Quantifying Cathepsin S Activity in Antigen Presenting Cells Using a Novel Specific Substrate*

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Cathepsin S (CatS) is a lysosomal cysteine protease belonging to the papain superfamily. Because of the relatively broad substrate specificity of this family, a specific substrate for CatS is not yet known. Based on a detailed study of the CatS endopeptidase specificity, using six series of internally quenched fluorescent peptides, we were able to design a specific substrate for CatS. The peptide series was based on the sequence GRWHTVGL-RWE-Lys(Dnp)-DArg-NH₂, which shows only one single cleavage site between Gly and Leu and where every substrate position between P-3 and P-3′ was substituted with up to 15 different amino acids. The endopeptidase specificity of CatS was mainly determined by the P-2, P-1, and the P-3′ substrate positions. Based on this result, systematically modified substrates were synthesized. Two of these modified substrates, Mca-GRWPP-MGLPWE-Lys(Dnp)-DArg-NH₂ and Mca-GRWPMGAPWE-Lys(Dnp)-DArg-NH₂, did not react with the purified cysteine proteases cathepsin B (CatB) and cathepsin L (CatL). Using a specific CatS inhibitor, we could further show that these two peptides were not cleaved by endosomal fractions of antigen presenting cells (APCs), when CatS was inhibited and related cysteine proteases cathepsin B, H, L and X were still active. Although aspartic proteases like cathepsin E and cathepsin D were also present, our substrates were suitable to quantify cathepsin S activity specifically in APCs, including B cells, macrophages, and dendritic cells without the use of any protease inhibitor. We find that CatS activity differs significantly not only between the three types of professional APCs but also between endosomal and lysosomal compartments.

Lysosomal cysteine proteases of the papain family were long believed to be exclusively involved in nonspecific proteolysis within the lysosome. However, the use of specific protease inhibitors and the study of gene knock-out mice suggested that some of them are involved in many other processes, such as cellular homeostasis, autophagy, apoptosis, and antigen presentation (1–3). Antigen presentation by MHC² class II molecule requires the entry of antigens into the endosomal-lysosomal compartment. These antigens are then processed by proteolytic enzymes, of which the lysosomal cysteine proteases of the papain family constitute an important subset. The generated peptides bind to MHC class II molecules, which are then displayed at the surface of professional APCs including macrophages, dendritic cells (DCs), and B cells. The variety of MHC class II peptide products that can activate CD4⁺ T cells is on the one hand determined by allelic variation in MHC molecule binding specificity and on the other by the identity and level of activity of processing proteases present in APCs. Although CatS is one of the major proteases involved in antigen processing (4–6), specific determination of its activity in antigen presenting cells is currently complicated because a specific substrate is not yet known. One reason for this is that CatB, CatL, and CatS show relatively similar endopeptidase specificities, and CatB and CatL possess a peptidyl-dipeptidase activity of relatively broad specificity as well (7–9). The development of a specific substrate for CatS should be useful to illuminate the complexity of the class II MHC-restricted pathway of antigen presentation, and knowledge of the protease specificity of enzymes involved in antigen processing, for example that of CatS, could help to create software for antigenic peptide prediction.

The mechanism of antigen presentation is regulated not only by antigen processing but also by the degradation of the invariant chain, a surrogate substrate and trafficking chaperone of MHC class II (10, 11). Experiments using human B cells treated with the CatS-specific inhibitor LHVS and the characterization of CatS knock-out mice have elucidated a clear, nonredundant role for CatS, in the late stages of invariant chain degradation (12–14). Blockade of the progressive cleavage of invariant chain causes an accumulation of invariant chain intermediates that occupy the MHC class II peptide-binding groove and prevent normal loading of antigenic peptides. Decreased surface expression of class II MHC products (15) and a reduced humoral immune response are the consequences (13). Furthermore, cathepsin S-deficient mice showed decreased susceptibility to collagen-induced arthritis (14), and rats with adjuvant-induced arthritis displayed significant decreases in inflammation after oral administration of the cathepsin S inhibitor LHVS (16), not only supporting a specific role for CatS...
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in rheumatoid arthritis but also validating CatS as an appropriate drug target in other autoimmune disorders. CatS has recently emerged as an important proteolytic enzyme in cancer development, and CatS inhibitors have been proposed as anticancer agents (17, 18). Compounds targeting CatS in rheumatoid arthritis, bronchial asthma, and psoriasis are already undergoing clinical evaluation, and the development of new CatS inhibitors is still a growing field (19). A specific substrate for CatS could be a useful tool to test whether newly developed inhibitors are able to inhibit CatS specifically in APCs.

In this study we examine the endopeptidase specificity of CatS at the substrate amino acid positions P-3 to P-3’ in detail, using six series of internally quenched fluorescent peptides. Based on this detailed study we were able to design a specific substrate for CatS. The substrate allows a clear differentiation between CatS activity and the activities of enzymatically similar proteases CatB, CatH, CatL, and CatX in APCs. Furthermore, the substrate is suitable for quantification of CatS activity specifically in all three types of professional APCs without the use of any protease inhibitor. Using the specific substrate, we find that CatS activity differs significantly between different types of APCs. Furthermore we show that CatS activity is mainly located in endosomes of the APCs investigated and only to a minor extent in lysosomes.

EXPERIMENTAL PROCEDURES

Peptides—Chemicals were obtained from commercial suppliers and used without further purification. All of the peptides were synthesized by a solid-phase technique, using the Fmoc strategy on a Syro II synthesizer (MultiSynTech, Witten, Germany). Fmoc amino acids were purchased from Novabiochem (San Diego, CA) or from MultiSynTech. Mca was from Sigma. Rink amide resin was purchased from Pep Chem (Reutlingen, Germany). 2-(1H-Benzotriazole-1-yl)-1,1,3,3-tetramethyluronium-tetrafluoroborat and 1-hydroxybenzotriazole were from MultiSynTech. The final de-protected peptides were purified by semi-preparative reverse phase HPLC, using a Reprosil C-8 column (5 µm particle size, 150 × 2 mm) from Dr. Maisch GmbH (Tuebingen, Germany), and a two-solvent system: (A) 0.055% (v/v) trifluoroacetic acid in water and (B) 0.05% (v/v) trifluoroacetic acid in 80% (v/v) acetonitrile in water. Products were detected by their UV absorbance at 220 nm and by fluorescence emission at 360 nm, following extinction at 280 nm. The percentage of digestion was calculated as 1 minus the UV area ratio of the undigested educt peak of the digestion mixture to the educt peak of an undigested negative control. All of the digestions were conducted in triplicate. The identity of cleavage products was verified by mass spectrometry.

In the quantitative assay of the substrate specificity, kinetic constants of eight peptides for each subsite were determined. The initial velocities of the CatS catalyzed reactions were measured at different substrate concentrations in a stopped enzyme assay. The peptides were subjected to CatS cleavage at pH 5.5 as described above, but this time the reaction was stopped by adding trifluoroacetic acid after 5 min. The product mixture was separated by analytical reverse phase HPLC as described above. The product concentration after 5 min was quantified by fluorescence emission of the N-terminal tryptophan at 360 nm, following extinction at 280 nm. The molar product concentration was calculated by multiplying the fluorescent peak area of the N-terminal fragment with a proportional factor, which was calculated for each peptide individually out of the fluorescent peak area after total substrate turnover. The kinetic parameters $K_m$ and $V_{max}$ were calculated by nonlinear regression based on the Michaelis-Menten function using the EKI3 software version 1.3 (Wiley-VCH, Weinheim, Germany), which uses the regression analysis described in Ref. 21. The value of $k_{cat}$ was calculated as the $V_{max}$/enzyme concentration ratio.

For the specific detection of CatS activity in APCs, 10 µl of subcellular fractions were added to 80 µl of digestion buffer (50 mM sodium acetate buffer, pH 5.5, containing 4 mM dithiothreitol) and preincubated for 1 h at 37 °C. When CatS was inhibited, the buffer additionally contained the specific CatS inhibitor LHVS at a final concentration of 10 nM. The inhibitor was freshly prepared as a 1 mM stock solution in Me2SO and diluted in water. The final MeSO concentration in the reaction mixture was 0.001%. The reaction was started by the addition of 10 µl of Mca-conjugated substrate solution (0.2 mM in water). The progress of fluorescent product formation was recorded over 2 h, using a fluorimeter (SLT Spectra Fluor, Tecan, Crailsheim, Germany) on kinetic mode at 37 °C ($\lambda_{ex}=340$ nm, $\lambda_{em}=405$ nm). All of the experiments were performed in triplicate.


**Cell Culture**—The Epstein-Barr virus-transformed human B cell line WT100 was cultured in RPMI 1640 medium (Invitrogen) supplemented with 10% (v/v) heat-inactivated fetal calf serum (Invitrogen), penicillin (final concentration, 100 units/ml; Invitrogen), and streptomycin (final concentration, 0.1 mg/ml; Invitrogen) at 37°C in tissue culture flasks (Nunc, Wiesbaden, Germany).

DCs and macrophages were generated from peripheral blood mononuclear cells. Peripheral blood mononuclear cells were isolated by Ficoll/Paque (PAA Laboratories, Pasching, Austria) density gradient centrifugation of heparinized blood obtained from buffy coats. Isolated peripheral blood mononuclear cells were plated (DCs: 1 × 10^6 cells/8 ml/flask; macrophages: 8 × 10^7 cells/10 ml/flask) into 75-cm² tissue culture flasks (DCs: Cell Star, Greiner Bio-One GmbH, Frickenhausen, Germany; macrophages: BD Primaria T 75, BD Biosciences, San Jose, CA) in RPMI 1640 (Invitrogen) under the same culture conditions as for WT100. After an incubation of 1.5 h for DCs and 2.5 h for macrophages at 37°C, nonadherent cells were removed, and adherent cells were cultured in complete culture medium. The macrophage medium was supplemented with granulocyte macrophage-colony-stimulating factor (Leukomax; Sandoz, Basel, Switzerland), the DC medium with granulocyte macrophage-colony-stimulating factor and interleukin-4 (R & D Systems, Minneapolis, MN) for 6 days as described previously (22–24). DCs were harvested, whereas macrophages were cultured for one more week in a specialized macrophage medium, consisting of 60% adoptive immunotherapy medium-V, 30% Iscove's modified Dulbecco's medium (Invitrogen), and 10% human AB⁺ serum. This resulted in a cell population showing the lowest V_max values in this series followed by the aromatic phenylalanine. The highest k_cat values were also obtained by these peptides. Interestingly, the amino acid isoleucine was not favored at the P-2 position. Although all other internally quenched fluorescent peptides with P-2 modifications showed relatively low k_cat/K_m values (<1 s⁻¹ μM⁻¹), no peptide was completely resistant to the hydrolysis reaction catalyzed by CatS. At the P-2 position the hydrophobic branched amino acids valine, norleucine, as well as methionine were clearly preferred by CatS. The substrates with valine and norleucine at the P-2 position showed the highest V_max values in this series followed by the aromatic phenylalanine. The highest k_cat/K_m values were also obtained by these peptides. The k_cat/K_m values at the P-2 position were more susceptible to hydrolysis than the others. Therefore, CatS was not fully resistant to the hydrolysis reaction catalyzed by CatS. The highest V_max and k_cat/K_m values not only in the P-1 series, but also among all tested peptides. Substrates with isoleucine and aspartic acid showed the lowest k_cat/K_m values. Together with valine these were the least well accepted amino acids at this subsite. Among all tested substrates, amino acid permutations at P-1’ affected the hydrolysis reaction most significantly. The substrates containing the hydrophobic branched amino acids valine, leucine, norleucine and isoleucine

**RESULTS**

**Endopeptidase Specificity of Cathepsin S**—Using six series of internally quenched peptides with single amino acid modifications, we analyzed the endopeptidase specificity of CatS at the substrate amino acid positions P-3 to P-3’ in detail. The peptide library was based on the CatS-sensitive substrate GRWHTVGLRWE-Lys(Dnp)-DArg-NH₂, which shows only one single cleavage site between Gly and Leu and where the fluorescence of the N-terminal tryptophan is quenched by the Dnp group. Fig. 1 shows the results for all the quantitative analyses of CatS specificity for all substrate positions between P-3 and P-3’. Table 1 shows the kinetic parameters of eight selected peptides per modified substrate position, determined in the quantitative assay. At the P-3 position, all of the 15 tested amino acids were accepted with nearly identical susceptibilities to CatS. Although no special group of amino acids was preferred by CatS at the P-3 position, the negatively charged aspartic acid showed the lowest V_max and k_cat/K_m values and was the least well accepted residue in this position. At the P-2 position the hydrophobic branched amino acids valine, norleucine, as well as methionine were clearly preferred by CatS. The substrates with valine and norleucine at the P-2 position showed the highest V_max values in this series followed by the aromatic phenylalanine. The highest k_cat/K_m values were also obtained by these peptides. Interestingly, the amino acid isoleucine was not favored at the P-2 position. Although all other internally quenched fluorescent peptides with P-2 modifications showed relatively low k_cat/K_m values (<1 s⁻¹ μM⁻¹), no peptide was completely resistant to the hydrolysis reaction catalyzed by CatS. At the S₁ subsite of CatS no clear discrimination was observed. In contrast to the P-2 series, all of the substrates with P-1 variations showed k_cat/K_m values around 1 s⁻¹ μM⁻¹ or higher. However, peptides with lysine and glutamine at the P-1 position were more susceptible to hydrolysis than the others. They showed the highest V_max and k_cat values not only in the P-1 series, but also among all tested peptides. Substrates with isoleucine and aspartic acid showed the lowest k_cat/K_m values. Together with valine these were the least well accepted amino acids at this subsite. Among all tested substrates, amino acid permutations at P-1’ affected the hydrolysis reaction most significantly. The substrates containing the hydrophobic branched amino acids valine, leucine, norleucine and isoleucine
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**FIGURE 1. Analysis of CatS specificity.** Library peptides with single amino acid exchanges and the general sequence GRWHTVGLRWE-Lys(Dnp)-DArg-NH₂ were processed at a concentration of 20 μM for 30 min by 1.3 nM CatS. The reaction mixtures were analyzed by HPLC, and UV peak areas were used for quantification of substrate digestion. All of the assays were performed in triplicate. The error bars denote the means ± standard deviation. The percentage of substrate digestion is shown for peptides with P-3, P-2, P-1, P-1’, P-2’, and P-3’ amino acid modifications. The x axis represents the amino acid at the corresponding substrate position, designated by the single-letter code (X, norleucine; Cit, citrulline). The basis peptide of the library experiment is marked with an asterisk. Preferred amino acids at each substrate position are marked dark gray.

It is noteworthy that the poorly digested peptides of the P-3’ series show relatively high \( K_m \) values at low or intermediate \( V_{max} \) values compared with other series. Hydrophobic amino acids at the P-3’ position result in substrates with higher affinity, in contrast to charged amino acids, which result in low affinity substrates. Taken together, these data show that the substrate specificity of CatS was mainly determined by the P-2, P-1’, and P-3’ substrate positions. The amino acid preferences of these positions are summarized in Table 2. The most restrictive position in our assay was the P-1’ substrate position.

**Design of CatS-specific Substrates**—Based on the conclusions on preferred CatS cleavage sites shown in Table 2, we synthesized various CatS-sensitive substrates, with multiple amino acid substitutions compared with the original peptide. To identify a peptide that is resistant to digestion by related cysteine proteases, these substrates were additionally subjected to CatB and CatL digestion for 1 h. With regard to a cellular application of a specific substrate, it was important to show that the peptide is not digested even under conditions where CatB and CatL concentrations significantly exceed that of CatS. Therefore we used CatB (11.6 nM) and CatL (13 nM) concentrations that were approximately 10 times higher compared with CatS (1.3 nM) in these assays. Another difficulty in creating a CatS-specific substrate was that CatB as well as CatL show a peptidyl-dipeptidase activity of relatively broad specificity, allowing C-terminal truncations of dipeptides (7, 8). To block this activity, substrates contained a protecting D-Arg residue at the C terminus. Compared with an unspecific substrate Mca-GRWHTVSVRWEK(Dnp)-DArg-NH₂ (KVSVR), which was digested equally well by all three enzymes, the peptide Mca-GRWHTVGLRWEK(Dnp)-DArg-NH₂ (TVGLR) we used as a basis for the library experiment was not completely digested by CatB and CatL within the given time (Fig. 2). The TVGLR peptide showed only one single CatS cleavage site between the Gly-Leu bond. The same cleavage products were also generated by CatB and CatL digestion, as identified by mass spectrometry of the collected HPLC fractions. In addition to these major cleavage products, CatB and CatL generated two minor cleavage products that were not identical for both enzymes (Fig. 2B). By changing the valine at the P-2 position of the original substrate to a methionine, the cleavage at the major cleavage site was reduced for CatB and L (not shown). However, the modified substrate was still cleaved at the minor cleavage sites. According to the experiments described above (Fig. 1) digestion by CatS was not affected by the valine-methionine exchange at the P-2 position. In these experiments we have also shown that the P-3 P-2’ position could be occupied by every tested amino acid without affecting the hydrolysis catalyzed by CatS. To prevent a CatB- and CatL-catalyzed cleavage at the minor cleavage sites, we chose proline as the new P-4, P-3, and P-2’ residue of the methionine peptide. The P-3 and P-2’ position of the CatS cleavage site are equal to the P-1 positions of the minor cleavage sites, which were generated by CatB and CatL. This modified substrate Mca-GRWPPMGLPLWEK(Dnp)DArg-NH₂ (PMGPL) was resistant to CatB and CatL digestion within the given time (Fig. 2C). Another peptide substrate Mca-GRWHPMGAPWEK(Dnp)DArg-NH₂ (PMGAP) without a third proline at P-4, was also resistant to CatB and CatL digestion (not shown). Oxi-
TABLE 1
Kinetic parameters for hydrolysis of six peptide series based on the sequence GRWHTVG ↓ LRWE-Lys(Dnp)-DArg-NH₂ by recombinant human cathepsin S for characterization of all subsites from S3 to S3’
The data represent the means ± S.D. of a triplicate experiment. ↓ represents the only cleavage site. X, norleucine; Cit, citrullin.

| Substrate position | Amino acid | \( K_m \) (μM) | \( V_{max} \) (μmol/min) | \( k_{cat}/K_m \) (1/s × μmol) |
|--------------------|------------|----------------|--------------------------|------------------------|
| P-2                | F          | 1.42 ± 0.32    | 0.450 ± 0.004            | 4.05                   |
| K                  | 1.94 ± 0.29| 0.349 ± 0.006  | 2.30                     |
| V                  | 2.76 ± 0.02| 0.752 ± 0.011  | 3.49                     |
| G                  | 5.14 ± 0.12| 0.626 ± 0.005  | 1.56                     |
| D                  | 1.88 ± 0.09| 0.154 ± 0.001  | 1.05                     |
| X                  | 1.60 ± 0.24| 0.367 ± 0.005  | 2.95                     |
| P                  | 3.24 ± 0.32| 0.478 ± 0.01   | 1.89                     |
| I                  | 0.48 ± 0.09| 0.216 ± 0.006  | 5.82                     |
| P-3                | F          | 2.01 ± 0.09    | 0.262 ± 0.008            | 1.67                   |
| K                  | 2.78 ± 0.54| 0.136 ± 0.006  | 0.22                     |
| T                  | 9.30 ± 0.17| 0.133 ± 0.004  | 0.18                     |
| V                 | 2.37 ± 0.17| 0.616 ± 0.009  | 3.34                     |
| G                 | 12.86 ± 0.27| 0.242 ± 0.005  | 0.24                     |
| D                 | 12.37 ± 0.35| 0.143 ± 0.002  | 0.15                     |
| X                 | 4.18 ± 0.26| 0.277 ± 0.003  | 0.85                     |
| P                 | 5.91 ± 0.29| 0.231 ± 0.004  | 0.50                     |
| M                 | 3.95 ± 0.44| 0.174 ± 0.003  | 0.57                     |

| Substrate position | Amino acid | \( K_m \) (μM) | \( V_{max} \) (μmol/min) | \( k_{cat}/K_m \) (1/s × μmol) |
|--------------------|------------|----------------|--------------------------|------------------------|
| P-1                | F          | 4.80 ± 0.44    | 0.206 ± 0.004            | 0.55                   |
| K                  | 5.35 ± 1.09| 0.071 ± 0.004  | 0.17                     |
| V                  | 2.37 ± 0.23| 1.005 ± 0.016  | 5.43                     |
| Cit                | 1.71 ± 0.38| 0.156 ± 0.003  | 1.17                     |
| X                  | 0.73 ± 0.05| 0.644 ± 0.027  | 11.24                    |
| R                  | 2.44 ± 0.26| 0.082 ± 0.001  | 0.43                     |
| I                  | 1.98 ± 0.22| 0.579 ± 0.011  | 3.75                     |
| P-1’               | F          | 5.27 ± 0.35    | 0.730 ± 0.014            | 1.78                   |
| K                  | 1.05 ± 0.12| 0.484 ± 0.019  | 5.91                     |
| H                  | 3.12 ± 0.01| 0.419 ± 0.002  | 1.72                     |
| Q                  | 2.59 ± 0.15| 0.637 ± 0.012  | 3.16                     |
| D                  | 3.14 ± 0.10| 0.455 ± 0.002  | 1.66                     |
| R²                 | 2.37 ± 0.17| 0.616 ± 0.009  | 3.34                     |
| P                 | 1.31 ± 0.10| 0.296 ± 0.004  | 2.91                     |
| I                 | 1.50 ± 0.30| 0.406 ± 0.009  | 3.47                     |
| P-3’               | F          | 8.20 ± 0.39    | 0.83 ± 0.010             | 1.30                   |
| K                  | 37.87 ± 3.16| 0.551 ± 0.031  | 0.19                     |
| H                 | 14.93 ± 0.78| 0.179 ± 0.004  | 0.15                     |
| G                 | 16.48 ± 0.39| 0.299 ± 0.005  | 0.23                     |
| W³                 | 2.37 ± 0.17| 0.616 ± 0.009  | 3.34                     |
| D                 | 16.68 ± 1.91| 0.353 ± 0.030  | 0.27                     |
| R                 | 11.54 ± 0.24| 0.400 ± 0.006  | 0.44                     |
| I                 | 8.20 ± 0.69| 0.897 ± 0.004  | 1.40                     |

\( ^a \) The basis peptide of the library experiment.

TABLE 2
Cathepsin S subsite specificities for cleavage sites
Favorable amino acids are marked +, less favorable are marked 0, and unfavorable are marked −. The ranking reflects the results shown in Fig. 1 and Table 1.

| Side chain | P-2 position | P-1 position | P-2 position | P-3’ position |
|------------|--------------|--------------|--------------|---------------|
| Hydrophobic | Aromatic | + (only Phe) | − | + |
|            | Aliphatic | + (not Ile) | − | + |
|            | Methionine | + | + | − |
|            | Proline | − | + | Not tested |
| Neutral | Amide | − | + | 0 |
|            | Small | − | + | 0 |
| Basic | Acidic | − | − | − |

Characterization of Endosomal Fractions of Antigen Presenting Cells—During the process of antigen uptake, endocytic vesicles of antigen presenting cells become enriched with many more proteases than only CatS, CatB, and CatL. Other cysteine proteases like CatH and CatX together with aspartic proteases like CatD and CatE as well as the asparagine-specific endopeptidase are also present (29, 30). To show that even under naturally occurring conditions the two methionine peptides PMGLP and PMGAP are specifically cleaved by CatS, it was important to use endosomal fractions for additional digestion experiments. Therefore, endosomal fractions of dendritic cells, macrophages, and the human B cell line WT100 were isolated, and existing cysteine proteases were characterized by active site labeling as described above. Using this method, the active forms of the enzymes are tagged and visualized (20). Earlier experiments in which we identified polypeptides in the 20–40-kDa range via immunoprecipitation with antibodies directed against individual cathepsins as well as by mass spectrometry-based sequencing after pulldown assay with streptavidin beads (31) allowed us to identify CatB, CatH, CatL, CatS, and CatX. Fig. 3 shows the active site label of the endosomal fraction of macrophages. The endosomal fractions of DCs and the WT100 B cell line showed similar patterns of cysteine proteases (not shown). Earlier experiments had shown that CatL was only visible when the concentration of the labeling compound DCGON was increased from 10 to 128 μM (21). Preincubation with the cysteine protease inhibitor E64 at a concentration of 10 μM led to an inhibition of all visualized cathepsins. In contrast to E64, the inhibitor LHVS at a concentration of 10 nM
can be used as a specific CatS inhibitor, although it is not impossible that other cathepsins could be inhibited when higher concentrations of LHVS are used.
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TABLE 3
Kinetic parameters of PMGLP digestion by cathepsins B, L, and S

| Enzyme | $K_m$ (μM) | $k_{cat}$ (1/s) | $k_{cat}/K_m$ (1/(s × μM)) |
|--------|------------|-----------------|-----------------------------|
| CatB   | 9.94 ± 0.67 | 0.044 ± 0.002   | 0.004                       |
| CatL   | 3.49 ± 0.79 | 0.112 ± 0.008   | 0.032                       |
| CatS   | 5.56 ± 1.33 | 5.666 ± 0.478   | 1.018                       |

FIGURE 3. Cysteine protease characterization in macrophage endosomal compartments. Equal amounts of total protein (0.67 μg) from macrophage endosomal fraction were labeled with the active site binding probe DCG0N. All of the samples were preincubated for 1 h at 37 °C. Left lane, without inhibitor (no Inh.); middle lane, with 10 μM of the general cysteine protease inhibitor E64; right lane, with 10 nM of the CatS-specific inhibitor LHVS. After SDS-PAGE and Western blot, proteins were visualized with peroxidase via streptavidin/biotin interaction. The molecular mass markers are indicated on the left, and identified cysteine proteases are on the right. In the right lane only the CatS band intensity is drastically reduced by LHVS, indicating a specific inhibition under these conditions.

FIGURE 4. Specific inhibition of CatS prevents digestion of designed substrates by endosomal fractions; comparison with an unspecific substrate. Time-progress curves of peptide substrate hydrolysis reactions catalyzed by macrophages endosomal fractions followed by fluorescence emission. 0.67 μg of total protein from macrophage endosomal fraction were preincubated for 1 h at 37 °C. ■, without inhibitor; □, with 10 nM LHVS. All of the assays were performed in triplicate at 37 °C in 50 mM sodium acetate buffer (pH 5.5). A, hydrolysis of the internally quenched fluorescent peptide Mca-GRWPPMGLPWE-Lys(Dnp)-DArg-NH$_2$, (PMGLP) at a concentration of 20 μM. Progress of product formation was recorded over 2 h by fluorescence emission of the N-terminal Mca fluorescence intensity to a maximum (Fig. 4, A and B). On the other hand, LHVS was not capable of inhibiting the hydrolysis reaction of the unspecific substrates Z-FR-AMC and KVSVR under similar conditions (Figs. 4C and 5C). It is true that the CatS inhibition reduced the velocity of the reaction, but nevertheless the unspecific substrate Z-FR-AMC was completely hydrolyzed after 2h. In addition to the results of the active site labeling, this indicates that related cysteine proteases remain active and are still capable of cleaving substrates in the presence of LHVS. When DCs and B cell endosomal fractions were used for comparable digestion experiments, similar reaction profiles were obtained (Fig. 6, A and B).

Quantification and Subcellular Distribution of CatS in Antigen Presenting Cells—Taken together, the above data shows that our designed substrates PMGLP and PMGAP were exclusively cleaved by CatS in subcellular fractions of antigen presenting cells and not by other present proteases. Therefore, they can be designated CatS-specific. Using one of these specific substrates, we quantified CatS activity in all three types of professional APCs (Fig. 6C). DCs and B cells showed significantly lower endosomal CatS activity compared with macrophages. The CatS activity in endosomal extracts from macrophages was ~10 times higher than in the corresponding extracts from the B cell line and even 30 times higher than in endosomal fractions from DCs. CatS activity differed not only between the different types of APCs but also between endo-
mal and lysosomal compartments of one cell type. We localized CatS activity mainly in endosomal compartments and only to a minor extent in lysosomal extracts (Fig. 7A). In WT100 B cells the endosomal CatS activity was ~7 times higher than the lysosomal CatS activity. On the other hand, the total activity of CatB, L, and S together, measured by digestion of the unspecific substrate Z-FR-AMC, was equally distributed between endosomes and lysosomes (Fig. 7B), indicating that CatS is more an endosomal than a lysosomal enzyme in APCs. Macrophage endosomal extracts even showed a 600-fold higher CatS activity compared with lysosomal extracts. Because of this high CatS activity in endosomal fractions of macrophages, it is not surprising that also the total activity of CatB, L, and S is higher in endosomes than in lysosomes. However, compared with the difference in CatS activity between the subcellular compartments the total CatBLS activity in endosomes was only five times higher than in lysosomes. These results confirm that CatS is mainly located in endosomes of APCs and show also the great advantage of a CatS-specific substrate in quantifying specifically the CatS activity compared with an unspecific substrate.

**DISCUSSION**

The specificity profile of CatS that we obtained from the library experiment highlighted clear amino acid preferences at the P-2, P-1, and P-3 positions. According to previously reported studies, the specificity of CatS is mainly determined by the amino acid at the P-2 position (9, 32–34). In those studies, conclusions on the CatS specificity were often drawn from the cleavage of fluorogenic substrates containing the fluorophore at the P-1 position. These substrates are relatively short and do not contain amino acids at the P position. In our strategy we used longer peptides and avoided the effects of a non-amino acid residue at the P-1 position. Although we have also seen a clear preference of branched hydrophobic amino acids at the P-2 position, we find that the P-1 position is even more restrictive than the P-2 position. Compared with studies with shorter substrates, no peptide with P-2 variations was completely resistant to CatS digestion. We have also seen that reduction of the peptide length results in a decrease of CatS activity (not shown), indicating that the CatS activity is not only dependent on amino acid composition of a substrate but also on its length.
were performed in triplicate at 37 °C in 50 mM sodium acetate buffer (pH 5.5). The results are representative of at least three independent experiments. The error bars indicate standard deviation.

The diversity of autoantigenic or tumor-specific MHC class II presented peptides is on the one hand determined by allelic variations of MHC molecule binding specificity and on the other by the substrate specificity of proteases involved in antigen processing. Therefore it would be useful to predict the MHC-presented peptides generated or destroyed by a given protease, such as CatS. The kinetic constants we determined for substrates with a distinct amino acid composition could be advantageous in predicting cleavage sites from the primary sequence of an antigen protein and could help to improve software for antigenic peptide prediction (35–37).

The substrates we designed on the basis of the specificity profile of CatS were resistant to CatB and CatL digestion under time-limited conditions when CatB and CatL concentrations were approximately 10 times higher than the CatS concentration. However, we cannot exclude that the substrates would be cleaved at higher CatB and CatL concentrations. Nevertheless, we have clearly shown that they are exclusively cleaved by CatS in subcellular fractions of professional APCs and not by other proteases present. Therefore they can be used as CatS-specific substrates in APCs.

These novel substrates offer new possibilities to study CatS-related physiologies and pathologies. For example, the role of CatS in cross-presentation of exogenous antigens on MHC class I molecules (6) or in the development of autoimmune disorders remains largely unclear. As described above, CatS is an appropriate drug target in cancer and autoimmune disorders. New compounds targeting CatS are currently being developed. Because therapeutic inhibition of CatS is linked to chronic diseases, irreversible inhibitors are less suitable as therapeutic drugs. Noncovalent and covalent reversible inhibitors, mimicking the substrate, should be more suitable because of their higher selectivity and the lower probability of an adverse immune response (19). The sequence of the developed substrate is highly selective toward CatS and could be helpful not only to create new inhibitors but also to test already existing inhibitors, as to whether they are able to inhibit CatS in APCs specifically.

Further applications of the internally quenched specific substrates, for example in fluorescence microscopy, are also possible.

Using one of these specific substrates, we have found a significant difference in activity levels of CatS between the different types of APCs. Whereas macrophages contained high levels...
of CatS, DCs showed significantly lower CatS activity. It is now well established that DCs are the most effective antigen presenting cells (38). In general DCs capture antigens in peripheral tissues, receive activatory signals, and migrate to the local lymph nodes, where they can activate T cells. During that maturation process antigens were captured long before the DCs encounter their specific T cells, and a rapid degradation of antigens by CatS and other lysosomal proteases might prevent an effective antigen presentation (39). The low activity level of CatS in DCs compared with macrophages might be one reason for their capacity to cross-present exogenous antigens on MHC class I by allowing them a greater chance to exit endosomal compartments to the cytosol. DC maturation is largely regulated by cytokines. Using our specific substrate, the influence of these cytokines on CatS activity during DC maturation can easily be measured. The finding that CatS is mainly located in endosomal compartments of antigen presenting cells is only one useful piece of information that can help to illuminate the complexity of CatS-related physiology.

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