaded into the binding cleft of MHC2 class II molecules for presentation on the surface of professional antigen-presenting cells (APCs) (1, 2). The degradation of antigenic proteins takes place in the endocytic compartments (endosomes and lysosomes) rich in proteolytic activity. The current list of protein-degrading enzymes in these compartments includes the group of cysteine cathepsins, aspartaginyl endopeptidase, the aspartic proteases cathepsins D and E, and thiol reductase GILT (3–5). This work is focused on cysteine cathepsins. Studies on gene knock-out mice showed that the pattern of antigenic peptide generation is not affected by the absence of cathepsins L and S (6), indicating redundant and overlapping roles within the group of cysteine cathepsins, although in certain cases specific roles in the degradation of antigens have been assigned to particular proteases (7, 8).

It is thought that the activity of proteases in the endocytic mixture is also regulated by protein inhibitors (4). A number of different inhibitors of cysteine cathepsins, including cystatins C and F and stefins A and B, have so far been localized in different types of APCs (9); however, direct and precise evidence regarding their involvement in the regulation of endosomal protease activity in APCs is still lacking (10, 11). Even more intriguing and less well investigated is the role of the inhibitory fragment of the p41 form of Ii (p41 fragment).

Ii associates with the α and β chains of MHC class II molecules in the endoplasmic reticulum and is responsible for their proper folding and trafficking to endosomes (12), where the MHC class II molecules are freed from li by proteolytic processing of li. li exists in mouse in two (and in human, four) alternately spliced forms, p31 and p41, distinguished by an additional 64-amino acid segment at the C terminus of p41 (13). The expression ratio between p31 and p41 forms of li depends on the particular type of APC and varies from 10% of the p41 form in B cells, 20–30% in macrophages and dendritic cells, up to 40% in Langerhans cells (14). The two forms of li have identical impact on the folding and trafficking of MHC class II molecules; however, they differ markedly in their functional effects on the presentation of antigens to T cells.

The p41 form can impose the degradation pattern to the p31 form when co-expressed in the same cell types, and its presence enhances the antigen presentation (15). The p41 form is also more resistant to endosomal proteolysis than the p31 form (16). These findings were explained in part when the spliced in fragment from the p41 form (p41 fragment) was found bound to cathepsin L (17, 18). At the time, however, it was thought that the fragment is an inhibitor specific for cathepsin L, because the activities of cathepsins S and B were not affected. Cathepsin L was therefore designated as the target protease of the p41 fragment in the regulation of endosomal protease activity. However, the more recently discovered cysteine cathepsins V, K, and F could also be potential targets of the p41 fragment, because they have been localized in the cells responsible for the endosomal immune response (19–26). Their interactions with the p41 fragment therefore merit investigation.

We show here that the p41 fragment has the ability to regulate and block the activity of a group of cysteine cathepsins in addition to cathepsin L. These findings suggest that the p41 form of li plays a wider role than previously thought by regulating the proteolytic activity of endocytic antigen presentation.

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1 To whom correspondence should be addressed: Jamova 39, Ljubljana, SI-1000, Slovenia. Tel.: 386-1-477-3857; Fax: 386-1-477-3984; E-mail: dusan.turk@ijs.si.

2 The abbreviations used are: MHC, major histocompatibility complex; PDB, Protein Data Bank; APC, antigen-presenting cell; DTT, dithiothreitol.
Inhibitory Properties of the p41 Fragment

EXPERIMENTAL PROCEDURES

The cDNA clones for mouse cathepsins L and S, human cathepsin V, and human and mouse p41 invariant chains were obtained at Deutsches Ressourcenzentrum fuer Genomforschung GmbH.

Expression of Human and Mouse p41 Fragments—The DNA transcripts encoding human and mouse p41 fragments were amplified from human and mouse full-length p41 invariant chain cDNAs by PCR and cloned into the bacterial expression plasmid pET-22b(+) in-frame with the N-terminal pelB leader sequence and C-terminal His<sub>6</sub> affinity tag sequence. The recombinant plasmids were transformed into Escherichia coli strain BL21(DE3) and grown at 37 °C with constant shaking in recombinant plasmids were transformed into Escherichia coli strain BL21(DE3) and grown at 37 °C with constant shaking in LB medium containing 100 μg/ml ampicillin and 1% glucose to an absorbance at 600 nm of 1.5–2.0. Expression of the p41 fragments was induced by adding isopropyl 1-thio-β-d-galactopyranoside to a final concentration of 1 mM. After 2 h of induction, the cells were harvested, and the bacterial periplasmic fraction was isolated according to the pET system manual (Novagen). p41 fragments were purified from clear periplasmic supernatants using nickel affinity chromatography on HisTrap HP column (GE Healthcare) and gel filtration on Superdex-75 HR column (GE Healthcare) according to the manufacturer’s instructions.

Expression of Cysteine Cathepsins—cDNAs for human and mouse procathepsins L and S and human procathepsin V were amplified by PCR. Site-specific mutations on the cover loops of human cathepsins L and S, namely human cathepsin S R141G and S157D/Q160E/N161E/N163E mutants and the human cathepsin L G139R mutant, were introduced as described (27). PCR products were cloned into a Pichia pastoris expression vector pPIC9K (Invitrogen) previously modified by insertion of a nucleotide sequence encoding a His<sub>6</sub> affinity tag between the SmaI and EcoRI recognition sites. Recombinant plasmids were electroporated into P. pastoris strain GS115 (Invitrogen). Recombinant clones were selected according to the Pichia Expression kit manual (Invitrogen). The clone with the highest protein expression level was grown overnight in BMGY medium at 30 °C with constant shaking. The next day the cells were transferred into BMMY medium containing 1% methanol, and growth was continued under the same conditions. Every 24 h a fresh supply of methanol was added to a final concentration of 1%. After 4 days of expression, yeast cells were removed by centrifugation, and the crude expression medium was induced by adding isopropyl 1-thio-D-galactopyranoside to a final concentration of 1 mM. After 2 h of induction, the cells were harvested, and the bacterial periplasmic fraction was isolated according to the pET system manual (Novagen). p41 fragments were purified from clear periplasmic supernatants using nickel affinity chromatography on HisTrap HP column (GE Healthcare) and gel filtration on Superdex-75 HR column (GE Healthcare) according to the manufacturer’s instructions.

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Inhibition Kinetics—All kinetic experiments were performed at 25 °C using the following assay buffers: 0.1 M potassium phosphate, 1 mM EDTA, 5 mM DTT, 0.1% (w/v) PEG, pH 6.0, for cathepsins S and F; and 0.1 M sodium acetate, 1 mM EDTA, 5 mM DTT, 0.1% (w/v) PEG, pH 5.5, for cathepsins L, V, K, and X. For cathepsins L, S, V, K, and F, benzoxycarbonyl-Phe-Arg-7-methylcoumarin was used as a substrate, and the release of 7-methylcoumarin was monitored at excitation and emission wavelengths of 370 and 460 nm, respectively, in a C-61 spectrophotometer (Photon Technology International). Cathepsin X activity was assayed with dinitrophenyl-Gly-Phe-Phe-Trp substrate, and tryptophan liberation was detected at excitation and emission wavelengths of 280 and 360 nm, respectively. Active concentrations of cathepsins were determined by active site titration, using the irreversible inhibitor E-64, and human and mouse p41 fragments by titration with cathepsin L.

Inhibition experiments were performed under pseudo first-order reaction conditions with inhibitor concentrations at least 10-fold higher than the enzyme concentrations with less than 10% substrate consumption during experiments. Based on previous results (18) we assumed a simple, competitive mechanism of inhibition without a pre-equilibrium step.

For every enzyme-inhibitor pair at least five measurements were performed with increasing concentrations of the p41 fragments mixed with the substrate solution (10 μM final concentration in the reaction buffer) to a final volume of 1900 μl. The reaction was started by the addition of 40 μl of enzyme. Progress curves for inhibition of cathepsins L and V showed an exponential approach to a final linear rate and were fitted by nonlinear regression to the corresponding equation (28).

Association and dissociation rate constants (k<sub>a</sub> and k<sub>d</sub>) for individual enzyme-inhibitor pairs were determined by linear regression analysis as described (29). K<sub>i</sub> values were calculated as K<sub>i</sub> = k<sub>d</sub>/k<sub>a</sub>.

The binding of the p41 fragment to cathepsins S, K, and F was too rapid to allow k<sub>i</sub> values to be determined. Therefore, only K<sub>i</sub> values were determined for these enzyme-inhibitor pairs using the equilibrium method according to Greco and Hakala (30).

The K<sub>i</sub> values were calculated from the steady-state velocities of substrate hydrolysis in the presence of increasing concentrations of inhibitor. The K<sub>i</sub> values were obtained by nonlinear regression analysis.

Molecular Modeling—The three-dimensional models of the human cathepsins O and W and mouse cathepsins L and S were generated by the computer program MODELLER (31) using the cathepsin L crystal structure (PDB entry 1ICF) from the complex with the p41 fragment (32) as the template. The mouse p41 fragment model was generated analogously using the human p41 fragment structure. Hypothetical models of complexes between cysteine cathepsins and the p41 fragments were made by superposition of the crystal structures of human cathepsins S (PDB entry 1GLO (33)), K (PDB entry 1MEM (34)), V (PDB entry 1FHO (35)), and F (PDB entry 1M6D (36)) and the three-dimensional models of other cathepsins to the structure of cathepsin L in complex with the p41 fragment. The position of the model of the mouse p41 fragment was obtained by superimposition on the p41 fragment in the crystal structure of the same complex. The superimposition was carried out by minimization of the sum of squares of the distances between pairs of the model of the mouse p41 fragment.
RESULTS

The p41 fragment fold and its binding mode are known from the crystal structure of the complex of p41 fragment bound to cathepsin L (32). The p41 fragment has a wedge-shaped structure that binds into the active site cleft of the target protease with its narrow edge (Fig. 1A). Its sequence contains numerous charged residues: six negatively (Glu199, Glu200, Asp217, Glu218, Glu243, and Glu257) and nine positively charged (Lys196, His203, His208, Arg213, Lys215, Arg248, Arg250, His252, and His253). All six carboxyl groups, plus the C-terminal carboxyl, are positioned at the top on both sides of the wide part of the wedge, whereas all positive charges except Leu196 are positioned on the lower part (Fig. 1B). The asymmetric distribution of the charged residues indicates a significant dipole moment of the molecule, which suggests its relevance in the binding to target proteases.

Comparison of the crystal structure with the putative complex between the p41 fragment and cathepsin S suggested that the tight binding to cathepsin L ($K_i = 0.0017 \text{ nM}$) and the lack of inhibition of cathepsin S (18) arise from unfavorable electrostatic and steric interactions between the p41 fragment and the loop covering the top of the right domain (Ile136 to Asp136 (cathepsin L numbering) and is further referred to as the cover loop). These interactions are absent in cystatin-like inhibitor binding and were thought to be specific to the p41 fragment (38). The structural comparison points to the cathepsin S residue Arg141, which stands out of the cathepsin S surface in contrast to Gly139 of cathepsin L, being the most perturbing factor of this interaction. Arg141 may clash sterically with the p41 fragment surface, as well as carrying the repulsive positive charge (Fig. 2A).

To verify the relevance of the role of Arg141, cathepsins L and S and

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**FIGURE 1. Structures of human cathepsin L and p41 inhibitory fragment.** A, three-dimensional structure of the complex between human cathepsin L and the p41 fragment (PDB entry 1ICF (27)). The chain traces of cathepsin L and p41 fragment structure are presented as cyan and gray, respectively. The side chain atoms of cathepsin L catalytic residue Cys25 are shown as yellow spheres. The cover loop on the top of cathepsin L right domain, corresponding to the cathepsin L sequence Ile136–Asp136, is shown in orange. B, chain trace of the p41 fragment. Negatively and positively charged residues are shown as red and blue balls and sticks. The asymmetric spatial distribution of six negatively and nine positively charged residues generates a molecular dipole moment. Figures were prepared with MAIN (37) and rendered with RENDER (54).

**FIGURE 2. Interaction of the p41 fragment with human cathepsins L and S.** A, close view of the p41 fragment binding to human cathepsin L (PDB entry 1ICF) and the putative complex with human cathepsin S. Residues important for the interaction are presented as balls and sticks and colored according to their charge as follows: negatively charged residues, red, and positively charged residues, blue. Positively charged residues from the p41 fragment (His208, Arg213, Lys215, and His253) located in close proximity to the cathepsins L and S cover loops are shown as blue balls and sticks. Neutral charged residues on the cathepsin S cover loop, whose positions overlap with cathepsin L negative charges, are shown as green balls and sticks. The figure was prepared with MAIN (37) and rendered with RASTER (54). B, kinetic and equilibrium data for the interaction of the p41 fragment with human cathepsins L and S and their cover loop cross-mutants.

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**TABLE**

| Inhibitory Properties of the p41 Fragment | $k_{cat} \times 10^7 \text{ (M}^{-1}\text{s}^{-1})$ | $k_{on} \times 10^9 \text{ (s}^{-1})$ | $K_i \text{ (M)}$ |
|-----------------------------------------|---------------------------------|---------------------------------|----------------|
| Human cathepsin L                        | 1.7 ± 0.61                      | 9.51 ± 1.03                     | (5.52 ± 2.03) $\times 10^{-2}$ |
| Human cathepsin L $g_{136}$             | 3.5 ± 0.42                      | 35.3 ± 4.0                      | (10.1 ± 1.67) $\times 10^{-2}$ |
| Human cathepsin S                        | nd                             | nd                             | (208 ± 25) $\times 10^6$ |
| Human cathepsin S $g_{141}$             | nd                             | nd                             | (30 ± 0) $\times 10^6$ |
| Human cathepsin S $g_{141}$             | nd                             | nd                             | (0.27 ± 0.05) $\times 10^0$ |

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Structurally equivalent C-α atoms using the program MAIN (37).
their mutants (cathepsin L G139R and cathepsin S R141G) were prepared. As expected, wild type cathepsin L binds the recombinant p41 fragment with high affinity ($K_d = 0.0055 \text{ nM}$) (Fig. 2B). The inhibition constant is in good agreement with previously published data (18); however, in contrast to expectations, a measurable affinity between the p41 fragment and cathepsin S was observed ($K_d = 208 \text{ nM}$). The human cathepsin S R141G mutant exhibited an ~7-fold greater binding affinity for the p41 fragment ($K_d = 30 \text{ nM}$) than native cathepsin S. The reverse substitution G139R in cathepsin L, however, resulted in only a 2-fold decrease in binding affinity ($K_d = 0.01 \text{ nM}$) (Fig. 2B). The arginine side chain thus appears to be flexible enough to escape the steric clashes with the p41 fragment residues, and its repulsive charge is not sufficient to reduce the binding affinity substantially. These data demonstrate that the arginine side chain plays a minor role in determining the total strength of interactions in binding. Hence, the contribution of a single residue is one reason, but not the only reason, for the observed differences in binding. The contribution of a single residue plays a minor role in determining the total strength of interactions, suggesting that the observed differences arise from a group of interactions with the cover loop residues of the enzymes.

As seen in Fig. 2A the cover loop on the R-domain of cathepsin L contains eight residues with negatively charged (Asp$^{139}$, Glu$^{147}$, Glu$^{148}$, Glu$^{153}$, Asp$^{155}$, Glu$^{159}$, Asp$^{160}$, and Asp$^{162}$) and two with positively charged groups (His$^{140}$ and Lys$^{147}$). We assume that the histidine residues are positively charged in the acidic conditions mimicking the endosomal environment.) Of these, Glu$^{141}$, Asp$^{160}$, and Asp$^{162}$ are positioned in the immediate vicinity of the positively charged residues of the p41 fragment, His$^{208}$, Arg$^{213}$, and His$^{253}$, and the slightly higher positioned Lys$^{215}$ (Fig. 2A). In contrast to human cathepsin L, human cathepsin S can offer only two negative charges, Asp$^{129}$ and Glu$^{135}$, whose positions correspond to the cathepsin L residues Glu$^{137}$ and Glu$^{153}$, and three positively charged residues, Arg$^{141}$, His$^{142}$, and Lys$^{149}$, whose positions correspond to cathepsin L residues His$^{140}$, His$^{147}$, and Lys$^{147}$. Because the effects of arginine/glycine substitutions only partially explained the differences in binding of the two enzymes to the p41 fragment, we have tried to transform the cathepsin S cover loop into that of cathepsin L. Although we were unable to express the cathepsin S mutant with all the cathepsin L negatively charged groups introduced, we were successful in expressing the 4-fold mutant (S157D, Q160E, N161E, and N163D). This mutant was inhibited by the p41 fragment with $K_i = 0.27 \text{ nM}$, which is 770 times stronger than the wild type enzyme, demonstrating that the negative charges on the surface of the cover loop indeed play a crucial role in determining the strength of interactions of cathepsins with the p41 fragment.

This finding prompted us to inspect the structures of other available cysteine cathepsins, endopeptidases F, K, and V, and exopeptidase cathepsin X. Comparison of their structures and sequences revealed that the composition of the cover loop of cathepsin V is the most similar to that of cathepsin L, whereas the cover loop of cathepsin F is least similar (Fig. 3, A and B). The cathepsin V cover loop contains a cluster of four negative charges Asp$^{137}$, Glu$^{153}$, Asp$^{161}$, and Asp$^{162}$, two with positively charged groups (His$^{140}$ and Lys$^{147}$, and Lys$^{159}$), Glu$^{153}$, Asp$^{157}$, and Asp$^{162}$ of cathepsin L, and three positively charged residues, His$^{140}$, Lys$^{147}$, and Lys$^{159}$, equivalently positioned to His$^{140}$, Lys$^{147}$, and Glu$^{159}$ of cathepsin L (Fig. 3A). Cathepsin K also has four negatively charged residues Asp$^{152}$, Asp$^{155}$, Glu$^{153}$, and Asp$^{156}$, however, they are positioned differently from those of cathepsin V and lack the aspartate at position Asn$^{161}$ (equipositioned to cathepsin L Asp$^{162}$) and the glutamate at position Ser$^{154}$ corresponding to the Glu$^{153}$ residue of cathepsin L. The two aspartates, 160 and 162, of cathepsin L, which are positioned in the complex structure in the vicinity of the p41 fragment, are neutral asparagines in the cathepsin K sequence. Cathepsin F, however, has only a single negatively charged residue Asp$^{158}$, equivalent to Asp$^{162}$ of cathepsin L, directly involved in interactions with the p41 fragment. Its four positively charged residues Arg$^{155}$, His$^{146}$, Arg$^{152}$ and R155A point away from the p41 fragment and are unlikely to severely disturb the formation of the complex, as in the case of cathepsin S. In addition, the helix of cathepsin F starts a residue earlier by turning right instead of left, making the active site cleft of cathepsin F wider by more than 3 Å than in the other cathepsins. Cathepsin F has actually no equivalent residue to cathepsin L Gly$^{159}$; its sequentially equivalent Pro$^{135}$ side chain coincides with the side chain of His$^{140}$, whereas the Phe$^{142}$ side chain points away from the sequentially equivalent Phe$^{146}$ of cathepsin S. This comparison suggests that cathepsin F can also be inhibited by the p41 fragment. The analysis of the cover loop of carboxypeptidase cathepsin X does not appear relevant because the p41 fragment, like the cystatins (39), would not be able to bind to the active site cleft because of steric clashes with the mini-loop residues positioned inside the active site cleft.

The inhibition constants of the p41 fragment with cathepsins V, K, and F (Table 1) reveal the same order of inhibition as suggested by visual inspection. To our surprise however, cathepsin V, the strongest inhibitor among them, is inhibited by the p41 fragment with a $K_i = 0.0072 \text{ nM}$ almost equal to that of cathepsin L. Cathepsin K inhibition was intermediate ($K_i = 0.02 \text{ nM}$), and the cathepsin F inhibition constant was an additional order of magnitude higher ($K_i = 0.51 \text{ nM}$); however, it was still significant and lower than the $K_i$ value with cathepsin S. Cathepsin X, like exopeptidases cathepsin B and C, was not inhibited at all (18). The similar $K_i$ values for cathepsins L and V suggest that the four acidic residues of cathepsin V are important mediators of the binding affinity of the p41 fragment. The absence of the two negatively charged residues in cathepsin K (Ser$^{154}$ and Asn$^{161}$) and the presence of the two different negatively charged ones (Glu$^{153}$ and Asp$^{159}$) appear to compensate for most of the lost interaction strength. The absence of the negative charges, other than Asp$^{158}$ in cathepsin F, however, results in the $K_i$ being increased by 2 orders of magnitude when compared with cathepsin L.

Comparison of the sequences of the cover loop region of the only two yet uncharacterized cathepsins, O and W (Fig. 3, A and B), suggests that cathepsin O could be inhibited by the p41 fragment with a $K_i$ value comparable with that of cathepsin K. The two (Asp$^{139}$ and Glu$^{159}$) out of three negatively charged residues in the third region (Asp$^{139}$, Asp$^{145}$, and Glu$^{159}$) are presumably in direct contact with the positively charged residues of the p41 fragment. The cover loop of cathepsin W, how-
ever, resembles more the loop of cathepsin S, suggesting that the inhibition of cathepsin W is poor, if measurable at all. This suggestion is only relevant subject to the assumption that the two insertions specific for the cathepsin W sequence do not modify the fold of the cover loop and the active site cleft structure.

FIGURE 3. Structural analysis of p41 inhibitory fragment binding to cysteine cathepsins. A, putative interaction of human p41 fragment (shown at the top as a gray chain trace) with the cover loop residues of cathepsins V, K, F, O, and W (shown at the bottom as an orange chain trace). The attachment of the loop to the body of the enzyme is indicated by the two gray chains. The side chains of the charged residues are shown as ball and stick models in colors corresponding to their charges as follows: blue for positive and red for negative. Neutral cover loop residues that overlap with the position of cathepsin L negatively charged residues are shown in green. B, amino acid sequence alignment of human cathepsins in the cover loop region. Negatively charged residues are colored red and positively charged residues blue. The beginning and end of the cover loop regions, as well as the positions of cathepsin L charges, use cathepsin L numbering.
Inhibitory Properties of the p41 Fragment

Do Mouse and Human Cathepsins and p41 Fragments Behave the Same?—Mouse is the most commonly used animal model to study the immune response mechanisms of mammals. It is therefore important to know to what extent the findings from mouse are applicable to humans. Comparison of human and mouse p41 fragments indicates that they should exhibit the same properties; they both have the same pronounced asymmetric distribution of charges at essentially the same positions (Fig. 4, A and C). Kinetic data indeed show that the mouse p41 fragment inhibits human and mouse cathepsins L with very similar inhibition constants; the $K_i$ values for cathepsins L and S are 0.007 and 85.0 nM, respectively (Table 1).

Cross-inhibition experiments in which interactions were measured for human and mouse cathepsins with p41 fragments gave similar results (Table 1), again confirming that the amino acid differences between the mouse and human p41 fragments do not affect their inhibitory properties. The data do, however, reveal that the sequences of mouse and human proteins have been optimized to fit each other better than across the species. An interesting observation, reflected also in the $K_i$ values, is that the distribution of charges in the cover loop of mouse cathepsin L is more similar to the distribution of charges in the human

| Interaction of human and mouse p41 fragments with cysteine cathepsins |
|---------------------------------|-----------------|-----------------|
| $k_a \times 10^{-7}$ | $k_d \times 10^{-7}$ | $K_i$ |
| Human p41 fragment | Human cathepsin V | 0.66 ± 0.03 | 4.75 ± 0.63 | (7.21 ± 0.03) $\times 10^{-12}$ |
| Human p41 fragment | Human cathepsin K | ND | ND | (90 ± 8) $\times 10^{-12}$ |
| Human p41 fragment | Human cathepsin F | ND | ND | (0.51 ± 0.09) $\times 10^{-9}$ |
| Human p41 fragment | Human cathepsin X | ND | ND | >1000 $\times 10^{-9}$ |
| Mouse p41 fragment | Mouse cathepsin L | 1.13 ± 0.03 | 1.98 ± 0.18 | (1.49 ± 0.09) $\times 10^{-12}$ |
| Mouse p41 fragment | Mouse cathepsin S | ND | ND | (113 ± 8) $\times 10^{-9}$ |

* ND indicates not determined.

**TABLE 1**

**FIGURE 4.** Comparison of p41 inhibitory fragments and cathepsins L and S from different species. A, location of charged amino acids in mouse p41 fragment. Positively and negatively charged residues are presented as blue and red balls and sticks. B, binding of the mouse p41 fragment to the active site of mouse cathepsins L and S. C, sequence alignment of cathepsins L and S cover loops and p41 inhibitory fragments from different species. Negatively charged residues are colored red and positively charged residues blue. Positions of cathepsin L negatively charged residues are numbered.
cathepsin V cover loop sequence than to that in human cathepsin L, indicating that perhaps mouse cathepsin L and the human cathepsin V are the real orthologs and not mouse and human cathepsins L (Fig. 4B). This would be consistent with the suggestion based on the physiological role of cathepsin V (40). Comparison of $K_i$ values for cathepsins S shows that the mouse pair of proteins exhibits a lower $K_i$ value than the human pair. Presumably the difference in $K_i$ is at least in part a consequence of the sequence diversity at position 141, which in the mouse cathepsin S is a serine and in the human enzyme is an arginine (Fig. 4B).

Comparison of the cover loop sequences of cathepsins L and S from various species showed that out of eight negative charges present in the human cathepsin L, mouse cathepsin L retains three negatively charged residues (Asp$^{136}$, Glu$^{154}$, and Asp$^{162}$), whereas the rat enzyme sequence in addition contains Asp$^{160}$, and the sequences of bovine, sheep, and porcine cathepsins L contain Asp$^{152}$ as well as Asp$^{160}$, which makes their cover loops closer to human cathepsin V than to L (Fig. 4C). Rat cathepsin S has two more negatively charged residues and one less positively charged residue than the human ortholog, suggesting that in rats the p41 fragment could seriously interfere with its activity, whereas the bovine cover loop appears similar to that of the human. This overview of the cover loop sequences suggests that a similar but not equivalent interaction behavior of the p41 fragment is present in human and mouse and probably in a variety of other mammals. Further investigations may deliver new surprises, as demonstrated by the stefin A studies that revealed that the inhibitory properties of human and mouse stefins A differ markedly (41). In contrast to the human stefins, mouse stefins A differentiate between endo- and exocysteine cathepsins.

**DISCUSSION**

*How Can the p41 Fragment Regulate the Processing of Ii and/or Antigens?*—It has been known for some time that the presence of the p41 form of Ii enhances murine antigen presentation (16) and that co-expression of the p31 and p41 forms of Ii results in a changed pattern of degradation of the p31 form (15). It has been discovered that the p41 fragment, which is spliced into the sequence of the p31 form of Ii, co-purifies with cathepsin L and is also its strongest inhibitor, but it does not inhibit cathepsin S (17, 18). This suggests that cathepsin L is the likely target of the p41 fragment and not cathepsin S, which is considered as the major Ii processing enzyme in bone marrow-derived APCs (43) and in murine primary B cells and lymph nodes (44). For the mouse it was shown that cathepsin L is probably the enzyme responsible for degradation of Ii in thymic epithelial cells (45), whereas in humans this role appears to be performed by cathepsin V (46). The inhibition constants presented here show that, in addition to human cathepsin L, mouse cathepsin L and human cathepsin V are also strongly inhibited by the p41 fragment, suggesting that the p41 fragment can regulate the processing of Ii performed by these enzymes. As shown here and earlier (18), the p41 fragment inhibits cathepsins K, F, H, and, presumably, O. It is not surprising that these enzymes are present in macrophages (20–26, 47), which further suggests that the p41 fragment can regulate their activity too. Intriguingly, the $K_i$ values against cathepsin S reveal that the p41 fragment can also inhibit the activity of this cathepsin, which is considered as the major processing enzyme of Ii in certain APCs. The rather high $K_i$ value, however, indicates that the p41 fragment most likely modulates, but does not block, the activity of cathepsin S. This behavior is typical of the buffer-type inhibitors, which rapidly bind the proteases but also rapidly release them in the presence of substrates (48). By regulating the activity of cathepsin S, the p41 fragment may slow down the processing of Ii in early endosomes. This role is reminiscent of the chaperone role of the p41 isoform of Ii for cathepsin L (49, 50). Taken together, the finding that the p41 fragment can inhibit most of the cysteine cathepsins, apart from cathepsins B, C, X, and presumably W, strongly suggests that the p41 form, in addition to regulating the processing of Ii, also modulates proteolytic processing of protein antigens, thus influencing the antigenic peptide selection and production.

It has been demonstrated, using cathepsin S- and L-deficient mice, that these two enzymes play an indispensable role in invariant chain degradation and thus the antigen presentation process, whereas other cysteine cathepsins appear to be redundant (51). The redundancy of cathepsins has however not been studied in detail. The redundancy is a consequence of the structure of their rather unconstrained active site cleft, which endows cysteine endopeptidases with broad specificity profiles (52). Nevertheless, the enzymes are not the same and may, in different environments and combinations, exhibit different degradation patterns. This indicates that the amounts of proteolytic enzymes and their combination with their inhibitors in endocytic compartments result in different degradation patterns of the target proteins. Because other cysteine cathepsin inhibitors, such as cystatins C and F (11, 53), are not known to control antigen presentation, the p41 fragment appears to be the primary candidate for regulating the activity of cysteine...
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cathepsins during Ii maturation and antigen processing in antigen presenting cells. The variability in sequences and Kᵢ values between the mouse and human enzymes so far investigated further suggests that the proteolytic environments in which the p41 fragment and substrates compete for binding to cysteine cathepsins may provide the basis for the observed variations in antigen presentation between species.

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