Destructive processing by asparagine endopeptidase limits presentation of a dominant T cell epitope in MBP

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Little is known about the processing of putative human autoantigens and why tolerance is established to some T cell epitopes but not others. Here we show that a principal human HLA-DR2–restricted epitope—amino acids 85–99 of myelin basic protein, MBP(85–99)—contains a processing site for the cysteine protease asparagine endopeptidase (AEP). Presentation of this epitope by human antigen-presenting cells is inversely proportional to the amount of cellular AEP activity: inhibition of AEP in living cells greatly enhances presentation of the MBP(85–99) epitope, whereas overexpression of AEP diminishes presentation. These results indicate that central tolerance to this encephalitogenic MBP epitope may not be established because destructive processing limits its display in the thymus. Consistent with this hypothesis, AEP is expressed abundantly in thymic antigen-presenting cells.

Myelin basic protein (MBP) is a candidate autoantigen in the inflammatory demyelinating disease multiple sclerosis and can induce the multiple sclerosis–like disease experimental autoimmune encephalomyelitis (EAE) in susceptible strains of mice and rats[1–3]. Like other autoimmune diseases, multiple sclerosis occurs at higher frequency in individuals with certain major histocompatibility complex (MHC) molecules—in particular, those of the DR2 haplotype. Several studies have shown that T cells isolated from DR2-positive individuals with multiple sclerosis recognize amino acids (aa) 84–102 from MBP as a dominant epitope, MBP(84–102)[4,5]. Both DR2a (DRB5*0101 β chain) and DR2b (DRB1*1501 β chain) heterodimers bind MBP peptides from this region (for example, aa 81–99) with high affinity[6–8].

Although human T cell responses to MBP also occur to other regions of MBP[9], there has been considerable focus on the 84–102 region. Peptides in this region can induce EAE in SJL mice[10] and Lewis rats, and an MBP(85–99)-DR2b complex has been found in multiple sclerosis–affected brain tissue[11]. A variant peptide based on aa 85–101 of MBP has been tested in two separate phase II clinical trials designed to ameliorate the symptoms of multiple sclerosis in affected people[12,13]. (Note that the MBP(85–101) peptide used in those studies uses an alternative MBP numbering system and is described as MBP(83–99).)

The relevance of this MBP epitope and its interaction with MHC class II molecules in the DR2 haplotype are strengthened by data from a "humanized" transgenic mouse; this mouse expresses DR2b and a T cell receptor (TCR) specific for MBP(84–102) and shows evidence of spontaneous EAE-like disease[14]. Thus, in the context of certain human and murine MHC class II molecules, T cells specific for this MBP epitope persist in spite of the presence of MBP in the thymus[15–17] and, under appropriate conditions, can be activated and may contribute to inflammatory disease. Why such T cells are not subject to tolerance induction in the thymus remains a puzzle, particularly because of the tight binding of these MBP peptides to disease-associated MHC class II molecules[7,8].

Here we show that MBP contains a major processing site for the endosomal–lysosomal protease asparagine endopeptidase (AEP)[18,19]. MBP is a very good substrate for AEP, which cleaves at an asparagine residue in the encephalitogenic peptide core, and there is an inverse relationship between AEP activity in human antigen-presenting cells (APCs) and MHC class II molecule–restricted presentation of the MBP(85–99) epitope. We found that AEP is expressed in thymic APCs that are likely to be responsible for negative selection. Our results show that protease action on antigen and autoantigen substrates can destroy T cell epitopes as well as generate them, and indicate a mechanism by which autoreactive T cells may escape tolerance induction.

Results

AEP cleaves MBP at Asn[94]

In spite of its importance as a putative autoantigen, little is known about the processing of MBP by APCs. To address this issue, we subjected MBP to *in vitro* digestion with lysosomal protein isolated from a human Epstein-Barr virus (EBV)–transformed B cell line. For MBP, we expressed a histidine-tagged recombinant protein of the major 18.5-kD form of either mouse or human MBP. MBP was digested by the lysosomal extracts to yield several cleavage products (**Fig. 1a**). We used different approaches to identify the proteases involved. Initial studies with protease inhibitors indicated that both leupeptin, a broad spectrum

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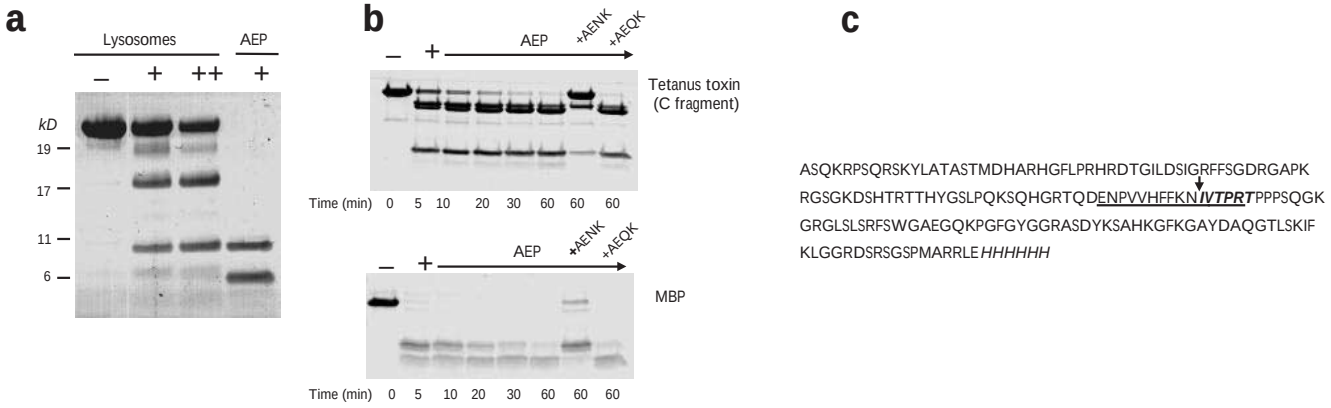

Figure 1. AEP cleaves after asparagine[94] in MBP. (**a**) Recombinant mouse MBP was digested *in vitro* either with 2 μg (+) or 5 μg (++) of purified, disrupted lysosomes from EBV B cells or with pig kidney AEP (1 mU) for 1 h at 37 °C. Digestion was done in 50 mM citrate buffer at pH 5.5. The reactions were separated on a Bis-Tris Nupage gel (Novex) and stained with Coomassie blue. (**b**) Time course of *in vitro* digestion of either TTCF or MBP with purified AEP (1 mU). An AEP inhibitor peptide (AENK) or a control peptide (AEQK) was included at the 60-min time-point. (**c**) Sequence of recombinant COOH-terminal His-tagged murine MBP (18.5-kD form). Residues 85–99 are underlined, and the sequence obtained from NH_2-terminal sequencing of the main digestion product of either AEP or lysosomal digestion is shown in bold italics. Arrow indicates the Asn[94] cleavage site.

inhibitor of papain-like lysosomal cysteine proteases, and inhibitors of AEP partially blocked lysosomal digestion of MBP (data not shown).

The endopeptidase AEP is insensitive to common cysteine protease inhibitors, such as leupeptin and *trans*-epoxy succinyl-L-leucylamido-(4-guanidino)butane (E64)[18], and has strict specificity for cleavage on the COOH-terminal side of asparagine residues. Its activity can be inhibited by small, blocked, asparagine-containing peptides such as Fmoc-AENK-NH_2 (AENK)[19]. As the 18.5-kD form of MBP contains only two asparagine residues, we were intrigued by the apparent involvement of AEP. Digestion of MBP with purified AEP gave two main fragments, one of which migrated with a lysosomal digestion product (**Fig. 1a**). We sequenced the MBP cleavage products generated by both lysosomal extracts and purified AEP. One product had the NH_2-terminal sequence IVTPRT (that corresponded to MBP(95–100)), indicating that cleavage had occurred after Asn[94] (**Fig. 1c**). Similar to the recombinant protein, MBP purified from human brain was also digested at Asn[94], which ruled out the possibility that conformational differences between these forms of MBP affected their processing by AEP (data not shown).

We compared the kinetics of digestion of MBP with that of the 50-kD COOH-terminal fragment of tetanus toxin (TTCF), an antigenic substrate of AEP[19]. Although TTCF is a good substrate for AEP, the faster kinetics of digestion of MBP by AEP indicated that MBP was a better substrate (**Fig. 1b**). Consistent with this, we found that it was more difficult to block digestion of MBP with the AENK competitor peptide. As expected, the glutamine-substituted tetrapeptide (AEQK) had no effect on AEP digestion of MBP (**Fig. 1b**). These results show that MBP has a marked sensitivity to AEP processing. The single MBP cleavage site is located at the Asn[94]-Ile[95] bond, which lies in the middle of the MBP(85–99) epitope bound by DR2 MHC class II molecules.

AEP inhibits MBP presentation

These results raised several questions concerning the possible sensitivity of MBP to AEP processing in living cells. Because AEP is expressed in human APCs such as EBV-transformed B cells[19], it seemed possible that AEP action might adversely affect presentation of the MBP(85–99) epitope to human T cells. We tested this idea using T cell hybridoma Ob.15, which is specific for MBP(85–99) and expresses a chimeric human-mouse TCR restricted to the DRB1*1501 MHC class II molecule; Ob.15 was derived originally from clone Ob.1A12, which was isolated from an individual with multiple sclerosis[7,14]. First, we established that the core sequence recognized by this TCR included Asn[94] and Ile[95] by finely mapping the specificity of the TCR using the EBV B cell MGAR, which expresses DR2* 1501. Only peptides containing the core sequence (aa 87–96; PVVHFFKNIV) caused stimulation of either fixed or living DR2-expressing MGAR cells (**Fig. 2**).

We next assessed whether the recognition of this epitope by Ob.15 T cells was affected by AEP digestion of MBP. Preincubation of MBP(85–99) peptide with AEP (**Fig. 3a, b**) completely abolished the Ob.15 response, as measured by the production of interleukin 2 (IL-2). Similarly, predigestion of full-length MBP with AEP also abolished its subsequent presentation by MGAR cells (**Fig. 3c**). In contrast, AEP

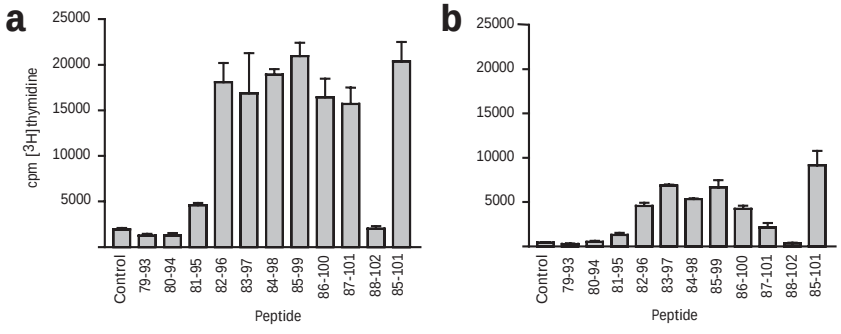

Figure 2. The minimal epitope for the Ob.1A12 TCR spans residues 87–96 (PVVHFFKNIV). Mouse splenocytes transgenic for the chimeric human-mouse TCR Ob.1A12 were cultured with either mitomycin C–treated (**a**) or paraformaldehyde-fixed (**b**) MGAR cells in the presence of the indicated peptides (50 μg/ml). Proliferation was assessed by uptake of [3H]thymidine after 72 h in culture.

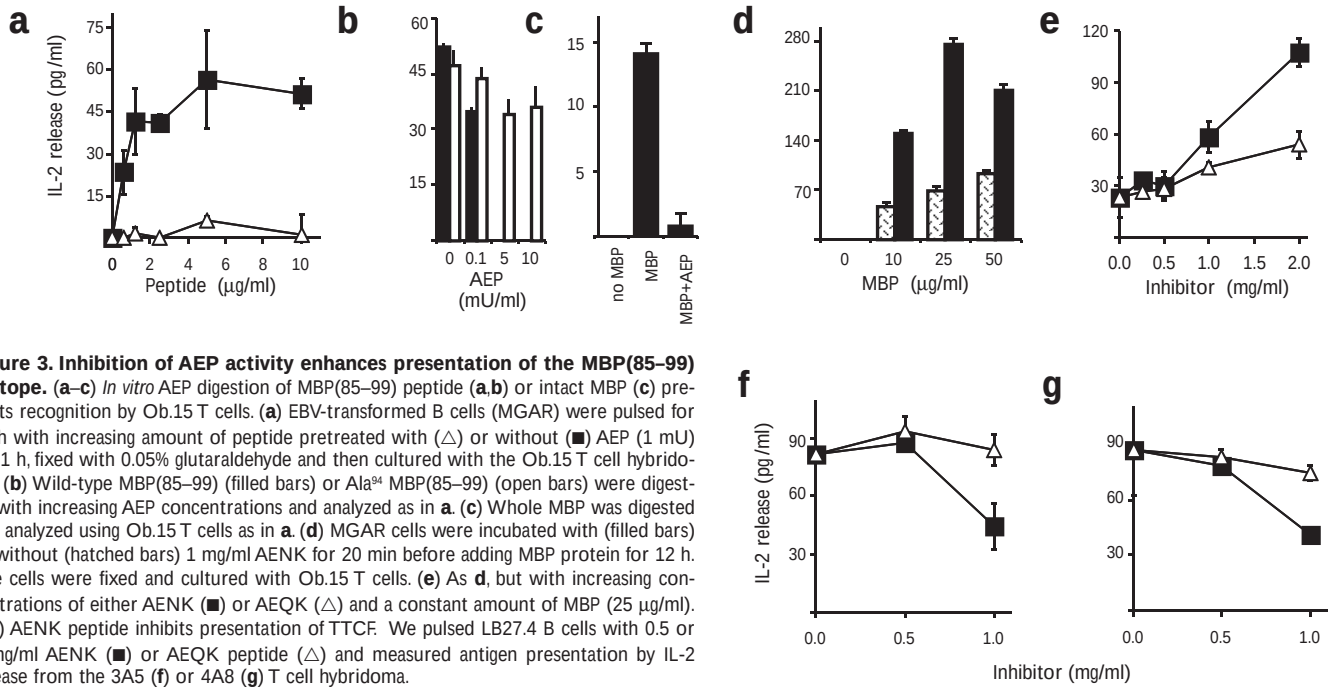

Figure 3. Inhibition of AEP activity enhances presentation of the MBP(85–99) epitope. (**a**–**c**) *In vitro* AEP digestion of MBP(85–99) peptide (**a**,**b**) or intact MBP (**c**) prevents recognition by Ob.15 T cells. (**a**) EBV-transformed B cells (MGAR) were pulsed for 12 h with increasing amount of peptide pretreated with (△) or without (■) AEP (1 mU) for 1 h, fixed with 0.05% glutaraldehyde and then cultured with the Ob.15 T cell hybridoma. (**b**) Wild-type MBP(85–99) (filled bars) or Ala^{94} MBP(85–99) (open bars) were digested with increasing AEP concentrations and analyzed as in **a**. (**c**) Whole MBP was digested and analyzed using Ob.15 T cells as in **a**. (**d**) MGAR cells were incubated with (filled bars) or without (hatched bars) 1 mg/ml AENK for 20 min before adding MBP protein for 12 h. The cells were fixed and cultured with Ob.15 T cells. (**e**) As **d**, but with increasing concentrations of either AENK (■) or AEQK (△) and a constant amount of MBP (25 μg/ml). (**f**,**g**) AENK peptide inhibits presentation of TTCF. We pulsed LB27.4 B cells with 0.5 or 1 mg/ml AENK (■) or AEQK peptide (△) and measured antigen presentation by IL-2 release from the 3A5 (**f**) or 4A8 (**g**) T cell hybridoma.

had no effect on an altered peptide in which Asn^{94} was substituted with alanine (**Fig. 3b**). Thus, AEP preprocessing *in vitro* destroys the MBP epitope recognized by Ob.15. This contrasts with previous findings on TTCF, for which preprocessing by AEP actually boosts the presentation of epitopes[19].

AEP inhibition enhances presentation of MBP(85–99)

We thought that an inverse relationship might exist between the cellular activity of AEP and presentation MBP(85–99); that is, inhibiting AEP in APCs *in vivo* might boost the presentation of MBP(85–99), whereas overexpressing AEP might reduce it. We therefore tested both of these predictions.

First, we pulsed MGAR cells with native MBP in the presence and absence of AENK and other peptide-based inhibitors of AEP. Although MGAR cells stimulated Ob.15 cells when pulsed with sufficient quantities of full-length MBP, the presentation of MBP was greatly enhanced when AENK was included (**Fig. 3d**). This enhanced presentation was dependent on the amount of AEP inhibitor present. At a fixed concentration of MBP (25 μg/ml), the increase in Ob.15 T cell stimulation was more or less linear as the concentration of AENK was increased from 0 to 2.0 mg/ml. At the highest concentration of inhibitor, the enhancement in IL-2 production by the Ob.15 T cells was almost fivefold (**Fig. 3e**). In contrast, the AEQK analog had some enhancing effect but was much less effective than AENK (**Fig. 3e**). Similar enhancements of presentation to Ob.15 T cells were seen with other asparagine-containing blocked peptides (B. M. and C. Moss, unpublished data).

To confirm that AENK could inhibit AEP *in vivo*, we tested its effect on presentation of two T cell epitopes in the TTCF antigen. With human T cell clones, it has been shown that inhibition of AEP interferes with the presentation of many epitopes in TTCF[19]. We obtained essentially the same result with two murine T cell hybridomas, 3A5 and 4A8, that recognize distinct epitopes in TTCF[20]. Presentation was inhibited as expected by AENK (**Fig. 3f,g**). Higher concentrations of AENK were toxic in this system, but 1.0 mg/ml of AENK inhibited the presentation of two different epitopes in TTCF by about 50%. Conversely,

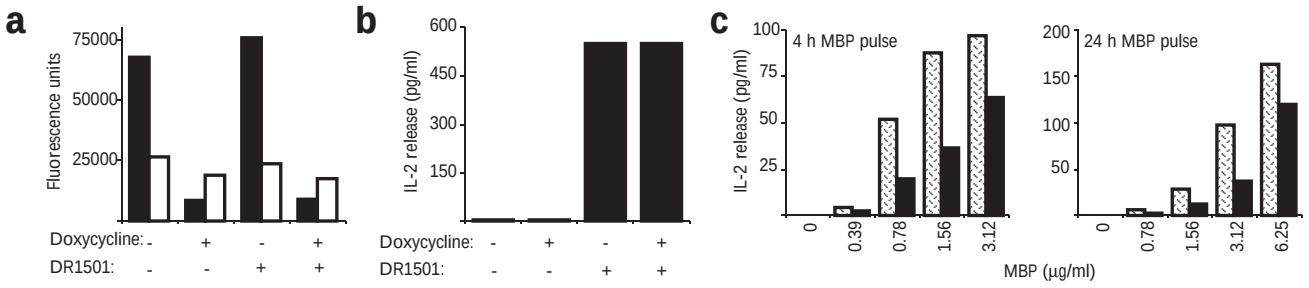

Figure 4. Overexpression of AEP in Mel Juso cell lines inhibits presentation of MBP. (**a**) Mel Juso clones SD1 and SD1 transfected with DRB1*1501 β chain exhibit Dox-regulated expression of AEP. Lysates from either SD1 (+/– Dox) or SD1/DR1501 (+/– Dox) cells were incubated with Z-Ala-Ala-Asn-NHMec (filled bars) or Z-Phe-Arg-NHMec (open bars) for 30 min, and NHMec release was measured at 460 nm. (**b**) DRB1*1501 transfected or untransfected SD1 cells grown in the presence or absence of Dox were pulsed with 5 μg/ml of MBP(85–99) and incubated with Ob.15 T cells for 24 h. IL-2 release was measured as above. (**c**) DRB1*1501-transfected SD1 cells grown in the absence (filled bars) or presence (hatched bars) of Dox were pulsed for 4 h or 24 h with increasing concentration of recombinant human MBP, and then fixed and cultured with Ob.15 T cells.

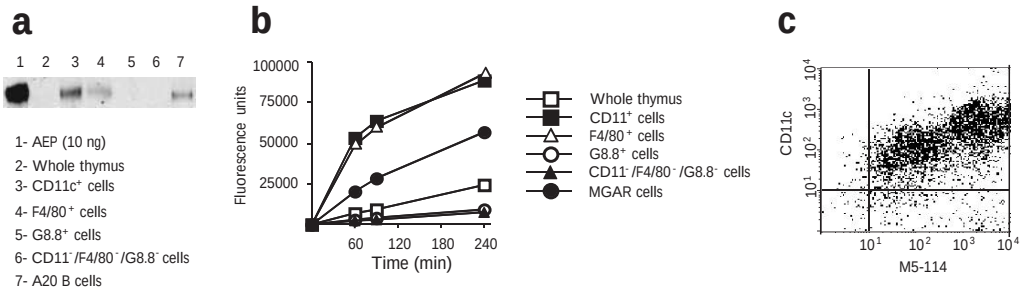

Figure 5. CD11c⁺ MHC class II⁺ thymic DCs express AEP. (**a**) Immunoblot showing expression of AEP in different thymic subpopulations and in murine A20 B cells for comparison. Note that the antibody was raised against human AEP but cross-reacts with murine AEP; murine A20, rather than human MGAR, cells were included for comparison. (**b**) Lysates of different thymic subpopulations and MGAR B cells were incubated with Z-Ala-Ala-Asn-NHMec substrate and NHMec release was measured at 460 nm after 60, 90 and 240 min. (**c**) CD11c⁺ cells isolated from the thymus were double stained using a mouse CD11c antibody and a rat MHC class II H-2^d (M5-114) antibody and analyzed by flow cytometry.

AEQK had little if any effect at this concentration (**Fig. 3f,g**). Thus, AEP action has different effects on the presentation of T cell epitopes in different protein antigens, with both constructive (for TTCF) and destructive (for MBP) outcomes.

AEP overexpression reduces presentation of MBP(85–99)

We next tested the second of our predictions and examined the effect of overexpressing the AEP protease on the presentation of MBP(85–99). We established cells in which overexpression of AEP was under the control of tetracycline or doxycycline (Dox) using the Tet-Off system. Although we were able to establish some regulated overexpression of AEP in EBV-transformed B cells, overexpression was most reliably and reproducibly achieved in the melanoma Mel Juso, which has been used previously to analyze MHC class II biosynthesis and antigen presentation[21–23]. A subclone of Mel Juso SD1 expressed normal amounts of AEP in the presence of Dox. On removal of the drug, a tenfold enhanced expression of AEP was observed, as measured by hydrolysis of the AEP substrate Z-Ala-Ala-Asn-NHMec (**Fig. 4a**, black bars). In contrast, hydrolysis of Z-Phe-Arg-NHMec, which can be cleaved by cathepsins B and L, was unaffected by the presence or absence of Dox (**Fig. 4a**).

We transfected SD1 cells either transiently or stably with the DRB1*1501 β chain and confirmed that a functional DR2b molecule was expressed by examining the presentation of MBP(85–99) to Ob.15 T cells (**Fig. 4b**). Presentation of MBP(85–99) peptide was unaffected by alterations in expression of AEP (**Fig. 4b**). To test the effect of AEP overexpression on MBP presentation, we grew Mel Juso SD1–DRB1*1501 with or without Dox for 2–7 days and then tested the cells in a dose-response assay after different times of MBP pulsing. Cells grown in the absence of Dox required at least twice as much MBP to trigger the same amount of IL-2 release compared with those grown with Dox (**Fig. 4c**). As expected, higher doses of MBP began to overcome the effect of AEP overexpression (**Fig. 4c** and data not shown). Together, these data showed that there is a inverse correlation between activity of the AEP protease and presentation of the MBP(85–99) epitope.

AEP activity in thymic APCs

Our results were consistent with a role for AEP in limiting the expression of a dominant region of MBP in the human DR2 haplotype, which might explain why T cells specific for this region avoid tolerance induction. Such a role would require AEP to be expressed in the thymic cells that are thought to determine negative selection. As very limited information is available on the cell types that express AEP *in vivo*, we isolated murine thymic dendritic cells (DCs), macrophages and medullary epithelial cells[24] and looked for the presence of AEP in cell lysates generated from both the starting thymic population and from the sorted populations.

Notably, AEP was only detectable by immunoblotting in CD11c⁺ and F4/80⁺ populations sorted from the thymus (**Fig. 5a**). We also detected AEP in a mouse B cell hybridoma, A20, that is used commonly as an APC for T cell assays. To determine whether the thymic AEP was active, we did fluorometric protease assays on the different cell populations. AEP activity was detectable after longer incubation times in the unfractionated thymic population (**Fig. 5b**) and in MGAR cells, as expected. But the sorted CD11c⁺ DCs and F4/80⁺ macrophages showed the highest activity of AEP, and hydrolyzed the AEP-specific Z-AAN-NHMec substrate at least ten times faster than the initial whole thymic extract. In contrast, G8.8⁺ medullary epithelial cells had low AEP activity, as did the cell population depleted of CD11c⁺, F4/80⁺ and G8.8⁺ cells (**Fig. 5b**). We confirmed that the AEP-rich CD11c⁺ population were also MHC class II⁺ (**Fig. 5c**). Thus, cell types that are likely to mediate negative selection expressed an active form of the AEP protease.

Discussion

Various mechanisms have been proposed to explain the failure of autoreactive T cells to be deleted in the thymus[25]. In addition to the low affinity of the TCR for peptide-MHC ligands, these include the failure of certain antigens to be expressed in or transported to the thymus and the failure of thymic antigen processing to generate peptide-MHC complexes in sufficient amounts to trigger negative selection. Several studies have shown that MBP and other myelin proteins are expressed in the thymus[15–17]. Presentation of MBP epitopes in the thymus seems to be responsible for the deletion of MBP-specific T cells, because T cell responses to MBP in *shiverer* mice, which lack MBP, show a distinct pattern of dominance compared with their MBP-expressing counterparts[26,27]. So why do some potentially pathogenic T cell responses to myelin antigens persist when others are eliminated effectively?

Two distinct mechanisms have been proposed to explain how some encephalitogenic T cells escape central tolerance. For the myelin-associated proteolipid protein (PLP), tolerance to the dominant peptide containing aa 139–151 (PLP(139–151)) is not established because the predominant form of PLP expressed in embryonic thymus (DM20) lacks residues 116–150. Re-expression of PLP(139–151) in embryonic thymus results in a reduction of PLP(139–151)-reactive T cells[28]. For Ac(1–9), a well-studied encephalitogenic NH_2-terminal epitope of MBP, the failure of tolerance induction can be attributed to the very low

affinity of the Ac(1–9) peptide for the A^u MHC class II molecule[29]. Consequently, even high-affinity T cells that are specific for this region fail to be deleted and can, under appropriate conditions, be activated and cause disease. Analogs of Ac(1–9) that bind more tightly to A^u can drive elimination of these autoreactive T cells and protect against the subsequent induction of EAE[30].

But neither of these mechanisms for failure of tolerance induction seems applicable to the DR2-restricted aa 85–99 region of MBP. These residues are present in the forms of MBP that are expressed in embryonic thymus, and peptides from this region of MBP bind tightly to DR2a and DR2b MHC class II molecules, which permitted the crystallization of MBP(86–105) with DR2a[31] and the generation of a monoclonal antibody specific for DR2b complexed with MBP(85–99)[11]. Our data indicate that there is a third distinct mechanism by which tolerance induction may fail: destructive processing by endosomal–lysosomal proteases.

In support of this hypothesis, we have shown that the Asn^{94}-Ile^{95} bond in MBP is a principal target for AEP digestion, that this enzyme destroys the dominant encephalitogenic T cell epitope in this region and that modulation of the activity of this protease in living cells controls presentation of the MBP(85–99) epitope. Inhibition of AEP activity resulted in an augmentation of MBP(85–99) presentation, in contrast to the inhibition of two epitopes in tetanus toxin C fragment, which requires AEP processing. Conversely, overexpression of AEP led to a diminished presentation of MBP(85–99). These findings show that the same protease can be required to generate some T cell epitopes and yet be destructive for others.

Our studies do not address directly the issue of whether presentation of this region of MBP *in vivo* is limited by AEP activity. At least two indirect observations indicate that this is likely to be the case, however. First, we found high AEP activity in thymic DCs and in thymic macrophages. Given the low amounts of MBP likely to be available for presentation by thymic APCs, it seems unlikely that this epitope will be displayed efficiently. Second, a new analysis of T cell responses to this region of MBP in the SJL mouse is presented in the accompanying paper[32], which indicates that AEP may have a role in controlling the display of naturally processed peptides from this region of MBP *in vivo*. Three overlapping T cell epitopes are defined in the aa 81–111 region of MBP, one of which is cryptic and contains Asn^{94} as a centrally located residue. Two other "naturally processed" epitopes flank the cryptic epitope with their core residues falling on each side of Asn^{94}. These data indicate that a processing event at Asn^{94} in the cryptic region (aa 92–98) may prevent presentation of this epitope but may be permissive for presentation of the two flanking "naturally processed" epitopes. It seems highly likely that AEP action at this site determines MBP presentation *in vivo* in the SJL mouse[32].

Activation of autoreactive T cells can be triggered by many mechanisms, and it is possible that even low amounts of the MBP(85–99) epitope may be sufficient to trigger T cells that have escaped tolerance induction if other conditions such as activation of appropriate professional APCs are in place[33]. But our data suggest ways in which the modulation of MBP processing *in vivo* may also contribute to T cell activation. Inhibition of AEP, even in the presence of normal concentrations of other endosomal–lysosomal proteases, resulted in an increase in the presentation of MBP(85–99). The activity of AEP on this region of MBP might be reduced *in vivo*, either because of lower intrinsic AEP activity or because of alterations in the MBP substrate. Changes in the MBP substrate might include conformational changes or associations with other proteins that are known to alter processing in other systems[34].

Another possibility, given the importance of asparagines for AEP action, is that Asn^{94} becomes post-translationally modified by deamidation. Nonenzymatic deamidation is the most commonly observed post-translational modification in proteins and causes the conversion of asparagine to aspartic acid and other minor products. Studies on the spontaneous deamidation of other proteins suggest that deamidation of Asn^{94} would be favored by the apparently flexible conformation that this region of MBP is thought to adopt[35] and by the very slow turnover of MBP *in vivo*. In contrast, an isoleucine at position 95 is not optimal because nonenzymatic deamidation proceeds most rapidly when glycine, serine or histidine are located COOH-terminal to Asn (ref. 36).

Although we have no direct evidence for deamidation of Asn^{94} in MBP, this post-translational modification is known to have effects on T cell recognition of other peptide and protein antigens. Deamidation of Asn^{59} in a peptide containing aa 48–62 from hen egg lysozyme (HEL(48–62)) may explain the existence of so-called type A and type B T cell responses to HEL[37]. Type B T cells stimulated by the HEL(48–62) peptide recognize a peptide in which Asn^{59} is deamidated to aspartate, but do not respond to naturally processed lysozyme because Asn^{59} is not deamidated in the more rigid HEL protein. Further work is needed to assess whether the deamidation of Asn^{94} in MBP occurs and how such a change might affect triggering of MBP-specific T cells. Notably, a study on molecular mimicry[38] identified viral and bacterial peptides that could trigger MBP(85–99)-specific T cell clones, and has found that four peptides that can trigger a DQ1-restricted T cell clone all have aspartate at the equivalent of position 94. In addition, this clone was much more potently stimulated when Asn^{94} was replaced with aspartate in the MBP(85–99) peptide, although several DR-2 restricted clones were not[38].

We propose a model whereby T cells specific for MBP(85–99) escape tolerization because AEP action at residue Asn^{94} in MBP prevents the effective presentation of epitopes in this region on thymic APCs, either because the epitope is simply destroyed or because residues on either side of Asn^{94} are essential flanking residues for efficient presentation of neighboring epitopes. Having escaped central tolerization, MBP(85–99)-reactive T cells may become activated in the periphery. The likelihood of this might be enhanced by alterations in the processing of MBP by AEP, either as a result of changes in AEP expression in APCs or modification of the MBP substrate. Combined with other triggering inflammatory factors, this change may be pathogenic as a result of activated MBP(85–99)-specific T cells entering the central nervous system.

Methods

Antigens and peptides. We expressed and purified recombinant mouse and human MBP (18.5-kD isoform) as described[39]. The residue numbering system is based on that in ref. 40. We prepared and purified a histidine-tagged derivative of the COOH-terminal domain of tetanus toxin as described[41]. Two human MBP(85–99) peptides (ENPVVHFFKNIVTPR) and (ENPVVHFFKAIVTPR) were synthesized using standard F-moc chemistry by G. Bloomberg (University of Bristol, UK). Epitope mapping of the Ob.1A12 T cell receptor was done using a set of 15-residue overlapping peptides with sequences in the MBP region between aa 79–93 and aa 85–101. We synthesized tetrapeptides AENK and AEQK as described[19].

MBP-processing activity. Lysosomal fractions from the human B-cell line Pala were prepared on 27% Percoll density gradients as described[42] using β-hexosaminidase activity to identify lysosomal fractions. MBP or TTCF (10 μg) was digested with lysosomal membranes (2 or 5 μg) or pig kidney AEP (1 mU) for various times as indicated. We separated the reactions by Nupage Bis-Tris gels (Novex, Groningen, the Netherlands) and stained them with Coomassie blue. The different thymic subpopulations and Mel Juso SD1 cells were lysed using a buffer containing 50 mM citrate, 5 mM dithiothreitol (DTT), 0.1% CHAPS and pH 5.5, 0.5% Triton X-100. We removed nuclei and cell debris by centrifugation at 14,000 rpm for 15 min and tested 5 μg of each lysate in a fluorometric assay.

Cell culture. We used RPMI medium supplemented with 2 mM L-glutamine, 5×10^{-5} M 2-mercaptoethanol, 100 U/ml of penicillin, 100 μg/ml of streptomycin (all from Life

Technologies, Paisley, UK) and 10% fetal calf serum throughout. After antigen pulsing, MGAR cells or Mel Juso cell lines (5×10^4 per well) were washed and fixed and cultured with the Ob.15 T cell hybridoma (5×10^4) for 24 h. We analyzed 50 µl of supernatant for the presence of IL-2 using an enzyme-linked immunosorbent assay kit (Pharmingen). For the splenocyte culture assay, we incubated 200 µl cultures containing 1×10^5 splenocytes transgenic for the chimeric human-mouse Ob.1A12, and 5×10^5 paraformaldehyde-fixed or mitomycin C–treated MGAR cells, at 37 °C for 72 h in medium containing 50 µg/ml of peptide. We assessed proliferation by uptake of [^{3}H]thymidine (0.5 µCi per well), which was added for the final 16 h, and measured incorporation using a liquid scintillation β-counter (LKB Wallac, Turku, Finland).

Fluorometric assays. Protease substrates (40 µM; Bachem, Saffron Walden, UK or a gift of G. Knight, University of Cambridge, UK) specific for cathepsin B/L (Z-Phe-Arg-NHMec) and for AEP (Z-Ala-Ala-Asn-NHMec) were incubated with postnuclear cell lysate (5 µg) for the times indicated. The buffer contained 50 mM sodium citrate (pH 5.5), 5 mM DTT and 0.1% CHAPS (AEP) or Brij 35 (cathepsin B/L). We measured release of 7-amido-4-methyl coumarin (NHMec) at various times on a Cytofluor 4000 Fluorimeter (Applied Biosystems, Warrington, UK).

Generation of stable transfectants. The AEP overexpressing Mel Juso cell line SD1 was established using the Tet-Off gene expression system (Clontech, Palo Alto, CA). Briefly, the cells were transfected first with the regulator plasmid pTet-Off, and then with the response plasmid pTRE containing the human AEP complementary DNA. We cloned DR1501 cDNA into the pcDNA3 Zeo+ vector (Invitrogen, Groningen, The Netherlands) and transfected 8 µg into SD1 cells using a standard calcium phosphate protocol[22]. Stable clones were established after 3 weeks of selection with 0.7 mg/ml of Zeocine (Invitrogen), 1 mg/ml of G418, 0.4 mg/ml of hygromycin B (all from Boehringer, Mannheim, Germany) and 50 µg/ml of doxycycline (Dox) (Sigma, Poole, England). We obtained the Mel JuSo clone SD1$^-$ that overexpresses AEP was obtained by removing the Dox drug for 7 days. Expression of DR1501 was confirmed using the Ob.15 T cell hybridoma.

Immunoblot analysis. Cells were lysed in 50 mM sodium citrate (pH 5.5), 5 mM DTT, 0.1% CHAPS and 0.5% Triton X-100. We determined protein content using a BCA assay (Pierce, Rockford, IL). Samples (5 µg) were fractionated by SDS-PAGE and transferred to nitrocellulose. We detected AEP using a polyclonal primary antibody raised in sheep against recombinant human AEP and a donkey anti-sheep horseradish peroxidase secondary antibody (Jackson), followed by chemiluminescence assay (Amersham, Uppsala, Sweden).

Mice. We removed thymi from BALB/c mice and isolated the different subpopulations of the thymus (CD11c$^+$, F4/80$^+$, G8.8$^+$) as described[24].

Note added in proof

Following acceptance of this paper, the data presented in Figure 1 were indepently confirmed by Beck *et al. Eur. J. Immunol* **31**, 3726–372636 (2001).

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Competing interests statement

The authors declare that they have no competing financial interests.

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