Cathepsin G, and Not the Asparagine-Specific Endoprotease, Controls the Processing of Myelin Basic Protein in Lysosomes from Human B Lymphocytes[1]

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The asparagine-specific endoprotease (AEP) controls lysosomal processing of the potential autoantigen myelin basic protein (MBP) by human B lymphoblastoid cells, a feature implicated in the immunopathogenesis of multiple sclerosis. In this study, we demonstrate that freshly isolated human B lymphocytes lack significant AEP activity and that cleavage by AEP is dispensable for proteolytic processing of MBP in this type of cell. Instead, cathepsin (Cat) G, a serine protease that is not endogenously synthesized by B lymphocytes, is internalized from the plasma membrane and present in lysosomes from human B cells where it represents a major functional constituent of the proteolytic machinery. CatG initialized and dominated the destruction of intact MBP by B cell-derived lysosomal extracts, degrading the immunodominant MBP epitope and eliminating both its binding to MHC class II and a MBP-specific T cell response. Degradation of intact MBP by CatG was not restricted to a lysosomal environment, but was also performed by soluble CatG. Thus, the abundant protease CatG might participate in eliminating the immunodominant determinant of MBP. Internalization of exogenous CatG represents a novel mechanism of professional APC to acquire functionally dominant proteolytic activity that complements the panel of endogenous lysosomal enzymes. ***The Journal of Immunology*****, 2004, 172: 5495–5503.**

Proteolysis in the endocytic tract of APCs is performed by sophisticated molecular machinery that serves two essential tasks: it degrades the MHC class II-associated invariant chain (Ii)[3] in a stepwise fashion, a process that both guides class II transport and enables subsequent loading of antigenic peptide, and converts complex antigenic protein into fragments suitable for class II binding (1, 2). These two basic aspects of endocytic proteolysis in APC are essential for class II-mediated immunity in response to exogenous challenges as well as in class II-associated autoimmune disorders such as multiple sclerosis (MS). In recent years, we have gained considerable insight into the biochemistry of Ii processing and the proteases involved. The cathepsins S and L have emerged as key players that are responsible for the crucial proteolytic step in different types of APC (3–6).

Unraveling the rules of Ag processing has proven more difficult. Genetic elimination of the major cathepsins (Cats) D, L, B, and S did not lead to a general block in Ag turnover although these proteases were involved in the intracellular processing or presentation of selected Ags or epitopes (4–9). Recently, the asparagine-specific endopeptidase (AEP) legumain was identified in the endocytic compartment of B lymphoblastoid cells (BLC) (10) where it initialized the intracellular processing of the tetanus toxoid C fragment (TTCF) and myelin basic protein (MBP), an autoantigen implicated in the pathogenesis of MS, controlling the immune response against these Ags (11–13) as well as participating in Ii processing (14). Although AEP activity is required for efficient presentation of TTCF, its function for presentation of murine MBP in BLC is inverse: overexpression of AEP down-regulated the T cell response against the major immunogenic epitope MBP85–99 by cleaving after N^{92} and thus destroying this determinant, a feature that has been linked to the pathogenesis of MS (10, 12, 15). The identification of AEP as the functionally dominant protease in the processing of MBP and TTCF by BLC has substantiated the idea that an individual protease might control proteolytic breakdown and presentation of a given Ag by initializing its destruction.

Understanding the processing pathway of intact autoantigens by primary human APC is important for the rational design of therapeutic approaches such as the use of tolerizing peptides in autoimmune diseases. Both “natural” and “cryptic” T cell epitopes elicit a T cell response against the appropriate peptide; however, only natural epitopes that are processed from the intact Ag are relevant for mediating the disease and therefore represent potential targets for therapeutic approaches that aim at tolerizing the pathogenic T cell clone. In the murine model, proteolytic processing by AEP has been implicated as a molecular basis for the natural vs

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[3] Abbreviations used in this paper: Ii, invariant chain; MS, multiple sclerosis; Cat, cathepsin; AEP, asparagine-specific endopeptidase; BLC, B lymphoblastoid cell; TTCF, tetanus toxoid C fragment; MBP, myelin basic protein; IAA, iodoacetamide; LAMP, lysosome-associated membrane protein.

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cryptic functional characteristics of three distinct T cell epitopes present in the immunodominant region of MBP (15).

The panel of endocytic proteases in primary human B lymphocytes is poorly characterized and we do not know how this machinery handles complex Ag such as MBP. No mature CatB, CatD, Cat L, or CatS were found in resting human B lymphocytes at the polypeptide level (16). By contrast, human BLC contain active CatS, CatB, and CatD while they are negative for CatL activity (3, 17, 18). To what extent AEP, presumably the most important protease in Ag processing identified to date, is present and functional in primary human B cells has not yet been established. Our data reveal that primary human B lymphocytes lack significant AEP activity. Instead, CatG that is internalized into lysosomal compartments of human B lymphocytes dominates proteolytic processing of MBP. CatG destroys the major immunogenic epitope of MBP and abrogates both class II binding and T cell activation by MBP84–99, suggesting an important role for CatG during Ag processing by human B lymphocytes in vivo.

Materials and Methods

Protein expression and purification

Human rMBP (18.5 kDa, Swiss-Prot: P02686-5 minus M position 1) was produced in *Escherichia coli* and purified by ion exchange chromatography, as described (13). For generation of MBP*, the cDNA for human MBP was mutated AAC→CAA using a site-directed mutagenesis kit according to the manufacturer's advice (Stratagene, Amsterdam, The Netherlands) to replace N^{92} by Q^{92}. The complete resulting cDNA was controlled by sequencing (Sequencing Facility of the Interdisciplinary Centre of Clinical Research Tübingen, Tübingen, Germany). The purified proteins were controlled by matrix-assisted laser desorption ionization-mass spectometry for both purity (>98%) and correct mass. CatG from human leukocytes was purchased from Sigma-Aldrich (St. Louis, MO); AEP was purified as described (19).

Generation of lysosomal extracts and in vitro processing

Lysosomal extracts were generated from B lymphocytes ($CD22^+$) and BLCs by differential centrifugation and characterized as published before (13, 20). In brief, crude endosomal compartments were enriched by differential centrifugation from postnuclear supernatants, followed by short exposure of the membrane pellet to H_2O, which preferentially disrupts the membrane integrity of lysosomes due to their low osmotic resistance. This yielded virtually pure lysosomal extracts as judged by the distribution of cathepsin D, transferrin receptor and *N*-acetyl-glucosaminidase (20). For in vitro processing, substrate solution (0.04 μg/μl MBP, 0.1 M citrate, pH 5.0, 2.5 mM DTT) was incubated with lysosomal fractions, or isolated CatG and AEP, respectively, at 37°C (0.5 μg of total protein). Where indicated, protease inhibitors (30 mM iodoacetamide (IAA), 20 μM chymostatin; Sigma-Aldrich) were included 30 min before addition of the substrate.

To demonstrate processing by cell-bound CatG, CatG was incubated with HLA-DRB1*1501-homozygous BLCs (LD2B) on ice for 30 min, followed by extensive washing and confirmation of CatG-surface binding by FACS analysis using a mouse anti-human cathepsin G (Chemicon International, Temecula, CA) Ab. Cells were then transferred into prewarmed serum-free medium (Ex Vivo 15; BioWhittaker, Walkersville, MD) containing 200 μg/ml rMBP. After 60 min, cells were pelleted by gentle centrifugation and protein in the supernatant was resolved by HPLC and mass spectrometry.

Identification of processing products

Separation of in vitro processing products was achieved by microbore reverse phase HPLC using a C8 150 × 2 mm column (Wicom, Heppenheim, Germany) as published (13). Mass spectrometry was performed using an Esquire 3000^{plus} ion trap mass spectrometer (Bruker Daltonics, Bremen, Germany) equipped with a standard electron spray ionization interface (Bruker Daltonics). Mass spectra (400–1,700 mass per charge) were acquired in the positive ion mode (−4,000 V electron spray ionization voltage), loading of the trap was controlled by the instrument (ICC 100,000). Expected masses were calculated as average mass with statistical isotope distribution. The mass accuracy of the detected molecular ions was in the range of ± 200 parts per million. The identity of the major CatG-derived fragments generated in lysosomal fractions from primary B cells was confirmed by automated Edman protein sequencing (494A Procise; Applied Biosystems, Darmstadt, Germany).

Cells and Abs

The human EBV-transformed lymphoblastoid B cell line LD2B (DRB1*1501) was cultured in complete RPMI 1640 medium (10% FCS, 70 μg/ml gentamicin). Human PBMC were isolated from buffy coats of donor blood. Subpopulations of B lymphocytes and monocytes were positively selected using the MACS technique (Miltenyi Biotec, Auburn, CA) with CD22 and CD14 Abs, respectively. FACS analysis of the preparations revealed routinely 95% pure B lymphocytes (MHC class II positive, TCR negative, CD14 negative, CD20 positive, CD22 positive, CD86 negative. All FACS Abs were obtained from BD Biosciences, Heidelberg, Germany). Human whole thymus specimens were obtained from adult patients that underwent surgery for aortocoronary bypass procedures after informed consent. Samples were immediately subjected to the extraction protocol described above to isolate lysosomal contents.

Immunofluorescence

LD2B or CD22-purified peripheral blood B lymphocytes were seeded onto Biocoat poly-D-lysine eight-well culture slides (Biocoat Cell Environments; BD Biosciences) and allowed to adhere for 1–2 h at 37°C. Purified CatG was incubated with LD2B for 30 min up to 4 h (37°C). Slides were washed with PBS and the cells were fixed in ice-cold methanol/acetone (1:1, v/v) at −20°C for 10 min. After two additional washes, samples were blocked with 1 μg/ml human IgG (Bayer, Wuppertal, Germany) for 10 min. The primary Abs mouse anti-human cathepsin G (Chemicon International) and rabbit anti-lysosome-associated membrane protein (LAMP)-1 (Dianova, Hamburg, Germany) were added for 1 h, followed by washes with PBS. After blocking with 1 mg/ml normal goat serum (Dianova), cells were incubated with the secondary reagents goat anti-mouse Cy3 and goat anti-rabbit FITC (Jackson ImmunoResearch Laboratories, West Grove, PA) followed by washes and mounting with mounting medium for fluorescence (ICN Biomedicals, Costa Mesa, CA). Photographs were taken using a laser scanning confocal microscope every 0.5-μm optical section (Zeiss LSM 510l; Jena, Germany) using the company's software.

RT-PCR

Cells were lysed in TRIzol (Life Technologies, Grand Island, NY), and the RNA was extracted following the manufacturer's protocol. First-strand cDNA was prepared from total RNA by reverse transcription using the Superscript RNase H^- Reverse Transcriptase (Life Technologies) and random hexamers. For real-time PCR, gene expression was measured in the ABI Prism 7000 Sequence Detection System (Applied Biosystems). Primers (B&G Biotech, Freiburg, Germany) were selected with the Husar Genius software (Deutsches Krebsforschungszentrum, Heidelberg, Germany) to span exon-intron junctions to prevent amplification of genomic DNA and to result in amplicons <150 bp to enhance the efficiency of PCR amplification. Primer sequences for AEP and the housekeeping gene 18S rRNA are as follows: AEP forward primer 5′-GAAGCCTGTGAGTC TGGGTC-3′, reverse primer 5′-CAGTCCCCCAGGTACGTG-3′, 18S rRNA forward primer 5′-CGGCTACCACATCCAAGGAA-3′, and reverse primer 5′-GCTGGAATTACCGCGGCT-3′. Amplification of specimens and serial dilutions of the amplicon standards was conducted in a total volume of 15 μl in 2× SYBR Green Master Mix (Applied Biosystems) and primers at optimized concentrations. Standard curves were generated for each gene and the amplification was found to be 90–100% efficient as determined by the slopes of the standard curves. Relative quantification of gene expression was determined by comparison of threshold values as suggested by the manufacturers. All results are normalized with respect to the internal control 18S rRNA, and are expressed relative to the levels found in one of the samples.

For qualitative detection of CatG mRNA, the following sets of oligonucleotide primers (MWG, Ebersberg, Germany) were used to amplify cDNA in a conventional thermal cycler: human CatG GCTGAGGCAGGGGAGAT CATC and TCCCCCTTGAAGGCAGCCTTC (GenBank no. NM-001911), human β-actin TCAGAAGGATTCCTATGTGGGC and CCATCACGAT GCCAGTGGTA (GenBank no. NM-001101). The expected size of the CatG PCR product was 533 bp. Primer pairs amplifying a 300-bp segment from the human β-actin gene were chosen as a positive control.

Ag presentation assay

L466 cells (5×10^4/ml) transfected with human DRB1*1501 were incubated with Ag (native MBP or MBP digested with CatG in vitro in PBS) in the presence of the HLA-DR2-restricted $MBP_{84–98}$-specific chimeric T

cell hybridoma 08073 (provided by K. Wucherpfennig, Dana-Farber Cancer Institute, Boston, MA), 5×10^4 cells) in a final volume of 200 μl of medium DMEM/5% FCS for 24 h. The supernatant was then harvested and the IL-2 release was determined by measuring the [^{3}H]thymidine incorporation of the CTLL-2 cell line after coincubation with the supernatants during 12 h.

Determination of CatG and AEP activity

Hydrolysis of the synthetic compound Suc-AAPF-AMC (Bachem, Babendorf, Switzerland) that serves as an established substrate to determine CatG activity (21) was quantified fluorometrically. CatG (0.005 U) or total protein (50 μg) from lysosomal fractions of CD22$^+$, BLC, monocytes, or total thymus were added to 100 μl of Tris-HCl buffer, pH 8, containing 2% BSA and 0.5M $MgCl_2$ ($\pm$100 μM chymostatin or eglin C for 30 min) and the reaction was started by addition of 200 μM Suc-AAPF-AMC at 37°C. The liberated fluorescence was measured at 37°C over 5 h with a fluorescence reader (Tecan SpectraFluor, Crailsheim, Germany) at an excitation wavelength of 360 nm and an emission wavelength of 465 nm. The fraction of the emitted fluorescence that could be inhibited by addition of chymostatin as well as by eglin C was considered specific CatG activity.

AEP activity was quantified as published (22) using the fluorogenic substrate 45 μM Z-VAN-AMC in lysosomal fractions of LD2B and CD22$^+$ taken up into reaction buffer (40 mM citric acid, 120 mM Na_2HPO_4, pH 5.8, 5 mM mercaptoethanol, 4 mM EDTA, 10 μM E64, 6 μM aprotinin, 0.01% Triton X-100). Liberated fluorescence was measured every 10 min in a fluorescence reader for 1 h. The fraction of total fluorescence liberated that could be blocked by addition of the AEP inhibitor AENK, but not by the control peptide AEQK, was considered specific AEP activity.

Results

Primary human B lymphocytes lack significant AEP activity

To assess the presence and activity of AEP in primary human B lymphocytes, we compared freshly isolated peripheral blood B lymphocytes with the BLC line LD2B (BLC) both on the mRNA and the activity level (Fig. 1). Although significant amounts of AEP mRNA were present in lysates from BLC, the transcriptional activity for the enzyme was at least one order of magnitude lower and at the limit of detection in material from primary human B cells (CD22 positive). This translated into a similar difference on the activity level of the enzyme, as assessed in lysosomal extracts from either type of cell: the specific turnover of the fluorogenic AEP-substrate Z-VAN-AMC was ~10-fold higher in BLC compared with primary B cells, demonstrating that primary B cells lack significant AEP activity.

AEP does not initiate proteolytic processing of MBP by lysosomal fractions from primary human B cells

Because AEP is the dominant protease for breakdown of MBP in BLC, we assessed whether the lack of significant AEP activity in primary B cells would result in changes in the proteolytic breakdown of the autoantigen. Therefore, we analyzed the degradation pattern of recombinant human MBP incubated with lysosomal extracts from either primary human B lymphocytes or BLC in vitro. Lysosomes were prepared by a two-dimensional purification procedure that consisted of differential centrifugation to pellet high-density endocytic fractions followed by osmotic stress that exploits the high osmotic fragility of lysosomes and selectively releases lysosomal contents into the supernatant, a separation protocol that we have previously used to identify the dominant role of AEP in the destruction of MBP by BLC (13). The procedure yields a highly enriched lysosomal extract in the supernatant that is virtually free of contaminating plasma membrane, as we have previously shown in detail (20). The patterns of proteolytic products obtained with lysosomes from either cell type after 2 or 24 h were clearly distinct (Fig. 2*A* and Table I). Lysosomal extracts from BLC attacked intact MBP predominantly at the AEP-sensitive site ^{92}NI^{93} as well as at the ^{44}FF^{45} and ^{89}FF^{90} positions, resulting in

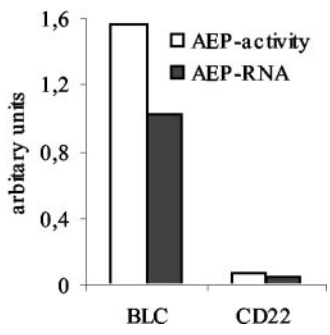

FIGURE 1. Human B lymphocytes lack significant amounts of AEP. Freshly purified human peripheral blood B lymphocytes (CD22, *right column*) and a human BLC line (BLC, *left column*) were assessed for AEP activity by incubation of lysosomal extracts with the AEP-specific fluorogenic substrate Z-VAN-AMC and for the expression of AEP mRNA using real-time PCR.

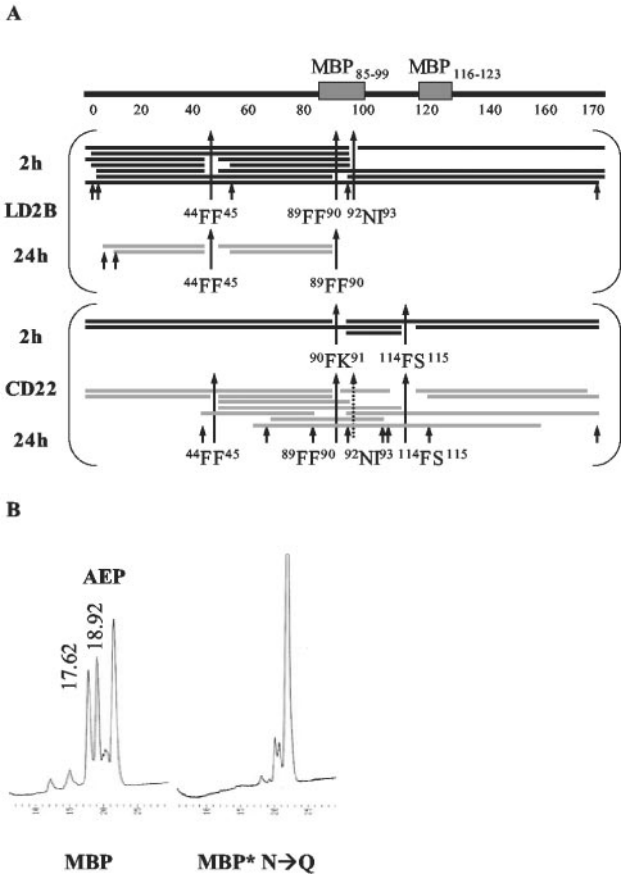

FIGURE 2. Proteolytic processing of MBP by lysosomes from BLC compared with primary human B cells. *A*, Intact MBP was incubated with lysosomal extracts from primary human B lymphocytes (CD22) or BLC (LD2B) for 2 and 24 h, respectively, and the proteolytic fragments obtained were resolved by HPLC and identified by mass spectrometry. Full-length MBP and the location of the two major immunogenic epitopes ($MBP_{85–99}$ and $MBP_{116–123}$) are indicated above. Large arrows indicate major cleavage sites, small arrows less dominant sites. *b*, To demonstrate the substrate specificity of AEP in MBP degradation, purified AEP was incubated in vitro with either wild-type MBP (*left*) or mutated MBP* which lacks the AEP cleavage site (*right*), followed by resolution of the fragments obtained by HPLC.

Table I. *Proteolytic fragments obtained after digesting MBP with CD22$^+$ B cell- or BLC (LD2B)-derived lysosomes, as deferred from mass spectrometry*

MBP LD2B					MBP CD22				
Ret.[a]	$[M + H]^+$	Expected mass $[H + H]^+$	ΔDa	Fragment	Ret.	$[M + H]^+$	Expected mass $[H + H]^+$	ΔDa	Fragment
2 h									
17.87	4,803.60	4,804.40	0.80	3–44	17.38	5,931.60	5,933.58	1.98	115–170
	4,891.10	4,891.47	0.37	2–44	18.71	8,511.60	8,509.58	2.02	91–170
	5,064.00	5,064.50	0.50	47–92	21.55	9,968.00	9,969.98	1.98	1–90
	5,267.50	5,268.73	1.23	45–92	22.54	12,550.40	12,545.98	4.42	1–114
19.02	8,266.50	8,267.30	0.80	93–170					
	8,379.30	8,381.40	2.10	92–170					
20.40	9,664.10	9,664.65	0.55	3–89					
	10,055.70	10,054.10	1.60	3–92					
	10,211.50	10,212.20	0.70	1–92					
21.68	13,516.00	13,517.01	1.01	45–170					
22.76	18,302.20	18,304.35	2.15	1–169					
	18,455.50	18,460.54	5.04	1–170					
24 h									
10.51	4,501.80	4,501.03	0.77	3–42	10.62	2,351.90	2,351.75	0.15	90–111
	4,674.10	4,675.05	0.95	47–89		2,594.10	2,595.01	0.91	91–114
	4,878.70	4,879.27	0.57	45–89		4,878.10	4,879.27	1.17	45–89
	5,268.30	5,268.73	0.43	45–92	12.88	4,301.60	4,301.68	0.08	42–81
16.96	4,803.80	4,804.40	0.60	3–44		5,267.30	5,268.20	0.90	45–92
	4,548.80	4,548.09	0.71	5–44	15.24	4,961.90	4,959.55	2.35	1–44
18.07	8,268.10	8,267.30	0.80	93–170		5,025.30	5,025.63	0.33	67–112
	8,379.70	8,381.40	1.70	92–170		5,419.90	5,418.94	0.96	115–166
19.46	9,664.10	9,664.65	0.55	3–89		5,660.10	5,656.88	3.22	117–170
20.87	13,516.00	13,517.01	1.01	45–170	16.39	5,932.90	5,993.58	0.68	115–170
					18.11	8,511.20	8,509.58	1.62	91–170
						9,694.60	9,695.76	1.16	63–152
					19.15	7,603.00	7,602.44	0.56	45–114
						9,824.10	9,822.81	1.29	1–89
					20.62	8,375.30	8,381.40	6.10	92–170
						9,970.70	9,969.98	0.72	1–90
					21.55	10,667.50	?		

[a] Ret., retention time.

destruction of the major immunogenic epitope MBP$^{85–99}$, as reported earlier (12, 13). In contrast, after 2 h of incubation, lysosomes derived from primary B cells led to a fragmentation pattern that was entirely the result of two distinct proteolytic cleavages at ^{90}FK^{91} and ^{114}FS^{115}, which were absent from the patterns observed with BLC. Of note, fragments indicative of AEP activity at ^{92}NI^{93} were not detected when MBP was digested with B cell-derived lysosomal fractions. After further processing for 24 h, the additional cleavage sites at ^{44}FF^{45} and ^{89}FF^{90} became also evident, similar to what had been seen in BLC, however, no cleavage after the AEP-sensitive position MBP92 was observed. Interestingly, processing of MBP at ^{90}FK^{91} destroyed the epitope MBP$^{85–99}$, similar to what had been observed for AEP. To demonstrate that proteolytic processing of MBP by AEP after position MBP92 was of little, if any, importance when MBP is degraded by B cell-derived lysosomal enzymes, we generated a mutant version of MBP where N92 was replaced by Q92 by site-directed mutagenesis, so that the mutated version MBP* lacked the AEP-sensitive site. Although processing of MBP with purified AEP in vitro resulted in the expected fragments (Fig. 2*B*: fragment 93–170 eluting with HPLC peak 17.62 and fragment 1–92 eluting at 18.92, as resolved by HPLC and identified by mass spectrometry), MBP* was resistant toward purified AEP. When MBP* was exposed to B cell-derived lysosomes, the proteolytic cleavage sites identified by mass spectrometry were identical to those observed with the wild-type protein (Table II), confirming that cleavage by AEP after position MBP92 was dispensable when MBP is degraded by B cell-derived lysosomal proteases.

A serine protease controls degradation of MBP in B cell-derived lysosomes

These results suggested the presence of proteolytic enzymes in the lysosomal compartment of primary human B cells that are absent from BLC and that attack intact MBP at the ^{90}FK^{91} and ^{114}FS^{115} processing sites. Comparison of the patterns of active papain-like Cats in both cell types using the active site-directed affinity probe JPM565 (18) did not reveal active papain-like cysteine proteases in B lymphocytes that were absent from BLC (data not shown). To further characterize the protease(s) responsible for destruction of

Table II. *Proteolytic fragments obtained after digesting MBP* with CD22$^+$ B cell-derived lysosomes, as deferred from mass spectrometry*

MBP* N → Q				
Ret.[a]	$[M + H]^+$	Expected mass $[H + H]^+$	ΔDa	Fragment
9.65	2,607.70	2,607.49	0.21	91–114
15.66	4,965.70	4,959.55	6.15	1–44
16.04	4,837.30	?		
	5,659.90	5,656.88	3.02	117–170?
	5,931.60	5,929.99	1.61	115–170
17.31	8,516.10	8,509.58	6.52	91–170
20.18	8,372.70	8,381.40	8.706	92–170
	9,970.70	9,964.01	0.69	1–90
21.26	10,668.80	?		

[a] Ret., retention time.

MBP in B cell-derived lysosomes, we tested the influence of protease inhibitors on the proteolytic events observed (Fig. 3). Addition of IAA virtually blocked degradation of MBP by BLC-derived lysosomes, consistent with the view that IAA-sensitive cysteine proteases like AEP initiated this process while inhibition of serine proteases using PMSF had no effect in this cell type. In contrast, IAA did not protect intact MBP from degradation by $CD22^+$ B cell-derived lysosomes but resulted in the accumulation of processing intermediates that were identical with the fragments obtained at the initial stage of degradation in the absence of inhibitors (retention time, 14.79 min: fragment 115–170; 16.48 min: 91–170; 19.98 min: 1–90; 20.50 min: 1–114, compare Fig. 2 and Table I), as confirmed by mass spectrometry. Strikingly, and in marked contrast to the results seen with BLC, processing was entirely blocked by PMSF. This suggested that the initial proteolytic attack on intact MBP by B lymphocyte-derived lysosomal extracts was mediated by a serine protease that cleaved MBP at $^{90}FK^{91}$ and $^{114}FS^{115}$, degrading the immunogenic epitope $MBP^{85–99}$, and resulting in distinct proteolytic intermediates that are then further degraded by lysosomal cysteine proteases.

CatG degrades MBP at $^{90}FK^{91}$ and $^{114}FS^{115}$

Given the extensive work on protease activity in cell biology, we reasoned that the proteolytic processing pattern observed in B lymphocyte-derived lysosomes would more likely be due to the activity of a known protease that had only not yet been recognized in the context of Ag processing, rather than by an entirely novel protease. The results so far obtained indicated that degradation of MBP by B cell-derived lysosomes was dominated by a 1) serine protease (PMSF-sensitive) with a 2) chymotrypsin-like activity

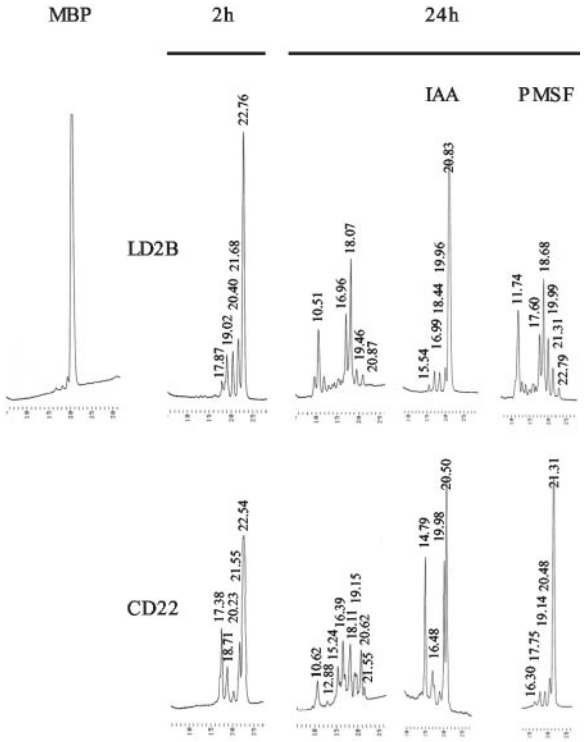

FIGURE 3. Intact MBP (*upper left*) was exposed to lysosomal extracts from either BLC (LD2B, *upper panel*) or primary B cells (CD22, *lower panel*) in the presence/absence of protease inhibitors (IAA, PMSF, 30 mM) in vitro, followed by resolution of the fragments by HPLC and identification by mass spectrometry. Expected mass values are expressed as nonprotonated (M^0) mass and calculated from average isotope distribution.

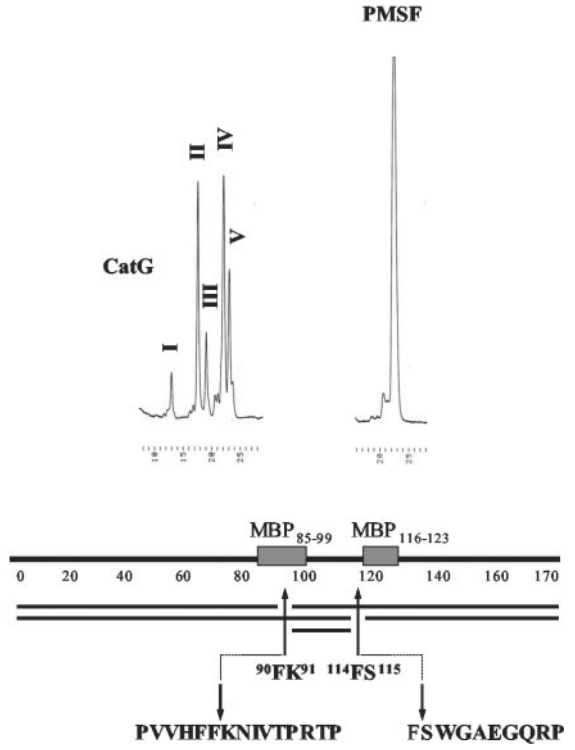

FIGURE 4. Purified CatG degrades MBP exclusively at $^{90}FK^{91}$ and $^{114}FS^{115}$. *Upper panel*, Purified rMBP was digested with CatG in the presence/absence of the serine protease inhibitor PMSF, followed by resolution of the emerging fragments (I-V) by HPLC. *Lower panel*, CatG-processing sites and the proteolytic fragments obtained aligned with the MBP sequence and the T cell epitopes that are immunodominant in the context of HLA DRB1*1501 ($MBP_{85–99}$) and HLA DRB1*0401 ($MBP_{116–123}$).

(cleavage at FS and FK). When we tested chymotrypsin and the aspartyl protease CatD, they were not able to reproduce the proteolytic fragments that we had observed with B cell-derived lysosomes after incubation with MBP in vitro (data not shown). A literature search revealed CatG as a potentially promising candidate enzyme, because it not only combined the desired functional criteria (serine protease with chymotrypsin-like activity), but had also been described in association with human B lymphocytes (23, 24). Strikingly, purified CatG attacked MBP selectively at $^{90}FK^{91}$ and $^{114}FS^{115}$ and thus generated a PMSF-sensitive fragmentation pattern that was identical to that obtained with B cell-derived lysosomes after 2 h of processing (Fig. 4 and Table III, compare Fig. 2 and Table I). This strongly suggested that CatG might be the functionally dominant endoprotease in lysosomes of human B lymphocytes that degrades the immunodominant epitope of MBP in the absence of significant AEP activity.

Table III. *Proteolytic fragments obtained after digesting MBP with purified CatG*

	CatG			
Ret.[a]	$[M + H]^+$	Expected mass $[H + H]^+$	ΔDa	Fragment
I	2594.20	2595.01	0.81	91–114
II	5932.73	5933.58	0.85	115–170
III	8507.48	8509.58	2.10	91–170
IV	9968.98	9969.98	1.00	1–90

[a] Ret., retention time.

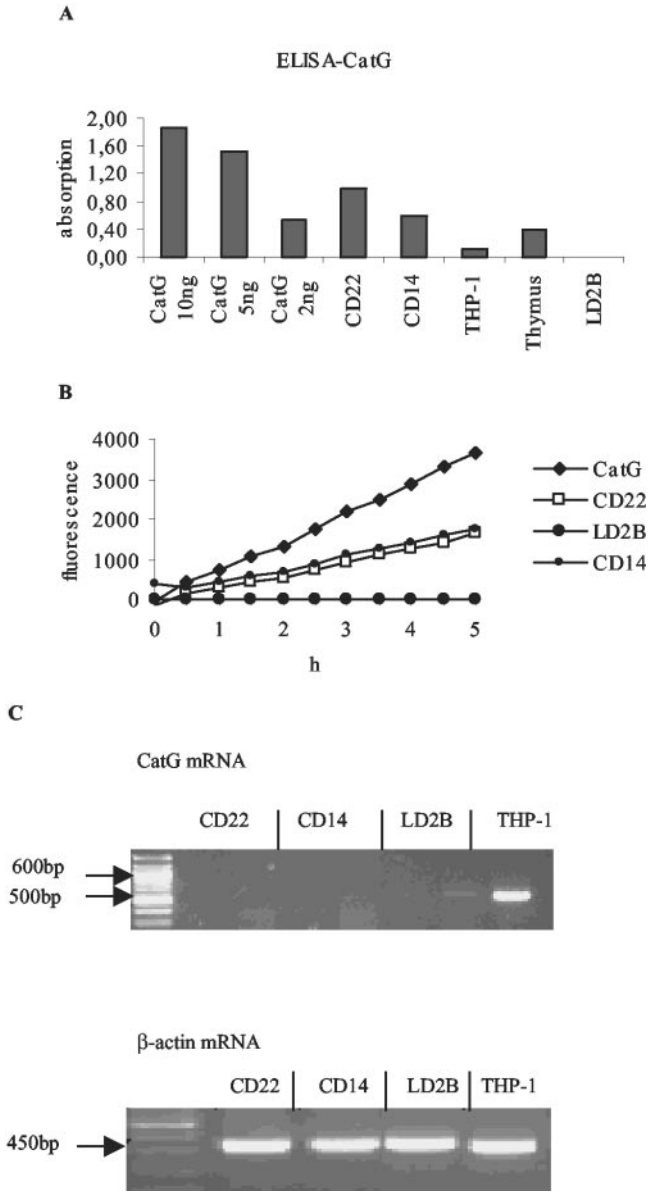

FIGURE 5. Visualization of CatG in lysosomal extracts. *A*, Lysosomal extracts from B cells (CD22), monocytes (CD14), the monocytoid cell line THP-1, whole thymus and BLC (LD2B; 2.6 μg of protein each) were analyzed for the presence of CatG protein by ELISA. Purified CatG served as an internal positive standard, LD2B cells as a negative control. *B*, Lysosomal extracts from B cells (CD22), monocytes (CD14), BLC (LD2B) were assayed along with purified CatG for turnover of the fluorogenic CatG substrate Suc-AAPF-AMC as described. Fluorescence was expressed in arbitrary units. *C*, Whole cell RNA from B cells (CD22), monocytes (CD14), BLC (LD2B), and a myelomonocytoid cell line (THP-1) was analyzed for the presence of mRNA for CatG; β-actin served as a positive control.

Lysosomal CatG activity in human B lymphocytes

To verify the presence of CatG in lysosomes from primary human B cells, we used a CatG-specific ELISA based on a mAb. Lysosomal extracts from human B cells were assayed along with purified CatG, lysosomal material from CD14-positive monocytes, and the THP-1 myelomonocytoid cell line (positive controls) and BLC (LD2B, negative control) as well as lysosomal fractions derived from whole human thymus. Purified CatG as well as both primary B cells, monocytes and THP-1 cells resulted in a CatG-specific signal (Fig. 5*A*), while BLC-derived lysosomes were negative for CatG protein. Interestingly, human thymus-derived lysosomal material was also positive for CatG. We further used the fluorogenic substrate Suc-AAPF-AMC to demonstrate the catalytic activity of CatG present in lysosomes of $CD22^+$ primary B cells. Although not absolutely specific for CatG, Suc-AAPF-AMC represents an established method to assess CatG activity in lysates from PBMC (21). Incubation of purified CatG with this substrate resulted in a linear substrate turnover over several hours, as expected (Fig. 5*B*). Although incubation of BLC-derived lysosomes (LD2B) with the substrate did not generate a fluorescence signal, significant CatG substrate turnover was detected when the same amount of $CD22^+$ lysosomal material or monocyte-derived fractions was assayed, in good agreement with our observation that cathepsin G protein was present in lysosomes from primary B cells and monocytes, but absent from the B cell line.

When both types of B cells ($CD22^+$ and BLC) and monocytoid cells (CD14 and THP-1) were assayed for the presence of CatG mRNA by RT-PCR, only the myelomonocytoid precursor cell line THP-1 was positive, as published (25). Thus, no transcriptional activity for CatG was observed in primary human B lymphocytes although CatG protein is present in lysosomal extracts of this cell type.

To confirm the presence of CatG in primary human B cells by an independent method and at the same time directly demonstrate the lysosomal location of the protease, $CD22^+$ B cells were processed for confocal microscopy and stained with the anti-CatG (red) and the lysosomal marker anti-LAMP-1 (green) reagents in comparison with untreated BLC grown in serum-free medium. Optical sections were taken in 0.5-μm slices to largely exclude optical artifacts (Fig. 6*A*, *upper panels*). $CD22^+$ cells strongly revealed the presence of CatG both at the surface and in intracellular pools near the plasma membrane (red, *middle*) while untreated BLC were negative for CatG (not shown). Merger of both images (CatG red, LAMP-1 green, colocalization yellow, *right*) revealed that the majority of LAMP-1-containig compartments were positive for CatG while some intracellular CatG, as well as CatG at the cell surface, did not colocalize with the lysosomal marker. Thus, CatG is present in lysosomal and nonlysosomal intracellular compartments of primary B lymphocytes in significant amounts.

Active CatG is present in human serum and binds to the surface of human B cells in a receptor-mediated fashion (23). Because CatG mRNA is not transcribed by human B cells, we speculated that exogenous CatG might be internalized following surface binding to supplement the endocytic Ag processing machinery of B cells. To this end, we incubated BLC with purified CatG followed by processing for confocal microscopy after up to 4 h of chase. LAMP-1 (green) and CatG (red) were visualized using appropriate primary Abs and fluorochrome-coupled secondary reagents (Fig. 6*A*, *lower panels*). At 0 h, LAMP-1 was visualized by green fluorescence in intracellular compartments adjacent to the plasma membrane, while no cell-associated CatG was detected. After 0.5 h, the plasma membrane stained red indicating the presence of CatG at the cell surface, while the bulk of lysosomal compartments remained unchanged. However, little colocalization of CatG and LAMP-1 in lysosomal compartments was already indicated by a discrete appearance of yellow fluorescence signals in lysosomes. After 4 h of chase, a major portion of lysosomal compartments was visualized in yellow revealing a strong colocalization of CatG and LAMP-1, while some noninternalized CatG was still present at the cell surface. These results were supported by biochemical data (Fig. 6*B*): when BLC were incubated with CatG for 30 min, followed by preparation of lysosomal extracts, no fluorescence signal was detected when the fluorogenic CatG substrate Suc-AAPF-AMC was coincubated with these extracts. By contrast, when BLC

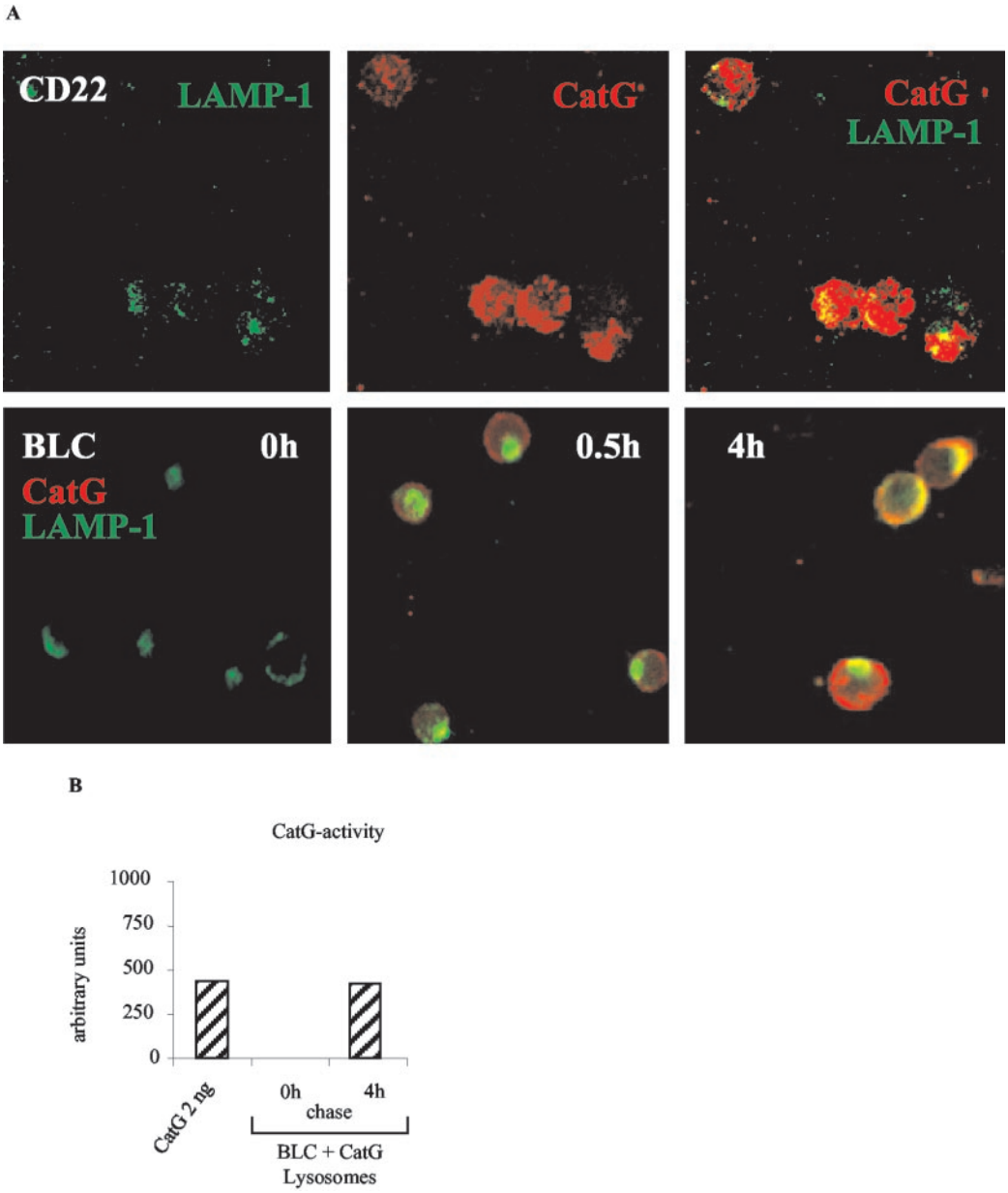

FIGURE 6. *A*, Internalization of CatG into lysosomal compartments. *Upper panel*, Visualization of CatG (red) and LAMP-1 (green) that colocalized (yellow) in lysosomal compartments of CD22-purified human peripheral blood B lymphocytes (CD22). *Lower panel*, BLCs (LD2B) were incubated with purified CatG for 0, 0.5, and 4 h, respectively. At each time point, cells were processed for confocal microscopy, stained for CatG (red) and LAMP-1 (red) and analyzed in 0.5-μm optical sections to reveal colocalization of LAMP-1 and CatG (yellow). *B*, BLC were incubated with purified CatG for 30 min, followed by a chase for 0 and 4 h, respectively. At each time point, lysosomal extracts were prepared and assessed for CatG activity using the fluorigenic substrate Suc-AAPF-AMC, along with purified CatG as a positive control.

were chased for 4 h after incubation with soluble CatG, a robust fluorescence signal could be retrieved from lysosomal material, indicating the internalization of CatG activity into lysosomes of BLC after incubation with the soluble enzyme. Together, these data demonstrated the transport of exogenously added CatG from the surface of B lymphoblastoid cells to lysosomes.

Processing of MBP by CatG interferes with T cell activation and is not restricted to a lysosomal environment

Binding of MBP85–99 to HLA DRB1*1501 has been suggested to protect this region from proteolysis, contributing to the immunodominance of this epitope (13). To assess whether the MBP fragment 91–114, that is generated from intact MBP by CatG, was still able to bind to HLA DRB1*1501, we compared the binding of either peptide to purified HLA DRB1*1501 in vitro using a competition assay (data not shown). Although close to 90% of the fluorescence-labeled reporter peptide was competed by MBP85–99, indicating strong binding of this peptide, MBP91–114 showed virtually no binding to HLA DRB1*1501 in vitro. This implicated that processing of MBP by CatG might interfere with T cell activation by the autoantigen. To test this hypothesis, we performed Ag presentation assays using the T cell line 08073 transfected with a TCR recognizing MBP84–98 in a HLA-DRB1*1501-restricted context, together with DRB1*1501-transfected L466 fibroblasts as APC (Fig. 7). Because L466 fibroblast cells do not bind CatG to their surface, possibly due to the lack of the appropriate receptor, we incubated MBP with CatG in vitro (2–50 ng) before addition of APC and T cells. MBP-specific IL-2 production was determined

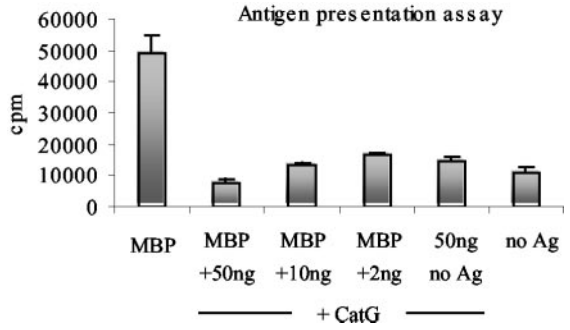

FIGURE 7. CatG converts soluble MBP into MBP91–114 which interferes with class II binding and T cell activation by the major immunodominant region. Activation of the MBP86–100-specific chimeric T cell hybridoma 08073 by HLA DRB1*1501-transfected L466 fibroblasts incubated with intact MBP (*left bar*) or by MBP preincubated with various concentrations of CatG. IL-2 secretion was measured by [^{3}H]thymidine incorporation (cpm) of CTLL-2 cells incubated with the culture supernatants. Comparison of supernatants from unstimulated cultures incubated in the presence/absence of supplemental CatG (50 ng/noAg, noAg) excluded a toxic effect of supplemental CatG.

by measuring the [^{3}H]thymidine incorporation of CTLL-2 cells grown in the supernatant. Addition of CatG in the absence of MBP did not affect baseline proliferation of CTLL-2 cells grown in the supernatant, indicating that CTLL2 cells were not compromised by the presence of CatG in the culture. Although stimulation with untreated MBP induced a strong proliferative response of the IL-2 reporter cells, preincubation of MBP with CatG reduced IL-2 production to background levels, indicating that the destruction of MBP by CatG functionally inactivated the autoantigen.

Discussion

The functionally important endocytic proteases in primary human B lymphocytes and the way they handle a potential autoantigen are largely unknown. The Cats S, B, H, D, as well as AEP, are considered the proteolytic enzymes likely to be involved, based on data mostly obtained with murine APC and human BLC (6, 8, 9, 12, 18).

Because AEP is functionally involved in both Ag destruction and Ii processing in different types of cells, it has emerged as a central endocytic protease in the class II pathway (11, 12, 14). AEP-mediated processing of MBP in BLC has been shown to control the turnover of intact MBP in vitro, eliminating the immunodominant epitope, which has been linked to the pathogenesis of MS: due to AEP activity present in the thymus, destruction of the immunodominant MBP epitope by AEP might impair local presentation of this Ag, which has been suggested to result in the survival of MBP-specific autoreactive T cells.

As our results show, freshly isolated human B lymphocytes lack significant amounts of AEP, and consequently, neither were major proteolytic fragments indicative of AEP activity present when MBP was incubated with B cell-derived lysosomes, nor did site-directed mutagenesis of the AEP cleavage site alter the pattern of proteolytic MBP fragments obtained. Consistent with this, the elimination of AEP activity using IAA failed to block proteolytic breakdown of MBP in lysosomes from this type of cell. In contrast, our results suggest that cathepsin G is present and active in APC including primary B cells, where it has functional implications for MBP processing that were originally described for AEP in BLC: CatG is the rate-limiting protease for the degradation of intact MBP in B cell-derived lysosomal extracts, it directly destroys the major immunodominant MBP epitope and interferes with T cell activation, while its inhibition blocks processing of MBP. Furthermore, CatG is present in human thymus in significant amounts. Thus, CatG is a major lysosomal protease in human B lymphocytes that must be added to the list of Ag processing enzymes in the class II pathway likely to be involved in the processing of exogenous Ags, including MBP, by B lymphocytes as well as other types of primary APC. The absence of significant AEP activity in B cells suggests that the prominent role of AEP in Ag processing could be restricted to certain cell types (e.g., lymphoblastoid cells) that may be of little functional relevance in vivo, while CatG could mediate a similar biological effect in primary B cells.

CatG is a serine protease that is not endogenously synthesized by B cells but is released by granulocytes and monocytes in vivo. It is present on the surface of native untreated B lymphocytes in the peripheral blood where it is bound via an unknown, thrombin-like receptor in an activity-dependent manner (23, 24). This cell membrane-bound CatG is proteolytically active, down-modulating the activity of the chemokine stromal cell-derived factor-1 by endoproteolytic processing (24). Lysosomal endoproteases in primary APC transit the endoplasmic reticulum/Golgi as zymogens and are routed to the endocytic compartment where they are activated (26). The transport of active surface-bound CatG to lysosomes and the presence of active CatG protein in lysosomes of primary human B cells described here represent a novel and unique way of professional APC to acquire lysosomal endoprotease activity in vivo. Although the catalytic optimum for CatG in vitro is in the mildly basic range (pH 8.5), purified CatG is stable and active also under acidic conditions (T. Burster, unpublished data). CatG is not a minor component of the proteolytic machinery present in lysosomes of human B lymphocytes, but represents the dominant and rate-limiting enzyme when intact MBP is processed by B cell-derived lysosomal fractions at pH 5.0: the fragmentation pattern obtained after 2 h could be entirely explained by CatG activity. Consistent with this, addition of PMSF blocked turnover of MBP, in contrast to BLC-derived lysosomes, where it had no effect. The functional relevance of the internalization of exogenous proteases into the MHC II pathway is supported by earlier data demonstrating that exposure of class II-transfected Chinese hamster ovary cells to high concentrations of soluble CatL in vitro abolished T cell activation by a model Ag via a mechanism that depended on the functional integrity of the endocytic machinery (27).

What are the implications of the destruction of MBP by B cell-associated CatG for the pathogenesis of MS? MBP-autoreactive T cells are believed to escape negative selection because the major immunogenic epitope 85–99 is destroyed by AEP present in thymic dendritic cells (12). As we show, CatG interferes with T cell activation by the MBP85–99 epitope, not unlike AEP, and is furthermore present in lysosomal extracts from human thymus. Degradation of MBP by CatG in the thymus might therefore contribute to the poor intrathymic presentation of this epitope, which may enable MBP-specific autoreactive T cells to escape negative selection.

The interaction between Ag-specific TH2 cells and B lymphocytes presenting the appropriate epitope contribute to the secretion of specific Abs and lead to increased IL-4 production, thus supporting the TH2-promoting environment. Based on our results, it is unlikely that intact MBP85–99 is presented by human primary B lymphocytes in the periphery. The absence of such presentation might adversely affect Ab formation and bias the immune response toward a TH1 type. Both, a TH1 bias of the immune response and the lack of affinity maturation of the Ab response compared with cerebrospinal fluid are features that characterize patients with MS (28).

With respect to presentation of MBP in the CNS, there is little information available regarding the presence of CatG in the putatively APC populations. Astroglia is known to carry a surface receptor for CatG in complex with α1-antichymotrypsin (29). Apart from its possible role in Ag presentation by professional APC in the brain, CatG might have an important regulatory effect in controlling the amount of intact soluble MBP in the CNS, because our data demonstrate that destruction of the MBP immunodominant epitope by CatG is not restricted to a lysosomal environment but can also be performed by soluble CatG. CatG is present and active at the cell surface of human neutrophils and is associated with granulocytes in primary human brain sections, both in the parenchyma and in perivascular regions (30). Thus, CatG not only complements the proteolytic machinery in primary human B lymphocytes, but is also present in human CNS, where it might contribute to the elimination of the MBP immunodominant determinant.

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