Cathepsin S and an asparagine-specific endoprotease dominate the proteolytic processing of human myelin basic protein *in vitro*

Hermann Beck[1], Gerold Schwarz[1], Christian J. Schröter[1], Martin Deeg[1], Daniel Baier[1], Stefan Stevanovic[2], Ekkehard Weber[3], Christoph Driessen[1,4] and Hubert Kalbacher[1]

[1] Medical and Natural Sciences Research Center, University of Tübingen, Tübingen, Germany
[2] Department of Immunology, Institute of Cell Biology, University of Tübingen, Tübingen, Germany
[3] Institute of Physiological Chemistry, University of Halle, Halle, Germany
[4] Department of Medicine II, University of Tübingen, Tübingen, Germany

The biochemical characterization of antigen degradation is an important basis for a better understanding of both the immune response and autoimmune diseases mediated by MHC class II molecules. In this study we used high-performance liquid chromatography and mass spectrometry to analyze the processing of myelin basic protein (MBP), a potential autoantigen implicated in the pathogenesis of multiple sclerosis. We resolved the kinetics of MBP processing by lysosomal extracts or purified endocytic proteases, identified the major cleavage sites during this process and assigned them to the activity of proteolytic enzymes. Proteolytic processing of MBP is mostly guided along the hydrophobic regions of the protein. It is initiated by two proteolytic steps (after N^{92} and S^{110}) that are performed by an asparagine-specific endopeptidase (AEP) and by cathepsin (Cat) S, respectively. The resulting processing intermediates are converted into more than 60 different species of 20–40-mers due to the activity of endopeptidases including CatS, D and L. The fragments thus generated are subsequently degraded by C- or N-terminal trimming. Strikingly, the initial cleavages during MBP processing affect two immunodominant regions of the potential autoantigen [MBP(85–99) and MBP(111-129)] in an inverse manner. CatS directly generates the N terminus of the epitope MBP(111–129) in large quantities during the initial phase of processing, which might explain the immunogenicity of this region in spite of its relatively poor binding to HLA-DR4. In contrast, the dominant cleavage by AEP mediates the destruction of MBP(85–99) unless the epitope is protected, *e.g.* by binding to HLA-DR. Our results thus characterize the proteolytic events during processing of MBP on a molecular level and suggest a biochemical basis for the immunogenicity of the immunodominant epitopes, which could serve as a guideline for future therapeutic strategies.

Key words: Antigen processing / Myelin basic protein / Cathepsin / MHC class II / B lymphocyte

Received	15/12/01
Revised	12/9/01
Accepted	9/10/01

1 Introduction

While many details of the assembly, transport, and processing of MHC class II molecules have been resolved in recent years, the generation of antigenic peptide from complex protein and its binding to MHC class II are still poorly understood [1–3]. Cathepsins (Cat) represent the most abundant family of endocytic proteases and new members of this group continue to be discovered [4, 5]. CatB, D, E, L and S have been implicated in antigen processing and the generation of immunogenic epitopes [6]. However, cathepsins represent by no means the entire spectrum of endocytic proteases and unrelated proteases might be equally important. Recently, the asparagine-specific endopeptidase (AEP) has been identified as the key enzyme that mediates the rate-limiting step in the intracellular processing of tetanus toxoid C fragment (TTCF) [7]. For TTCF, the generation of dominant processing intermediates by AEP precedes the

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Abbreviations: AEP: Asparagine-specific endopeptidase **Cat:** Cathepsin **TTCF:** Tetanus toxoid C fragment **AMCA:** 7-amino-4-methyl-coumarin-3-acetic acid **B-LC-line:** Lymphoblastoid B cell line **ESI-MS:** Electrospray ionization source mass spectrometry **E-64:** Trans-epoxysuccinyl-L-leucylamido-(4-guanidino)butane **HPSEC:** High-performance size exclusion chromatography **MALDI-MS:** Matrix-assisted laser desorption ionization mass spectrometry **MBP:** Myelin basic protein **TFA:** Trifluoroacetic acid **Z:** Benzyloxycarbonyl

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formation of the immunogenic epitopes [8]. Whether such initial "unlocking" of intact antigenic protein is a more general characteristic of the processing of exogenous antigen, whether it is performed by a single enzyme, a class of enzymes or by unrelated proteases, whether the sites of such dominant processing steps are determined by the specificity of certain proteases or merely by the structure of the antigenic protein itself, remain largely open questions.

Several lines of evidence suggest an important role of myelin basic protein (MBP) as an autoantigen in the pathogenesis of the MHC class II-associated autoimmune disease multiple sclerosis (MS). A dominant autoreactive T cell response against certain regions of human MBP can be found in a large portion of patients with the disease [9, 10]. The immunodominant epitope in the context of HLA-DR2, the haplotype associated with the strongest susceptibility for MS, has been localized to MBP residues 82–100, with MBP(85–99) constituting the minimum T cell epitope [11–13]. Similarly, DRB1*0401, the most common DR4 allele in the Caucasian population, is associated with susceptibility for MS. DRB1*0401restricted autoreactive T cells were found to recognize MBP(111–129), with the major T cell epitope corresponding to residues MBP(116–123) [10]. Processing of MBP and the generation of the antigenic determinants remain poorly characterized to date. A better understanding of the processing of MBP should not only provide new insights in the function of the antigen-processing machinery in general, but might also open ways to interfere with the presentation of epitopes implicated in the pathogenesis of MS.

We here show that proteolytic processing of MBP by lysosomal proteases *in vitro* is dominated by two initial cleavages performed by CatS and AEP. Further proteolysis of the resulting intermediates is largely guided along the hydrophobic regions of the protein. Surprisingly, one of the major processing sites lies within the immunodominant region MBP(85–99). Binding to MHC class II adequately protects this epitope from destruction, highlighting the importance of determinant capture by class II molecules for the preservation of the major immunogenic epitope of MBP.

2 Results

2.1 The initial stage of proteolytic processing of MBP is controlled by AEP and CatS

The endocytic compartment of APC contains a complex mixture of individual proteases each of which is potentially involved in antigen processing. To characterize the processing of MBP *in vitro*, lysosomes were isolated from a human B cell line by differential centrifugation and recombinant human MBP was digested with lysosomal extracts. At different time points the resulting pattern of degradation products was resolved by RP-HPLC and UV absorption was detected at 214 nm (Fig. 1a). During the initial phase (<80% of substrate turnover) of proteolytic *in vitro* processing, the bulk of protein eluted in four distinct peaks (marked I-IV) shortly before intact MBP. After prolonged incubation intact MBP was no longer detected, and peaks I-IV slowly converted into poorly resolved material that eluted after shorter retention times. Human MBP, obtained from human white matter preparation by semipreparative HPLC as described [14], represents a mixture of the unmodified protein along with its monomethylated (6%) and dimethylated (60%) species. This results in more complex HPLC profiles after processing with lysosomal extracts (Fig. 1b), where the kinetics and the ratio of the MBP degradation products II, III and IV were altered compared to recombinant MBP. However, dominant molecular masses corresponding to identical degradation fragments were identified from peaks I-IV by matrix-assisted laser desorption ionization mass spectrometry (MALDI-MS) when either recombinant MBP or MBP purified from human CNS was subjected to *in vitro* proteolysis in the same manner (see also below), confirming that the initial phase of proteolytic processing of purified MBP *vs*. recombinant MBP *in vitro* followed a similar cleavage pattern.

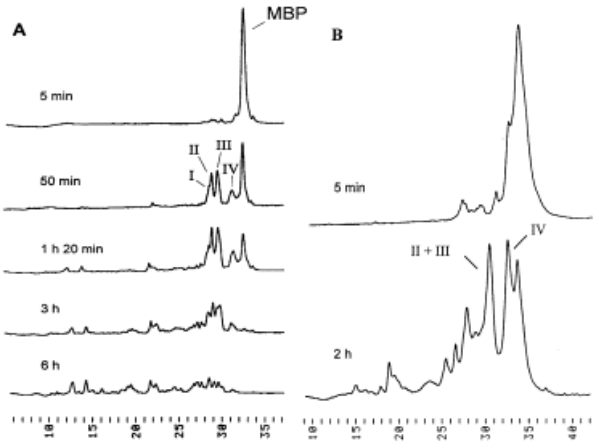

Fig. 1. Reversed-phase HPLC profiles of human MBP after different incubation times with disrupted lysosomes at pH 5.4. Substrate (0.1 mM) was incubated with disrupted lysosomes equivalent to 5×10^5 cells/μmol substrate. (A) Recombinant MBP, (B) MBP purified from human CNS. The peaks indicated consist of: I, MBP(111–170); II, MBP(93–170); III, MBP(1–92); IV, MBP(1–110), as inferred from microsequencing.

The degradation pattern observed suggested that processing of MBP *in vitro* occurred in a stepwise fashion: during an initial phase MBP was converted into a limited number of dominant processing intermediates that were degraded into multiple smaller fragments as processing proceeds. Using protease inhibitors, we tried to identify the enzymes responsible for the generation of the dominant degradation products at the first stage of processing (50–80% of substrate turnover). While inhibition of serine-, metallo- and aspartate-proteases had no detectable influence on the *in vitro*-degradation pattern resolved by HPLC (data not shown), the addition of trans-epoxysuccinyl-L-leucylamido-(4-guanidino) butane (E-64), a generic inhibitor of cysteine proteases, interfered with formation of HPLC peaks I and IV, while peaks II and III were still present (Fig. 2). This strongly suggested that peaks I and IV were the result of MBP processing mediated by a cysteine protease. CatS, L and B represent the most abundant cysteine proteases in the endocytic compartment of antigen-presenting cells. While the former has a rather broad pH optimum ranging from pH 4.5–pH 7, the latter two are inactivated by exposure to neutral pH [15–17]. To narrow down the proteases possibly involved in the generation of peaks I and IV, we performed a similar set of *in vitro* processing experiments at neutral pH. Under these conditions, peaks I and IV remained unchanged, consistent with their possible generation by CatS, while peaks II and III disappeared. Finally, when MBP was digested with purified CatS, only protein peaks eluting at the retention times of peaks I and IV were observed, which strongly supported that CatS is responsible for one of the initial cleavages of MBP during processing *in vitro*.

Based on the results described above, the endocytic protease(s) responsible for the generation of the dominant peaks II and III was/were characterized by (i) inactivity at neutral pH and (ii) resistance to common generic protease inhibitors. The legumain-type protease AEP that was recently discovered as the key protease involved in the unlocking of TTCF [7] matched these criteria. We therefore synthesized a competitive inhibitor of AEP activity, benzyloxycarbonyl (Z)-AENK-NH_2, along with its control analogue Z-AEQK-NH_2, to test whether specific inhibition of AEP during *in vitro* proteolytic processing of MBP would affect the formation of HPLC-

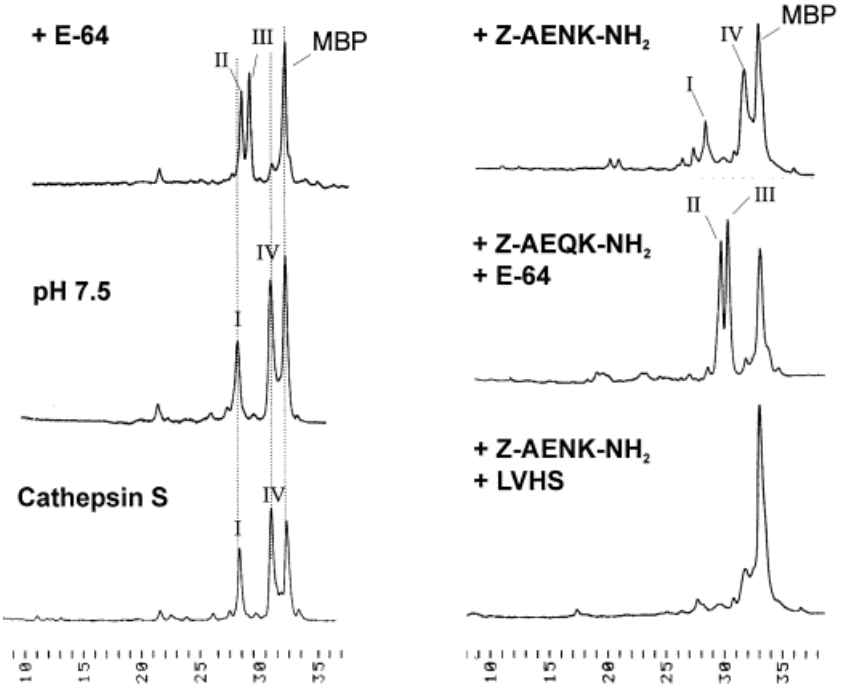

Fig. 2. Processing of human MBP under various conditions. Left panel: top: disrupted lysosomes preincubated with 10 μM E-64; middle: disrupted lysosomes preincubated at pH 7.5 for 1 h, 37°C; MBP incubated with Cathepsin S at pH 5.4. Right panel: top: co-incubation with 5 mg/ml Z-AENK-NH_2; middle: co-incubation with 5 mg/ml Z-AEQK-NH_2, 10 μM E-64; bottom: co-incubation with 5 mg/ml Z-AENK-NH_2, 5 nM LVHS. The experiments were run in parallel and processing was stopped at the same time point. Fragments of human MBP in the indicated peaks: I, MBP(111–170); II, MBP(93–170); III, MBP(1–92); IV, MBP(1–110). Substrate (0.1 mM) was incubated with disrupted lysosomes equivalent to 5×10^5 cells/μmol substrate. In all cases, identification was achieved by sequencing.

peaks II and/or III. Indeed, formation of both peaks was antagonized by addition of AENK, consistent with a dominant role of AEP for their generation. In contrast, addition of AEQK and E-64 left peaks II and III unaffected, which strongly supported that AEP was the protease responsible for generation of the MBP fragments resolved in peaks II and III after proteolytic processing *in vitro*.

To confirm that exclusively these two proteases were responsible for processing of MBP during the initial phase of degradation *in vitro*, we combined the AEP antagonist AENK with LVHS, a compound that specifically inhibits CatS at nanomolar concentrations [18]. As shown in Fig. 2, MBP processing was minimized and formation of peaks I–IV was essentially blocked under these conditions, as expected. These results strongly suggested that two major proteases, CatS and AEP, attack MBP during the initial phase of its degradation by lysosomal proteases and thus dominate the first phase of proteolytic MBP processing *in vitro*.

2.2 Degradation of human MBP by purified cathepsins

To dissect the processing of MBP by cathepsins on a molecular scale we mapped the main cleavage sites of human MBP for the cathepsins B, D, L and S. To this end, recombinant MBP was incubated with the respective enzyme and the degradation products were resolved by RP-HPLC followed by identification of the resulting fragments by mass spectrometry and microsequencing (Fig. 3).

The aspartate protease CatD cleaved with high specificity between two aromatic residues (FF) that are present twice in the sequence of MBP (44/45 and 89/90). Even with high enzyme concentrations and after prolonged incubation times only three fragments were generated by CatD, consistent with earlier observations [19].

CatS and CatL generated degradation patterns that were far more complex than those caused by CatD. CatS and CatL were similar to each other with regard to their cleavage sites within MBP, but differed in the relative contribution of each of these positions to the total turnover (Table 1 and Fig. 3). CatS preferentially cleaved between S^{110}/L^{111}, this cleavage accounted for 90% of the processing observed at $t_{1/2}$ of the substrate. After extensive proteolysis we observed more than 40 individual peptides that represented processing products of MBP by CatS. Similar to CatS, CatL had a major processing site at position S^{110}/L^{111}, however, this accounted for only 20% of the processing products observed after limited

Fig. 3. Preferential cleavage sites of human MBP after incubation at pH 5.4 with cathepsin D, L, S or disrupted lysosomes. After full substrate turnover, proteolytic products were separated by microbore RP-HPLC and characterized by MALDI-MS and Edman-sequencing as described in Sect. 4. Rate-limiting initial cleavage sites are indicated by bold arrows, cleavages probably caused by exopeptidases are indicated by dotted arrows. *For cathepsin L, only main cleavage sites were shown. The immunodominant regions of MBP in the context of HLA-DR2 and –DR4 are printed in bold.

digestion. For CatL, degradation of MBP was dominated by cleavage at K^{91}/N^{92}, accounting for about 50% of the processing at $t_{1/2}$ of the substrate. When MBP was digested with CatS, only minute amounts of degradation occurred at this position. CatB is reported to show low endopeptidase activity only at pH >5.5 [17, 20]. Some very weak processing activity of CatB was observed at pH 6.0, but even at high CatB concentrations and incubation times of more than 20 h we were unable to detect significant endopeptidase activity of CatB on MBP (not shown). At pH 5.4, low peptidylpeptidase activity of CatB was observed, as expected. Thus, CatB is unlikely to be involved in endoproteolytic processing of MBP.

Table 1. Relative distribution of cleavage sites after processing with purified CatL and CatS[a)]

Cleavage site	Cathepsin C	Cathepin L
$K^{91}//N^{92}$		50%
$S^{110}//L^{111}$	90%	20%
others	10%	30%

a) Purified recombinant MBP was incubated with the respective protease and the contribution of either processing site to the total protein turnover by each protease was determined based on UV 214 nm signals of the HPLC peaks containing the respective fragments.

2.3 Initial processing of MBP *in vitro* occurs mainly at positions N^{92}/I^{93} and S^{110}/L^{111}

To identify the proteolytic fragments that arise after incubation of MBP with disrupted lysosomes, the digested material was resolved by RP-HPLC, followed by analysis using mass spectrometry and Edman microsequencing. The rate-limiting cleavages that initialized processing generated large fragments of 60 to 110 amino acids in length. Entirely consistent with the results described above, positions N^{92}/I^{93} and S^{110}/L^{111} were identified as the dominant sites of initial processing of MBP (Fig. 3 and 4a). N^{92}/I^{93} matches the substrate specificity known for AEP, while S^{110}/L^{111} was identified above as the preferential cleavage site for CatS. Based on UV214 nm-signals in RP-HPLC, we estimated that at $t_{1/2}$ of substrate turnover cleavage after position N^{92} accounted for about 50–60% and after position S^{110} for about 30–40% of processing products. All other fragments identified after $t_{1/2}$ represented minor amounts visible in the MALDI spectra but below the threshold of UV-spectrometry or Edman sequencing.

The second phase of processing with subsequent destruction of the initially formed processing intermediates occurred much slower and with a considerable lower specificity than the initial steps, but was still dominated by endoproteolytic processing. At the time of >90% MBP substrate turnover, nearly 60 different species of peptide of 20–40 amino acids in length could be detected. At this time peptides with intact N^{92}/I^{93} bond were no longer observed, suggesting that fragment 1–110 is an intermediate species with a short half-life time. When aligned with a map of the estimated hydrophobicity of MBP, the ends of the fragments generated during the second phase of processing strikingly correlated with the predicted hydrophobic regions within the protein, suggesting that processing is largely guided by properties of the antigen itself. Of note, identical major proteolytic processing sites were detected when MBP purified from human brain was used instead of its recombinant version.

After extensive processing at hydrophobic regions, exoprotease activity became dominant. Most processing products isolated after digestion with lysosomes at later time points showed some N- or C-terminal trimming by exopeptidases. In general, exopeptidase activity was stronger at the N terminus of the peptides than at their C-terminal end. For some of the processing products this trimming allowed a direct identification of the fragments by MALDI spectra without the need of further sequencing (Fig. 4b). Peptides of only few amino acids in length or free amino acids represented the final products of MBP-processing with lysosomal fractions.

2.4 Cleavage after aspagine residues guides processing of α-lactalbumin, but not myoglobin

The dominant proteolytic step after N^{92}/I^{93} in MBP processing, which is most likely mediated by AEP, strikingly resembles the processing pathway recently described for TTCF [7]. We therefore hypothesized that asparagine-specific processing might be important to guide antigen degradation in the endocytic compartment in a more generalized way that could apply to a wider selection of proteins. Using the same approach as described above, we, therefore, analyzed the *in vitro* processing of two additional proteins, α-lactalbumin (α-lac) and myoglobin. Little is known about the proteolytic processing and the T cell epitopes generated from α-lac. Degradation of myoglobin by endocytic proteases of porcine macrophages had been demonstrated to release the major T cell epitopes in a CatD-dependent fashion [21, 22], suggesting that AEP might be of less importance for this particular antigen. However, because the protease content and the processing machinery between different types of APC as well between species may well differ, experiments with human B cell-derived endocytic extracts are required to draw more definite conclusions.

The results obtained for α-lac were strikingly similar to those observed for MBP processing: degradation during the initial phase of α-lac processing was dominated by cleavages after asparagine residues (Fig. 5). The combination of asparagine in P1 with a hydrophobic amino acid in P1 (NI for MBP, NI and NY for α-lac) characterized major sites for initial processing. A second dominant cleavage site (F^9/R^{10}) did not contain an asparagine residue. The subsequent processing of α-lac during the second stage of processing followed a number of endoproteolytic cleavage sites, preferentially after N. As is the case for MBP, the N-P bond remained stable during α-lac processing.

In stark contrast, AEP activity was dispensable for both the normal course of destruction and the generation of antigenic fragments of myoglobin by B cell-derived lysosomal fractions (Fig. 6a). Cathepsin D clearly dominated the initial proteolytic steps of myoglobin processing, generating the fragments 1–29, 33–69 and 73–153 as stable intermediates. This pattern could be blocked by addition of pepstatin A and was reproduced using purified cathepsin D, entirely consistent with earlier data from porcine macrophages [21]. Neither had the inhibition of AEP using iodoacetamide or the AEP-specific competitor Z-AENK-NH_2 a major effect on the fragmentation pattern observed, nor was purified AEP able reproduce any of the dominant fragments observed after incubation with lysosomal fractions.

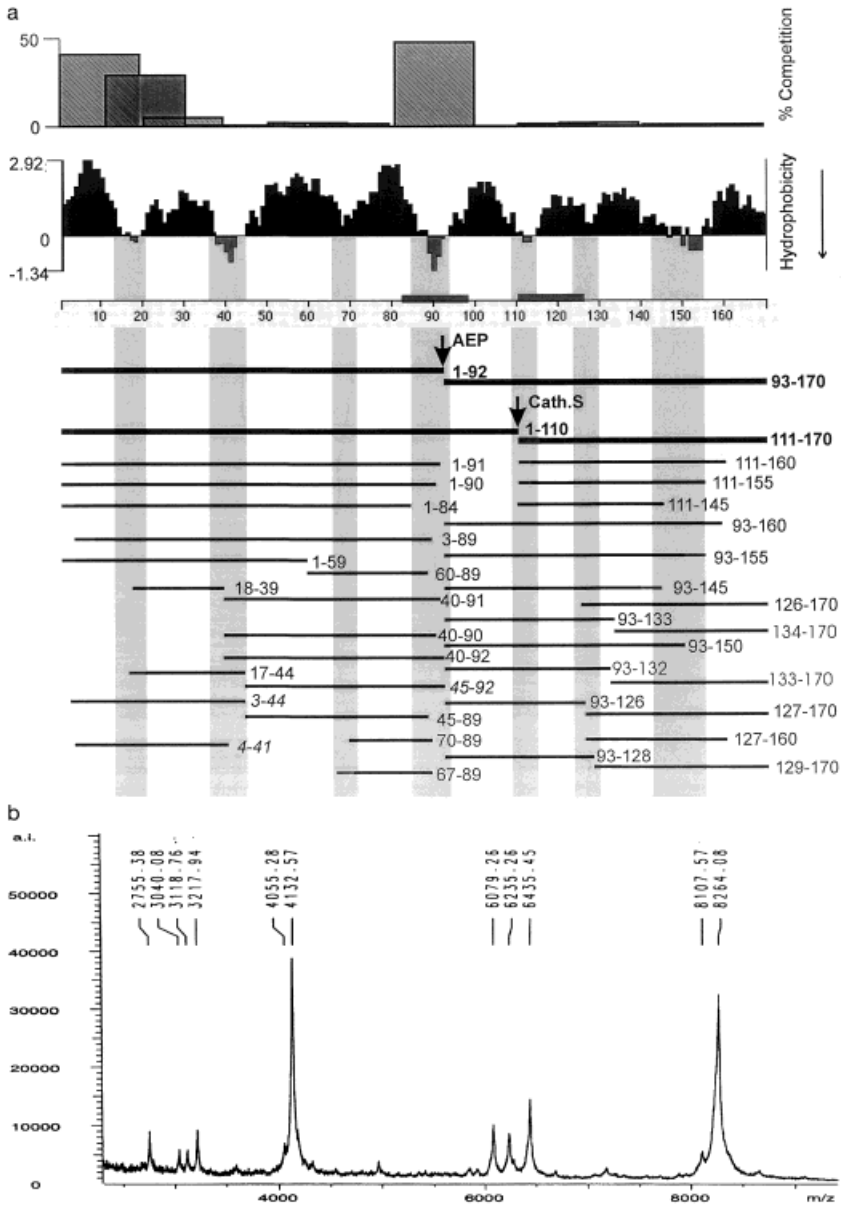

Fig. 4. (a) Fragments of human MBP released by disrupted lysosomes at pH 5.4 after 90% of substrate destruction. The peptides identified are symbolized by black bars, exopeptidase activity is indicated in gray. The dominant initial fragments are printed in bold. Cleavage sites are aligned with the hydrophobicity plot after Kyte and Doolittle and the immunodominant regions in context of HLA-DR2 and -DR4 are indicated. Top: competitive binding-assay of overlapping 19mer MBP peptides to AMCA-HA(307–319) and HLA-DRB1*0401 deferred from Muraro 1997 [10]. (b) MALDI-spectrum of peptides in peak I of Fig. 1. Mass $(M+H)^+$ 8264.1: MBP(92–170). $(M+H)^+$ 6435.5, 6235.3, 6079.3: MBP(111–170), MBP(113–170), MBP(114–171) The difference between observed and calculated mass accounts to about 0.02%).

Further proteolytic processing of the initial degradation intermediates converted the fragment 1–29 into 1–13 and 14–29, while 73–153 yielded 73–111, 112–137 and 138–153 in a CatD-dependent fashion (Fig. 6b). Thus proteolytic processing of myoglobin is clearly dominated by CatD, while AEP plays no essential role. We conclude that unlocking of intact protein antigen by AEP initiates processing of MBP and lactalbumin, but is not a general characteristic for the proteolytic pathway mediated by human B cell-derived lysosomal proteases.

Fig. 5. Cleavage sites of α-lactalbumin after *in vitro*-processing with disrupted lysosomes. Rate limiting initial cleavage sites at pH 5.4 at about 90% of substrate destruction were indicated by bold arrows, the AEP-sensitive amino acid Asn is stressed.

3 Discussion

Myelin basic protein is believed to be a major autoantigen in the pathogenesis of multiple sclerosis, a demyelinating autoimmune disease associated with the MHC class II alleles HLA-DR2 and HLA-DR4 [10, 11]. T cell activation by native human MBP relies on the activity of cysteine proteases and on the internalization of MHC class II [14, 23]. In contrast, preparations of lipid-free denatured MBP led to T cell activation independent of cysteine proteases or endocytic transport [14]. MBP exposed to APC *in vivo* is much more likely to resemble native MBP than to match a lipid-free denatured MBP preparation, which argues that intracellular processing of MBP is presumably required for efficient activation of MBP-specific T cells *in vivo.* MBP is a very loosely folded protein that represents an easy target for all types of proteases once it has been purified from its adjoining lipids. Native MBP, however, is likely to reach a protease-accessible state similar to that of purified MBP after native MBP has been exposed to the denaturing environment of the endocytic compartment. To mimic this intracellular situation in late endocytic compartments, we chose denatured recombinant MBP and a reducing and acidic (pH 5.4) environment for our experiments *in vitro*.

It is not known how MBP is converted into antigenic peptides and which enzymes might be involved. Cathepsins are commonly believed to be the major proteases responsible for antigen breakdown in the MHC II compartment [2, 6]. However, other known as well as yet-unknown proteases could be at least of equal importance, perhaps best illustrated by the recent identification of AEP as the key protease in TTCF processing [7].

Our results show that processing of MBP occurs in three distinct phases: first, a rapid breakdown of intact MBP is achieved by dominant cleavages after N^{92} and S^{110}, generating large processing intermediates of 60–110 amino acids in length. These two cleavages account for more than 80% of all degradation products identified after $t_{1/2}$ of substrate turnover. Subsequent processing is driven

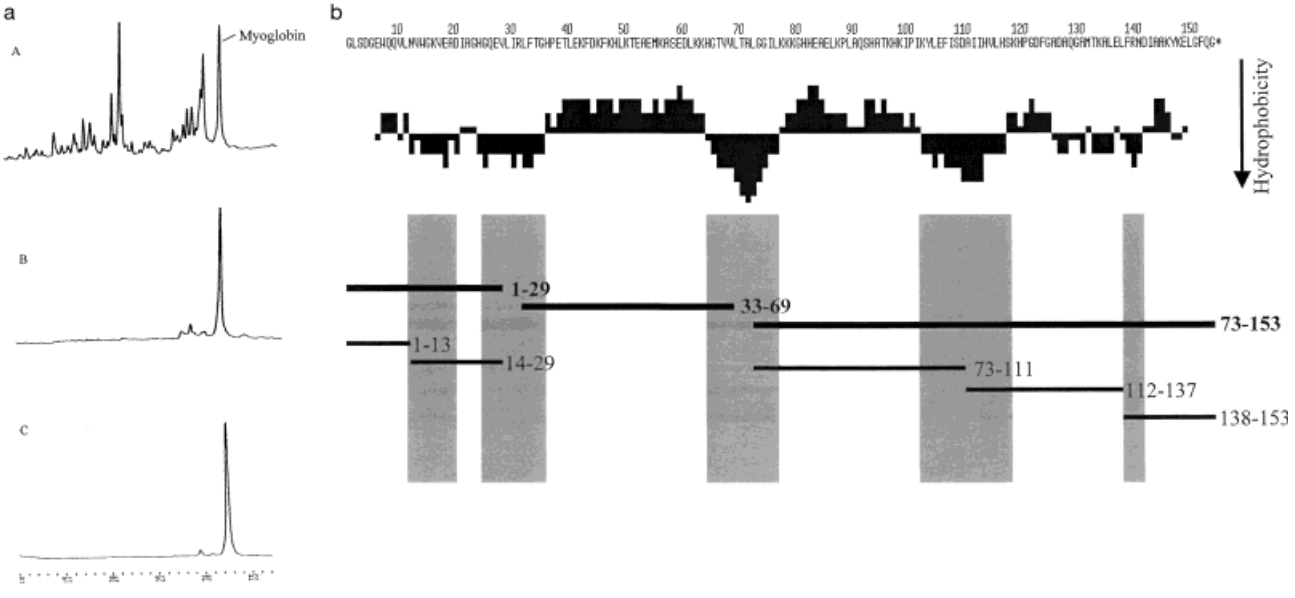

Fig. 6. Processing of myoglobin with disrupted lysosomes. Myoglobin was incubated with lysosomal fractions obtained from human B cells under reducing conditions at pH 5.4 as above. (a) HPLC-profiles of (A) the native digest, (B) the digest after addition of pepstatin A and (C) digestion with purified AEP. (b) Initial fragments released from myoglobin after incubation with lysosomal fraction. First observed cleavage sites are printed in bold.

by less specific endopeptidase activity at a much slower rate, which results in peptides that comprise 20–40 amino acids. Strikingly, endopeptidase activity is largely guided along the hydrophobic regions of MBP. This is not an artifact due to the use of recombinant MBP, since the same major processing sites were identified for purified human MBP. Similar patterns of processing along hydrophobic regions were found for additional antigens tested (*e.g.* α-lactalbumin, myoglobin, hen-egg lysozyme; Fig. 5, 6 and H. Beck, unpublished observations). This suggests that antigen processing is largely guided by structural properties of the protein itself, *e.g.* by the distribution of hydrophobic regions over the sequence. This is perhaps not surprising given that most lysosomal proteases prefer to cleave next to hydrophobic amino acids [24]. In the third phase of processing exopeptidase activity becomes dominant, with a significantly stronger activity at the N terminus compared to the C-terminal end of the fragments. This finally leads to a stage of almost complete degradation, where only short peptides or free amino acids are present.

According to our data the first stage of MBP processing is dominated by the cysteine protease CatS (cleavage after position S^{110}) and by the legumain-like AEP that cleaves after N^{92}. This is supported by (i) blockade of the generation of the respective dominant HPLC peaks by addition of specific inhibitors against AEP- and CatS-activity; (ii) generation of corresponding processing intermediates and HPLC fractions by the purified enzymes; and (iii) identification of major processing products that match the substrate preferences of either enzyme after incubation of intact MBP with lysosomal extracts. Thus CatS not only plays a unique role for Ii degradation in B cells and dendritic cells, but also represents one of the key enzymes that initialize MBP processing. Given that CatS is a major endopeptidase that has been shown to be active in early endocytic compartments of dendritic cells [25] we speculate that CatS may be involved in MBP processing in intact cells in a similar manner. AEP is not only the rate-limiting protease for TTCF processing [7], it also initiates degradation of MBP *in vitro* (this report). In contrast to TTCF processing, however, competitive inhibition of AEP does not halt processing of MBP, because proteolysis of MBP is still initiated by CatS. Thus the initial unlocking of antigenic protein for further processing can be performed by different proteases of distinct specificity at different sites. Our data further suggest that AEP activity could be of more general relevance during the initial events of antigen processing: *in vitro* processing experiments of a similar type, using α-lactalbumin as a model substrate, revealed a similar pattern of dominant initial cleavage sites that were entirely consistent with their generation by AEP. However, a third protein substrate tested (myoglobin) did not follow this pattern but was almost exclusively degraded by CatD, entirely consistent with earlier observations in other types of APC [21]. “Unlocking” by AEP might therefore be important for the initiation of processing in a variety of antigens, however, we do not consider it a general rule for antigen processing in the MHC class II compartment.

CatS and CatL showed a similar profile with respect to their specificity in MBP processing, although we observed clear-cut differences in the quantitative contribution of different processing sites between both enzymes. The shared specificity between CatS and CatL corresponds well with the similar role of both enzymes in Ii processing in B cells/dendritic cells, and thymic epithelial cells, respectively [26, 27]. CatD generates a relatively small set of degradation products, in agreement with its narrow substrate specificity [2]. Accordingly, fragments that match the major MBP-processing sites for CatD were only found in a low percentage in lysosomal extracts. This suggests that CatD is dispensable for processing of MBP, consistent with earlier results obtained in CatD -/- mice *in vivo* [28].

Strikingly, the dominant proteolytic events during the initial phase of MBP-processing affect the two immunodominant regions of MBP in an exactly inverse manner: while the initial cleavage by CatS (after S^{110}) directly contributes to the generation of MBP(111–129), the second dominant proteolytic event (cleavage after N^{92} by AEP) destroys the central immunodominant region MBP(85–99) eliminating the first two anchor positions of the epitope. In fact, MBP(85–99) represented the region with the fastest turnover, and peptides containing MBP(85–99) were exclusively found at early time points. How can these differences be reconciled?

The guidance of antigen processing by protective matrices like MHC molecules or specific receptors has been frequently suggested [29–31]. Intact MBP is able to bind to HLA-DR molecules already on the cell surface [14, 23]. Since MBP(85–99) binds to class II with a high affinity, binding of this region to class II might protect the central epitope from degradation by AEP. We confirmed that class II binding is indeed sufficient for this type of protection *in vitro*, in agreement with earlier observations (data not shown and [31]). Thus, we suggest that the high affinity of MBP(85–99) for class II rescues the epitope from degradation by AEP which in part could explain its immunogenicity. In this scenario, the generation of the immunogenic peptide is therefore largely guided by the MHC molecule itself. AEP here acts as an epitope-destructive protease, in contrast to the situation encountered for TTCF processing, where AEP is required for the generation of the immunodominant epitope.

The inverse situation is encountered in case of MBP(111–129): this epitope shows only a low affinity to HLA-DRB1*0401, but still represents an immunodominant region *in vivo* [10]. Our data demonstrate that the N terminus of this epitope is directly generated by one of the dominant proteolytic steps that initiate MBP processing, the cleavage by CatS after S^{110}. In addition, we observed a remarkable stability of the residues 111–126 against proteolytic attack. We suggest that generation of MBP(111–129) is not guided by MHC binding, consistent with its low affinity for class II, but by the substrate preference of CatS. The high cleavage rate of CatS after residue S^{110} guides the generation of relatively high amounts of MBP(111–129). This way MBP(111–129) can become an immunodominant region irrespective of its relatively poor HLA-DR4 binding.

Based on these data, what might represent a promising therapeutic strategy to possibly influence the generation of the immunodominant epitopes of MBP? The initial steps of processing largely predetermine the peptides generated and therefore represent attractive targets for intervention. Different strategies might be needed, depending on the way immunogenicity of a given epitope is achieved. In the case of MBP(85–99), peptidomimetics, that bind to HLA-DR with high affinity but are at the same time relatively protease resistant might be able to displace the immunodominant region from its protective binding to HLA-DR and thus might initiate its destruction by AEP. For MBP(111–129), pharmacological inhibition of CatS might be a promising strategy, too. Both types of agents, protease-resistant HLA-DR-binding peptidomimetics and a specific pharmacological inhibitor of CatS are available [25, 32].

In summary, we find that hydrophobic regions guide the processing of human MBP by lysosomal enzymes. Proteolytic processing of MBP is not a stochastic event, but dominated by rate-limiting initial cleavages by AEP and CatS, while the bulk of subsequent endoproteolytic processing is performed by cysteine proteases. While the proteolytic breakdown of α-lactalbumine appears to follow similar rules, the proteolytic processing of myoglobin is clearly distinct and largely dominated by cathepsin D.

The immunodominant epitope MBP(85–99) is likely to be only generated when efficient protection from destruction by AEP is provided, possibly via MHC class II binding. In contrast, MBP(111–129) is generated during the initial phase of MBP processing and may therefore become immunodominant even as a poor binder to HLA-DR4.

4 Materials and methods

4.1 Protein expression and purification

Human recombinant MBP (18.5-kDa form, Swiss-Prot: P02686) was produced by recombinant expression of a vector (pET 21, Novagene, Madison) containing the cDNA coding for human MBP in *E. coli* host BL21 (DE) (Novagene) according to the manufacturers advice. The crude product was purified by RP-HPLC using a Grom-Sil 120 Cyano-1 column (Grom, Herrenberg, Germany) with a gradient of 0.05% TFA/acetonitrile and monitoring at 214 nm. Peaks were collected and characterized by mass spectrometry. Product purity was assessed by SDS-PAGE and HPLC to be more than 98%. Purified human MBP was a gift from M. Vergelli and purified by HPLC as published [14]. AEP was purified as described by Chen et al. [33].

4.2 *In vitro* processing

Lysosomal fractions were obtained from the human EBV-transformed homozygous lymphoblastoid B cell line (B-LCL) BSM (DRB1*0401, DRw53) by differential centrifugation and hypotonic lysis as recently described [34].

CatL was prepared from human procathepsin L [35] by processing the latent proenzyme to the mature single-chain enzyme on a negatively charged surface. CatS was purified from human spleen according to the method described by Kirschke et al. [15] with minor modifications.

Substrate solution containing 2 mg/ml MBP (human α-lactalbumin, equine heart myoglobin) in 150 mM citrate/phosphate, containing 4 mM DTT, adjusted to the desired pH, was incubated at 37°C with different endo- and exopeptidases and subcellular fractions for 10 min to 20 h. Processing studies were performed with 20 μg/ml human CatB (Sigma, St. Louis, MO), 10 μg/ml bovine spleen CatD (Sigma), 1–3 μg/ml CatL, 1–5 μg/ml CatS, 1–3 μg/ml AEP and with lysosomal fractions equivalent to 0.1×10^6–1×10^6 cells/μmol substrate. In some experiments, specific protease inhibitors (0.2 mM PMSF, 1 μM pepstatin A, or 10 μM E-64 (Sigma)) were added to the substrate solution. In addition, an inhibitor cocktail also containing 10 μM chymostatin, 5 mM EDTA, and 5 μM leupeptin was used if maximal inhibition was intended. For competitive inhibition of AEP, processing studies were performed in the presence of 5 mg/ml Z-AENK-NH_2 (prepared by standard solid-phase synthesis and HPLC purification) or Z-AEQK-NH_2 as control.

For pH-induced enzyme inactivation, disrupted lysosomes were preincubated in 20 mM citrate pH 5.4, containing 8 mM DTT and 1 μM pepstatin A at 37° C. After 1 h, aliquots of the solution were diluted with 0.2 M phosphate pH 7.5 at 37°C for 1 h. For processing studies at pH 5.4, 0.2 M citrate pH 5.2 was added until the solution reached the desired pH.

For specific inhibition of CatS, an aliquot of lysosomal fraction was 1:4 diluted with 0.2 M citrate pH 5.3, containing 20 nM LVHS and incubated at 37°C for 30 min before use in processing experiments.

4.3 Analysis of *in vitro* processing products

In experiments with whole human MBP as substrate, proteolytic products were separated by microbore reversed-phase HPLC using a C_8 or C_4 column (protein and peptide, 2.1×150 mm, Vydac). The column was equilibrated with 100% system A. Elution was carried out at a flow rate 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, Weiterstadt, Germany). Dominant eluting peak fractions were collected and dried in a speed vac. Of each sample 1 μl was subjected to MALDI-MS (Bruker Reflex III, Bruker, Bremen, Germany). Some of the fractions were also subjected to Edman protein microsequencing (494A "procise", Applied Biosystems). The relative quantity of MBP processing products was estimated by UV signals in RP-HPLC and Edman sequencing.

Acknowledgements: We gratefully acknowledge Prof. Dr. H. Meyer (University of Bochum, Germany) for performing initial MALDI-ionization mass spectrometry studies and H.L. Ploegh (Harvard Medical School) for providing the Cathepsin S-inhibitor LHVS. This work was supported by grants from the Deutsche Forschungsgemeinschaft (SFB 510, Ka 767/4–2, DR 378/2–1). C.J.S. is a fellow of the Boehringer Ingelheim Fonds.

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Correspondence: Hubert Kalbacher, Medical and Natural Sciences Research Center, University of Tübingen, Ob dem Himmelreich 7, D-72074 Tübingen, Germany
Fax: +49–707187815
e-mail: kalbacher@uni-tuebingen.de