Specific role for cathepsin S in the generation of antigenic peptides *in vivo*

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To address the role of different proteases in degradation of antigen destined for MHC class II-restricted presentation, we generated cathepsin-deficient mice carrying a transgenic B cell receptor (BCR) specific for hen egg lysozyme (HEL). We demonstrate that degradation of HEL in B lymphocytes is highly processive and does not result in discrete processing intermediates. Moreover, degradation of HEL does not require initial unlocking of the antigen by any of the cathepsins tested. Using mass spectrometry and microsequencing, we show that all major cathepsins (CatS, CatL, CatB, and CatD) digest HEL *in vitro* with considerable redundancy, although some preferential cleavages are evident. These observations have a functional correlate: when triggered by cathepsin S-deficient antigen-presenting cells, T cells that recognize different HEL epitopes fail to present two HEL-derived epitopes, while a third epitope is presented independently of the activity of cysteine proteases. We conclude that the proteolytic processing machinery is redundant, and that several proteases can substitute for each other to degrade a given antigen. However, a certain degree of proteolytic specificity is demonstrable for the generation of particular epitopes, notably by CatS.

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1 Introduction

Proteolysis is key in antigen presentation by MHC class II molecules. Stepwise degradation of the invariant chain (Ii), the clas II-associated chaperone responsible for delivery of class II molecules to the endocytic pathway, renders the class II binding pocket available for peptide loading [1]. Furthermore, endosomal and lysosomal proteases fragment endocytosed proteins and generate antigenic epitopes (reviewed in [2, 3]). Optimal presentation of particular epitopes requires additional endosomal processing [4], although partially folded and/or reduced antigen may also bind to class II heterodimers [5, 6]. Depending on the sequence context of the epitope, such proteolysis presumably occurs in different endocytic compartments.

The identity of proteases that participate in class II antigen presentation is beginning to emerge. A large number of lysosomal proteases is known [7], some of which have been implicated in MHC class II function (reviewed in [8, 9]). Cathepsin S (CatS) and CatL are essential for the final steps in the degradation of Ii *in vivo* [10, 11]. CatS has also been suggested to be involved in the generation of certain antigenic epitopes [12]. In contrast, the most abundant lysosomal cysteine and aspartate proteases CatB and CatD may participate in MHC class II antigen processing, but neither is essential for presentation of a variety of different epitopes [13]. However, recent work suggests that CatB, as well as CatS and other cysteine proteases are responsible for the generation of discrete intermediates during processing of a model antigen [14]. To date, a single lysosomal protease has been attributed the specificity required for efficient processing of a defined antigen: Asparaginyl endopeptidase (AEP) carries out the rate-limiting cleavage in the processing of the microbial tetanus toxin C fragment TTCF [15].

Using hen egg lysozyme (HEL) as a model antigen, we here analyzed biochemically the processing of HEL *in vitro* and tracked the fate of internalized HEL in B lymphocytes. To this end, we made use of a combination of an anti-HEL Ig transgenic mouse and defined cathepsin disruption mutants. As antigen we used [^{35}S]-labeled HEL generated by *in vitro* translation, as a means of producing a native radiolabeled antigen of high specific activity.

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The first two authors contributed equally to this work.

Abbreviations: Cat: Cathepsin **HEL:** Hen egg lysozyme

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We observed extensive degradation of HEL both after BCR-mediated internalization and when digested with cathepsins *in vitro*, with little apparent specificity. The absence of individual cathepsins does not affect the rate of turnover of intact HEL. However, the generation of particular HEL epitopes involves a more discrete set of reactions. In particular, CatS is required for the efficient generation of the HEL epitopes HEL_{30-44} and HEL_{46-59}, but not HEL_{74-88}. The cleavage preferences of CatS within the HEL sequence could provide a biochemical basis for these observations.

2 Results

2.1 *In vitro* translation of HEL

To study antigen degradation biochemically, we sought to examine a native, homogeneously labeled antigen in order to be able to detect possible processing intermediates. We chose HEL since it is a well-characterized protein; the crystal structures of both free HEL and HEL in a complex with several antibodies have been solved. Whereas iodination of commercially available HEL is not quantitative and almost invariably entails oxidative damage in the course of labeling, the generation of radiolabeled HEL by *in vitro* translation suffers none of these drawbacks.

The presence of multiple disulfides in HEL necessitates the use of a translation system supplemented with microsomes to allow insertion into the lumen, where oxidation of the cysteines occurs yielding the folded, native molecule. We therefore equipped a HEL cDNA with a signal sequence derived from dog pre-pro-insulin (DPPISS) to ensure efficient insertion into microsomes. HEL mRNA was translated *in vitro* using rabbit reticulocyte lysate in the presence or absence of dog pancreas microsomes (DPM).

HEL contains two methionine residues (M12, M105) and eight cysteine residues (C6, C30, C64, C76, C80, C94, C115, C127), each of which was radiolabeled in the course of *in vitro* translation using either [^{35}S] methionine or [^{35}S] cysteine. Translation products were analyzed by SDS-PAGE. Translation of HEL mRNA without microsomes resulted in a polypeptide product of approximately 17 kDa, corresponding to the full-length HEL protein containing the signal peptide (Fig. 1 lanes 1–5). Translation in the presence of microsomes resulted in removal of DDPPISS and yielded a product of 14 kDa, the expected molecular weight of HEL (Fig. 1 lane 6). This polypeptide migrated slightly slower than iodinated commercial HEL (Fig. 1 lane 7), due to presence of a C-terminal YL1/2 tag in the *in vitro* translated protein. As

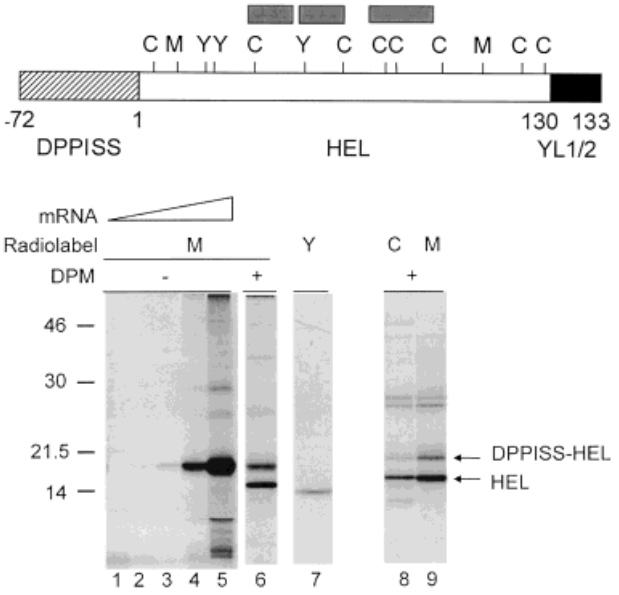

Fig. 1. (A) Sequence of HEL, containing the DPPISS signal sequence and a YL1/2 tag. Methionine (M), cysteine (C) and tyrosine (Y) residues that were radiolabeled using [^{35}S] or [^{125}I] are marked. Grey bars indicate the location of the three H-2^{b}-restricted epitopes analyzed. (B) *In vitro* translation of HEL. HEL mRNA was translated at increasing dilutions from the stock RNA in the absence (lanes 1–5) or presence (lane 6) of DPM at 30 °C for 1 h. Total translational material and pelleted microsomes, respectively, were analyzed by reducing SDS-PAGE (15 %). [^{125}I]-labeled HEL serves as control for the molecular weight of the *in vitro* translated HEL (lane 7). HEL radiolabeled with either [^{35}S] cysteine (lane 8) or [^{35}S] methionine (lane 9) was analyzed by non-reducing SDS-PAGE. Unprocessed (DPPISS-HEL) and mature HEL are indicated.

expected, HEL radiolabeled with either [^{35}S] methionine or [^{35}S] cysteine showed the same migration pattern on a non-reducing SDS-polyacrylamide gel (Fig. 1 lanes 8, 9). The cysteine residues of HEL form two stable (C64–C80; C76–C94) and two more labile (C6–C127; C30–C115) intrachain disulfide bonds [16]. Using IEF gel analysis in conjunction with alkylation of available free thiols, we could show that proper formation of disulfide bonds occurred in our microsome-supplemented translation system (data not shown). A mixture of the unprocessed and the mature form was always obtained after translation, due to incomplete cleavage of the signal sequence. However, only the mature form of HEL was recognized by the conformation-specific HyHEL-10 antibody exposed on the surface of the transgenic B cells (see below).

2.2 HEL is degraded *in vitro* by lysosomal cysteine proteases without the generation of discrete processing intermediates

We first assessed degradation of HEL *in vitro*. As a source of proteolytic activity we used whole cell lysates generated from bone marrow-derived APC. Preliminary data showed that virtually identical results were obtained using whole cell lysates or endosomal/lysosomal extracts prepared by differential centrifugation. Lysates were incubated with [^{35}S] cysteine-labeled HEL obtained by *in vitro* translation in the presence of 3 mM DTT, since endosomal/lysosomal proteolysis is more effective under reducing conditions [17].

HEL was efficiently digested in the cell lysates: indeed 50 % of the intact, mature HEL was degraded after 60 min. We examined the *in vitro* digests by SDS-PAGE and autoradiography, and observed no discrete breakdown intermediates. The inevitable loss of smaller fragments in the course of processing the gel for autoradiography (see Sect. 4) effectively precludes a quantitative estimate of HEL degradation. We therefore chose gel filtration chromatography on a Superdex peptide column as the analytical tool. This approach allows both size estimates and quantitative recovery of digestion products. Gel filtration analysis confirmed that HEL is not fragmented into distinct intermediates, but rapidly broken down into a plethora of short peptides and free amino acids. For all timepoints examined, radioactivity was associated with either intact HEL or with material eluting in fractions corresponding to peptides with a molecular mass of 400 Da, or roughly 4–5 amino acids and smaller (Fig. 2). No label was lost in the course of digestion and subsequent gel filtration.

To address the identity of the proteases responsible for degradation of HEL, we examined the effect of several protease inhibitors on proteolysis of HEL *in vitro*. As expected, incubation with lactacystin, an inhibitor of proteasomal degradation, did not affect the degradation of HEL (Fig. 3 lane 4). Digestion of HEL was inhibited by E-64 and leupeptin, both broad-spectrum inhibitors of lysosomal cysteine proteases (Fig. 3 lanes 6, 7). In agreement with this finding, alkylation of free thiols with iodoacetic acid abrogated proteolysis (Fig. 3 lane 5). Pepstatin, PMSF, EDTA, and Fmoc-AENK-NH_2 showed no effect on the degradation of HEL (Fig. 3 lanes 8–11), indicating that aspartyl, serine and metalloproteases, as well as AEP are unlikely to play a dominant role in this processing of HEL *in vitro*.

Taken together, these results demonstrate that HEL is degraded efficiently by endosomal/lysosomal cysteine proteases without generation of discrete processing intermediates.

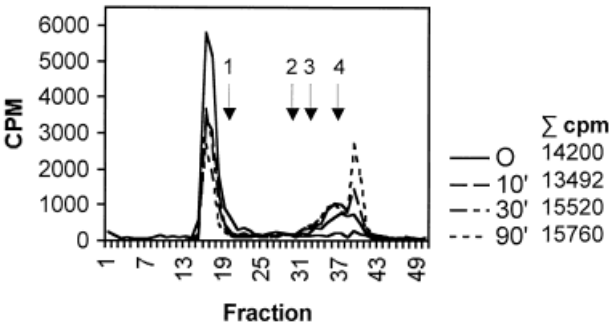

Fig. 2. Profile of HEL digested *in vitro* by lysates of bone marrow-derived APC for 0 min, 10 min, 30 min or 90 min in reducing lysis buffer at pH 5. The digests were resolved by gel infiltration chromatography using the Superdex peptide column HR 10/30. Fractions (0.5 ml) were collected and analyzed by liquid scintillation spectrometry to detect [^{35}S]-labeled degradation, products of HEL. Total recovered radioactivity (Σ cpm) is indicated. As markers, standard peptides were resolved according to the manufacturer's instructions as indicated: 1 cytochrome C (12,500 Da), 2: substance P (1,348 Da), 3: $(Gly)_6$ (360 Da), 4: Gly (75 Da).

2.3 Individual cathepsins attack similar regions of HEL but generate characteristic sets of degradation products

To examine the putative role of cathepsins in the degradation of HEL, intact HEL was digested with CatS, CatL, CatD, or CatB at pH5 *in vitro* under reducing conditions. Processing intermediates were separated by reversed phase-HPLC and identified using MALDI-TOF mass spectrometry and microsequencing. The identity of the vast majority of dominant proteolytic fragments could be established, and only few fragments remained unidentified (Fig. 3 B).

Each cathepsin digested HEL efficiently, consistent with earlier studies showing that CatB and CatS may participate in proteolysis of HEL [12, 17]. A complex mixture of degradation products was obtained for each digest. Nonetheless, a common pattern emerges: the cleavage preferences of the cathepsins tested correlated with the hydrophobic regions of the antigen, so that peptide bonds at residues 30–33, 56–59, 76–78, 85–88 and 108–112 were preferentially cleaved. Each individual cathepsin still yielded a characteristic pattern of proteolytic fragments. CatD showed a relatively high cleavage specificity with only two major targets within the HEL sequence (residues 59 and 110). CatL cleaved preferentially after residues 30, 57, 76 and 85, quite similar to the pattern observed for CatB. In addition to the full-length fragments created by the endoprotease-like activity of CatB, CatB-treated samples contained a number of sim-

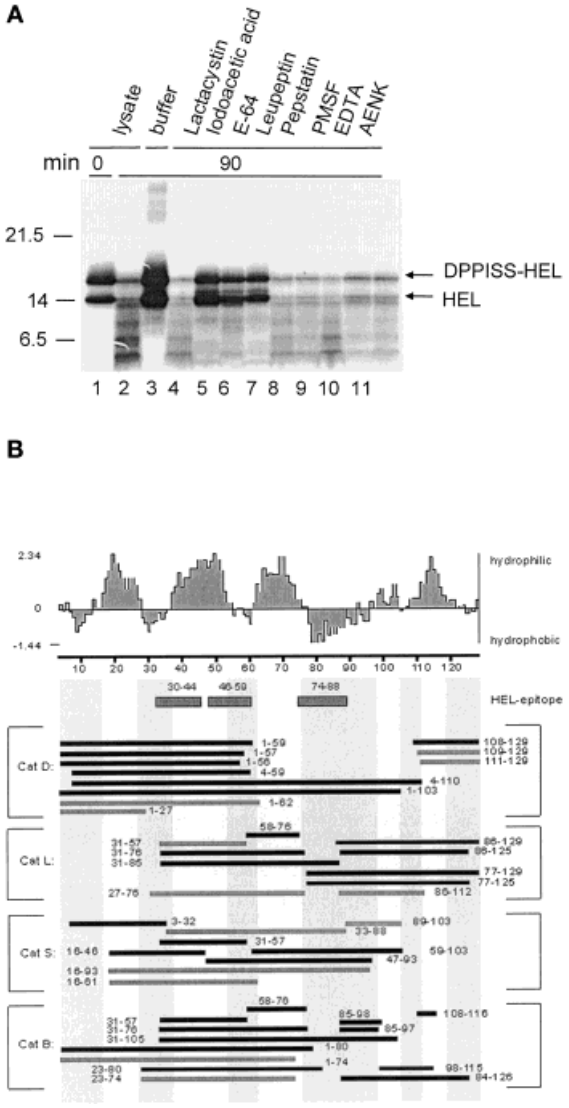

Fig. 3. Effect of inhibitors on degradation of HEL. Whole cell lysates of bone marrow-derived APC were incubated with *in vitro* translated HEL in reducing lysis buffer at pH 5 for 0 min (lane 1) and 90 min in the absence (lanes 2, 3) or presence of inhibitors: Incubation was performed in the presence of 10 μM lactacystin (lane 4), 1 mM iodoacetic acid (lane 5), 20 μM E-64 (lane 6), 1 mM leupeptin (lane 7), 10 μM pepstatin (lane 8), 1 mM PMSF (lane 9), 1 mM EDTA (lane 10), or 2 mg/ml Fmoc-AENK-NH_2 (lane 11). As control, HEL was incubated in lysis buffer alone (lane 3). (B) *In vitro* degradation of HEL by recombinant cathepsins. HEL was incubated with purified CatD, CatL, CatS, ad CatB *in vitro* under reducing conditions. The resulting peptides were separated by RP-HPLC and analyzed by MALDI-TOF mass spectrometry (mass error ± 0.04 %). Black bars represent dominant processing products, grey bars represent less abundant processing products. The top panel aligns the degradation intermediates observed with the hydrophobicity plot after Kyte and Doolittle as well as with three I–A^b-restricted epitopes examined in subsequent functional assays.

ilar fragments lacking 1–3 dipeptides at the carboxy terminus (not shown), in good agreement with the carboxypeptidase-like activity of the enzyme [18]. Samples digested with CatS shared some of the cleavage sites found for CatL or CatB (after 30 and 57). This observation is consistent with data obtained in intact cells, demonstrating overlapping or similar functional roles of CatS and CatL as well as CatS and CatB [2, 17, 19]. However, CatS displayed two rather unusual sites of cleavage (after residue 15 and 45, respectively) that are located outside the hydrophobic stretches of HEL. These cleavages may reflect a unique role of CatS within the processing machinery.

2.4 Intracellular degradation of HEL after BCR-mediated uptake does not result in discrete processing intermediates

We next sought evidence that the major known cathepsins are involved in processing of HEL in living cells. For this purpose, we used splenic B cells of transgenic mice bearing HyHEL-10, a BCR specific for HEL [20]. This high-affinity antibody recognizes a discontinuous epitope on conformationally intact HEL and binds to the antigen predominantly via hydrogen bonds and van der Waals interactions, as defined by the crystallographic structure of the HEL-HyHEL-10 complex [19, 21]. We took this approach to ensure that a population of conformationally homogeneous, native HEL molecules could be tracked in synchronous conditions.

To characterize binding and uptake of the antigen by transgenic B cells, splenocytes were incubated at 4 °C with radiolabeled HEL, washed and lysed, followed by direct analysis of the lysates on SDS-PAGE. Binding of HEL to the splenic B cells was 10^3–10^4 times more efficient for the transgenic BCR-bearing cells than for non-specific adsorption to wild-type splenocytes (data not shown). Interactions between HEL and HyHEL-10 were disrupted upon exposure of the splenocytes to buffer at pH 3 but not at physiological lysosomal/endosomal pH 4 or 5 (data not shown). This suggests that the pH in endosomes/lysosomes by itself is not sufficient to cause detachment of the antigen from the antibody. The fully reduced form of HEL, which was generated by treatment with DTT, was unable to bind efficiently to the HyHEL-10 receptor (Fig. 4 A). This implies that the reducing environment of the endocytic pathway promotes unfolding, possibly exacerbated by low pH, with attendant loss of the HyHEL-10 epitope and is most likely responsible for dissociation of the immune complex.

We next assessed degradation of HEL in the HyHEL-10 transgenic B cells. After binding of radiolabeled HEL to

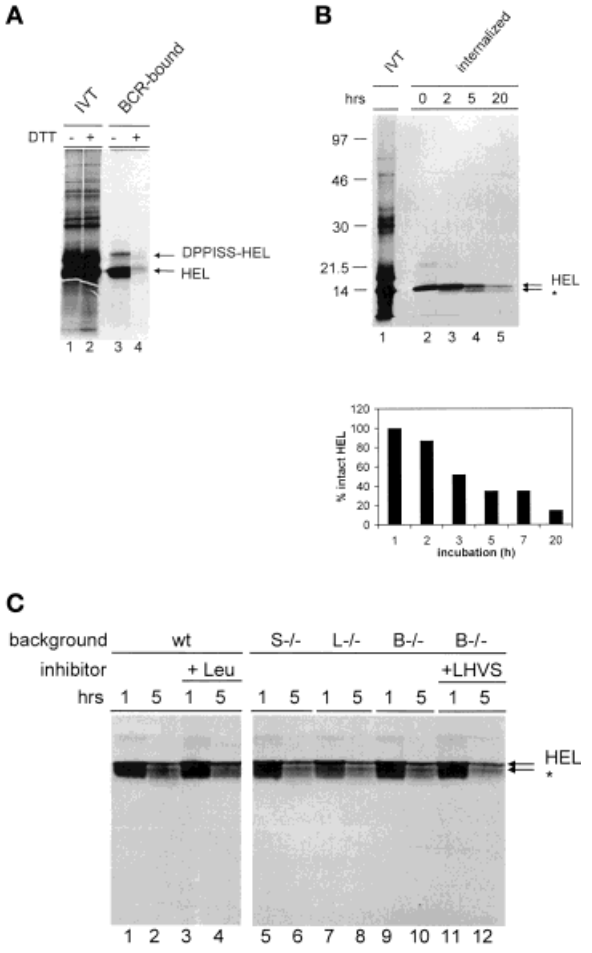

Fig. 4. (A) Binding of native and reduced HEL to the HyHEL-10 BCR. Splenocytes generated from HyHEL-10 transgenic mice were incubated with *in vitro* translated HEL for 1 h at 4 °C. HEL was translated in the absence (lane 1) or presence of 2 mM DTT (lane 2). After incubation with folded (lane 3) or reduced HEL (lane 4), splenocytes were washed in lysed. Samples were subjected to 15 % SDS-PAGE. (B) Degradation of HEL after BCR-mediated uptake. Splenocytes generated from HyHEL-10 transgenic mice were incubated with *in vitro* translated HEL for 1 h at 4 °C, washed, and subsequently incubated at 37 °C for the indicated periods of time. Except for timepoint 0 (lane 2), cells were incubated with PBS at pH 3 for 5 min on ice prior to lysis to remove surface-bound HEL. Whole cell lysates were subjected to 15 % SDS-PAGE. Starting material from IVT is shown in lane 1. The asterisk indicates the single processing intermediate HEL* reliably detected. The bar graph shows quantitation of the remaining intact HEL done by phosphorimaging. (C) Degradation of HEL after BCR-mediated uptake in wild-type and cathepsin-deficient mice. Cells and lysates were prepared as described above. Leupeptin was added during the incubation at a final concentration of 1 mM (lanes 3, 4). Incubations of splenocytes derived from CatB-deficient mice were done in presence of 5 nM LHVS (lanes 11, 12) for additional ablation of CatS. Samples were subjected to 15 % SDS-PAGE. HEL and HEL*(*) are indicated.

the BCR, cells were washed and then shifted to 37 °C for different periods to allow BCR-mediated uptake and subsequent processing of antigen. At each timepoint, cells were exposed to pH 3 to remove sufarce-bound antigen that had failed to be internalized. Whole cell lysates of these cells were analyzed by SDS-PAGE.

Uptake of the antigen by the BCR was detected as early as 15 min after binding, consistent with earlier studies [22]. Intracellular accumulation of HEL reached a maximum after 1 h (data not shown). Some 30 % of HEL retained after 5 h of incubation, as judged quantitatively by phosphorimaging (Fig. 4 B). The degradation pattern observed in intact B cells was very similar to that seen after *in vitro* digestion (see Fig. 2). No discrete processing intermediates of HEL could be detected using *in vitro* translated HEL labeled with either [^{35}S] methionine or [^{35}S]cysteine, with the exception of one degradation fragment migrating slightly faster than the native protein (HEL*). Similar observations were made when the degradation experiments were done using iodinated HEL (data not shown).

2.5 CatB, CatS, and CatL are not required for degradation of HEL *in vivo*

To investigate whether the major known cathepsins present in splenocytes are involved in degradation of HEL, experiments as described above were performed in cells from HyHEL-10 transgenic mice that had been crossed onto different cathepsin-deficient backgrounds.

Degradation of HEL by B cells from CatB-, S-, and L-deficient mice was indistinguishable from that seen in control cells. Incubation of CatB-deficient splenocytes with the CatS-selective inhibitor LHVS allowed the additional ablation of CatS and did not alter the degradation pattern. Incubation with leupeptin confirmed that cysteine proteases were not required for bulk degradation of HEL in intact B lymphocytes. However, it appeared that the quantity of HEL* is reduced in the presence of leupeptin, suggesting that particular cleavages are performed by (a) cysteine protease(s) different from the cathepsins analyzed here. Although AEP is a cysteine protease, it is not a likely candidate, because its activity is known not to be influenced by leupeptin [15, 22a].

Thus, the degradation of HEL in intact B lymphocytes does not follow a pathway that would generate distinct dominant intracellular processing intermediates, but proceeds in a highly processive manner, in agreement with the results obtained using cell lysates.

2.6 CatS is required for efficient presentation of HEL_{30-44} and HEL_{46-59}

The I-A^b background onto which the HyHEL-10 transgene and the cathepsin deficiencies had been introduced precluded a straightforward analysis of antigen presentation by B cells. The I-A^b haplotype is known to be a low responder towards HEL, in contrast to I-A^k and I-E^k [23]. Consistent with these observations, to stimulate I-A^b-restricted HEL-specific hybridomas, far higher concentrations of antigen were required than those reported for I-A^k (see below). Importantly, no differences were observed in presentation efficiency between the Ig-transgenic and wild-type animals. We nonetheless examined the possible effects of cathepsin deficiencies on presentation of HEL, and therefore used unfractionated splenocytes from the different HyHEL-10 transgenic cathepsin-deficient mice as APC. Antigen presentation assays were performed with I-A^b-restricted T cell hybridomas specific for distinct epitopes of HEL. We chose three different HEL epitopes, which are either located within the core of the native antigen (HEL_{46-59}) or surface-disposed (HEL_{30-44}, HEL_{74-88}) (see also Fig. 3 B). First, we examined whether the generation of these epitopes requires processing by cysteine proteases in general, and therefore antigen presentation assay were performed in the presence of leupeptin (Fig. 5 A). Stimulation of the HEL_{74-88}-specific T cell hybridomas was relatively resistant to leupeptin, consistent with earlier data for I-A^b-restricted presentation of HEL [4]. However, presentation of the HEL_{30-44} and HEL_{46-59} regions to the appropriate T cell hybridomas was impaired in presence of leupeptin in a concentration-dependent manner, implying the involvement of cysteine proteases in the generation of these epitopes. This is in line with data that report targeting of similar epitopes of HEL to late endosomal and lysosomal compartments in the I-A^k-background [24].

To elucidate the identity of these cysteine proteases, we examined the influence of individual cathepsins on presentation of the different HEL epitopes. Splenocytes derived from the BCR-transgenic CatS- or CatB-deficient mice were used as APC for the different T-cell hybridomas in the presence of various concentration of HEL and compared with APC derived from BCR-transgenic mice with intact cathepsin repertoire. As shown, lack of CatB did not affect the presentation of each of the three epitopes (Fig. 5 B). However, splenocytes lacking CatS showed impaired presentation of the epitopes HEL_{30-44} and HEL_{46-59}. In contrast, T cells specific for HEL_{74-88} responded normally to APC lacking either CatS or CatB. This experiment demonstrates that APC from CatS-deficient animals are fully functional and that the observed effect is epitope specific.

3 Discussion

Effective antigen presentation requires proteolytic processing of antigens in endocytic compartments. The major endosomal/lysosomal proteases implicated so far in antigen processing are CatS, B, D, E, L [2, 8], and AEP [15]. However, most of the experimental evidence in support of their involvement comes from *in vitro* studies, not from experiments involving living cells. Protease inhibitors, often used to invoke involvement of specific proteases, usually lack a high degree of specificity and target multiple proteases, which complicates the interpretation of their effects. Cathepsin-deficient mice overcome

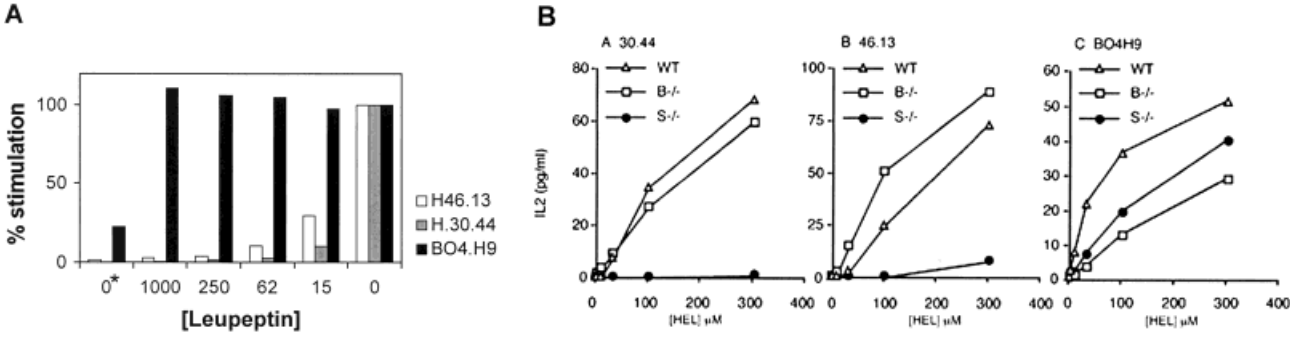

Fig. 5. Antigen presentation assays. (A) Splenocytes derived from wild-type mice were used as APC. Stimulation of the three T cell hybridomas was done with 100 μM HEL in the presence of the indicated concentrations (mM) of leupeptin. As control, HEL was omitted from the incubation (0*). (B) Splenocytes derived from BCR-transgenic cathepsin deficient mice were used as APCs. Cultures of splenocytes together with the indicated T cell hybridomas were incubated with the indicated concentrations (μM) of HEL. Levels of IL-2 in cultured supernatants were determined using an IL-2 ELISA. The results shown are representative of three independent experiments in which each value is the mean of duplicate determinations.

these limitations and allow a more accurate assessment of the role of these proteases in antigen processing and presentation.

To study antigen processing of HEL in live cells, we used splenic B cells derived from HyHEL-10 transgenic mice, which efficiently internalize HEL via their antigen-specific receptor. Degradation of the bulk of HEL internalized via the HyHEL-10 receptor appears to be highly processive. We did not observe dominant processing intermediates in the intracellular degradation of HEL. We used both external ^{125}I-labeling of tyrosine residues and internal biosynthetic labeling with [^{35}S] methionine and [^{35}S] cysteine. It is therefore unlikely that degradation fragments escaped detection due to loss of label during processing. Furthermore, we show that degradation of HEL does not uniquely rely on the presence of CatB, S, or L, and/or other cysteine proteases. It thus appears that the immune system has developed a fairly redundant processing machinery in which several proteases can substitute for each other. This conclusion is consistent with the rather broad substrate specificity of cathepsins *in vitro* [25]. The cleavages introduced by individual cathepsins correlate best with the more hydrophobic regions within the HEL sequence. The different proteases examined attack intact HEL and apparently generate very similar short-lived proteolytic intermediates.

The redundancy of the processing machinery as seen with T cells appears more limited for the generation of particular epitopes, than is observed for the overall degradation of antigen as visualized biochemically. While generation of the $HEL_{74–88}$ epitope is independent of cysteine proteases, production of the $HEL_{30–44}$, as well as the $HEL_{46–59}$ epitope requires their involvement.

Depending on their location within the folded protein, different epitopes probably require distinct levels of processing along the endocytic pathway. Surface-disposed epitopes are likely to require moderate endosomal processing, whereas epitopes deeply buried in the protein core are presumably released in later endosomal/lysosomal compartments for simple reasons of accessibility [24, 26]. HEL appears to be reduced and degraded rapidly. Both the cysteine protease-independent epitope $HEL_{74–88}$ and the cysteine protease-dependent epitope $HEL_{46–59}$ are localized at the surface of native HEL. Hence, the generation of a particular epitope is more likely determined by the protease content of different endosomal compartments than by the structural context of the epitope within the native HEL protein. In addition, the conditions under which dissociation of the internalized immune complex occur might be important for the generation of particular antigenic determinants. The HyHEL-10-HEL complex does not dissociate at endosomal/lysosomal pH, but will do so under reducing conditions. Hence, HyHEL-10 most likely rapidly releases the antigen in the reducing environment of endosomal/lysosomal compartments and might therefore be unable to protect (a) part(s) of HEL from proteolysis.

CatS is required for the presentation of $HEL_{30–44}$ and, to a lesser extent, $HEL_{46–59}$, but not $HEL_{74–88}$. Loading of $HEL_{74–88}$ on class II molecules may occur via the alternative pathway that exchanges Ii for antigenic peptide without involving CatS and HLA-DM [27]. However, this alternative pathway is restricted to early endosomes, while the peptide loading compartment for antigen delivered via surface Ig-receptor is distinct from early endosomes [28]. Our results provide evidence that CatS is not only important for degradation of Ii, but that it also plays a direct role in antigen processing. In contrast to AEP, which controls the initial rate-limiting proteolytic step during intracellular breakdown of intact TTCF, the function of CatS is largely redundant at this stage, and elimination of CatS does not alter the kinetics of intracellular degradation of HEL. We therefore favor the idea that CatS shapes the $HEL_{30–44}$ epitope from intracellular processing intermediates. The ability of CatS, but not CatB, L, or D, to cleave HEL after residue 45 *in vitro*, suggests that the crucial role of CatS for the generation of particular epitopes might be a result of the unique cleavage preference of CatS at this position.

Our model contrasts with the study of Castellino et al., which reports generation of a processing intermediate consisting of two isotypically distinct class II molecules bound to a large HEL fragment [29]. This discrepancy can be explained by the fact that antigen processing depends on the mode of delivery of antigen to the endocytic route (fluid-phase endocytosis *vs.* Ig receptor-mediated uptake) and might be influenced by the MHC haplotype (I-A^k *vs.* I-A^b).

We conclude that not only is the overall degradation of HEL carried out by a set of proteases of overlapping specificity, but also – to a lesser extent – the generation of particular immunodominant epitopes. The recent finding that for efficient presentation of several epitopes of TTCF an “unlocking” cleavage is required at one particular residue [15] might exemplify a unique rather than a general mechanism for antigen processing. Future studies must show whether other, as yet unknown, proteases can be implicated in MHC class II-mediated antigen presentation.

4 Materials and methods

4.1 Reagents and medium

Lactacystin was obtained from Dr. E. Corey (Harvard University, Cambridge, MA). Recombinant and purified cathepsins L and S were generously provided by Dr. R. Riese (Brigham and Women's Hospital, Boston, MA). N-morpholinurea-leucinyl-homophenylalanyl-vinylsulfone-phenyl (LHVS) was synthesized as published [30]. Fmoc-AENK-NH_2 and Fmoc-AEQK-NH_2 were prepared using standard Fmoc-based solid phase peptide synthesis and HPLC. All other chemicals were obtained from commercial sources and were of analytical grade.

Unless stated otherwise, cell cultures were maintained at 37 °C in 5 % CO_2 in air, in complete medium consisting of RPMI 1640 + HEPES, supplemented with 10 % fetal calf serum, 2 mM L-glutamine, penicillin, and streptomycin.

4.2 Mice

Cat B-, S-, and L-deficient mice have been described earlier [12, 13]. Wild-type C57BL/6J (I-A^b) mice as well as transgenic mice (I-A^b) expressing HEL-specific IgM and IgD on their B cells (Tg $BCR^{+/-}$), were purchased from Jackson Laboratories and bred onto the desired cathepsin-deficient background. All animals were maintained under pathogen-free conditions in compliance with institutional guidelines.

4.3 *In vitro* transcription and *in vitro* translation

HEL cDNA was kindly provided by Dr. P. Dempsey (University of Cambridge, Cambridge, GB). HEL cDNA contained HEL preceded by the dog prepro-insulin signal sequence and fused C-terminally to a YL1/2 tag. The cDNA was cloned into the pSP72 vector (Promega Corp.), linearized, and transcribed using T7 polymerase (Promega Corp.). The optimal amount of mRNA for translation was determined empirically for each separate batch of RNA. Before use in translation reactions, mRNA was heated at 80 °C for 2 min and then chilled on ice.

In vitro translations were performed for 1 h at 30 °C with rabbit reticulocyte lysate (Flexi®; Promega Corp.) in the presence of dog pancreas microsomes (DPMs) prepared as described [31]. After translation, microsomes were pelleted and solubilized in SDS-sample buffer for direct analysis of the translated material. Translation products subsequently used for *in vitro* digestion were lysed for 30 min on ice at pH 5, in lysis buffer (LB) (1 % Nonidet P-40, 5 mM $MgCl_2$, and 3 mM DTT). Microsomal pellets used for *in vivo* studies were lysed for 30 min on ice in NP-40 lysis buffer (0.5 % NP-40, 50 mM Tris-HCl, pH 7.4, 5 mM $MgCl_2$).

4.3 *In vitro* degradation of HEL in whole cell lysates from murine bone marrow-derived APC

APC were obtained essentially as described [32, 33]. For the generation of whole cell lysates, cells were solubilized at a concentration of 10^8 cells/ml for 30 min at pH 5 in LB. Whole cell lysates were incubated with microsome-associated HEL of a standard translation reaction and incubated at 37 °C for the indicated periods of time. Samples were analyzed by SDS-PAGE in a 15 % acrylamide resolving gel followed by autoradiography.

4.4 Analysis of digested HEL by gel filtration chromatography

Solubilized HEL obtained by *in vitro* translation, was subjected to ultrafiltration through a Centricon® concentrator (Cut-off 10,000 MW; Amicon, Inc.) to remove unincorporated label. This material was used for *in vitro* proteolysis in whole cell lysates for the indicated periods.

Gel filtration chromatography was performed on the Pharmacia FPLC® System (Amersham Pharmacia Biotech), on a Superdex peptide column HR 10/30 in 20 mM Tris-HCl, pH 7.4, 100 mM NaCl, at a flow rate of 0.4 ml/min. Fractions (0.5 ml) were collected and analyzed for [^{35}S]-labeled peptides by liquid scintillation spectrometry (LS 6500 Scintillation System; Beckman Instruments, Inc.).

4.5 *In vitro* degradation of HEL by purified cathepsins and analysis of the processing products

HEL was solubilized and reduced using 3-bromopropyl-trimethyl-ammoniumbromide, a method that had been demonstrated not to influence proteolytic degradation of HEL [34]. Substrate solution containing 2 mg/ml HEL in 150 mM phosphate, containing 4 mM DTT, adjusted to pH 5, was incubated at 37 °C with respectively 20 μg/ml human CatB, 10 μg/ml bovine spleen CatD, 1–3 μg/ml CatL, 1–5 μg/ml CatS for 10 min to 20 h. Digestion was terminated by addition of 0.5 M glycine pH 2.

Proteolytic products were separated by micropore reversed-phase HPLC using a C8 column (protein & peptide, 2.1 × 150 mm, Vydac) equilibrated with 100 % system A. Elution was carried out at a flow rat of 0.2 ml/min using an acetonitrile gradient: 0–5 min 0–15 % system B, 5–50 min 15–55 % system B (system A = 0.05 % TFA in water, system B = 80 % acetonitrile, 0.05 % TFA in water). The column effluent was monitored with a diode array detector (1000S, Applied Biosystems). Dominant eluting peak fractions were collected and dried in a Speedvac. Each sample was subjected to matrix-assisted laser desorption ionization mass spectrometry (Bruker Reflex III, Bruker) as well as Edman protein microsequencing (494A "procise", Applied Biosystems). The relative quantity of HEL processing products was estimated by UV signals.

4.6 Degradation of HEL in murine splenocytes

Translation material solubilized with detergent was incubated with ten volumes of 30 % bovine serum albumin for 10 min at 37 °C to complex the detergent and to allow exposure of the translated material to intact cells without cytolysis [35]. The detergent-depleted materials was incubated with isolated murine splenocytes for 1 h at 4 °C to allow binding of HEL to the BCR. Cells were then washed, resuspended in prewarmed medium, and incubated for the times indicated. At each timepoint, cells were harvested as described [33]. Cell lysates were analyzed by 15 % SDS-PAGE followed by autoradiography. A Storm 860 phosphorimagersystem (Molecular Dynamics, Inc.) and the appropriate software (ImageQuant™: Molecular Dynamics, Inc.) were used for quantitation of protein bands.

4.7 Antigen presentation assays

Splenocytes were γ-irradiated (3,300 rad) and plated at a density of 5×10^5 cells per well in 96-well plates. HEL was added to the splenocytes at the indicated final concentrations. T cell hybridomas (10^5/well) were co-cultured with the APC in a final volume of 200 μl. I-A^b-restricted HEL-specific T cell hybridomas used were H30.44 specific for HEL_{30-44}, H46.13 specific for HEL_{46-59}, and BO4H9 specific for HEL_{74-88} BO4H9 was a kind gift from Diane Mathis (Joslin Diabetes Center, Boston). Afer 24 h, 100 μl of the supernatants were transferred to a new 96-well plate, frozen and thawed. Ag-specific stimulation of the T hybridomas was assessed by measuring production of IL-2 in the supernatants, using a commercially available IL-2 ELISA kit (Pharmingen). ELISA were performed according to the manufacturer's specifications. Concentrations of IL-2 in individual samples were determined using the cytokine standard provided by the kit.

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