BIOCHEMICAL PHARMACOLOGY 74 (2007) 1514–1523

available at www.sciencedirect.com

ScienceDirect

journal homepage: www.elsevier.com/locate/biochempharm

Design of protease-resistant myelin basic protein-derived peptides by cleavage site directed amino acid substitutions

Timo Burster [a,b,d,*], *Viviana Marin-Esteban* [c], *Bernhard O. Boehm* [d], *Shannon Dunn* [e], *Olaf Rotzschke* [c], *Kirsten Falk* [c], *Ekkehard Weber* [f], *Steven H.L. Verhelst* [g], *Hubert Kalbacher* [b], *Christoph Driessen* [a,b,1,**]

[a] *Department of Medicine II, University of Tübingen, Tübingen, Germany*
[b] *Medical and Natural Sciences Research Center, University of Tübingen, Tübingen, Germany*
[c] *Max-Delbrück-Center for Molecular Medicine, Berlin, Germany*
[d] *Division of Endocrinology and Diabetes, Department of Internal Medicine I, Ulm University, Ulm, Germany*
[e] *Department of Neurology and Neurological Science School of Medicine, Stanford University, Stanford, USA*
[f] *Institute of Physiological Chemistry, Martin Luther University Halle-Wittenberg, Halle, Germany*
[g] *Department of Pathology, Stanford University School of Medicine, Stanford, CA 94305, USA*

ARTICLE INFO

Article history:
Received 18 April 2007
Accepted 16 July 2007

Keywords:
Antigen presentation/processing
Antigens/peptides/epitopes
Autoimmunity
MHC

ABSTRACT

Multiple Sclerosis (MS) is considered to be a T cell-mediated autoimmune disease. An attractive strategy to prevent activation of autoaggressive T cells in MS, is the use of altered peptide ligands (APL), which bind to major histocompatibility complex class II (MHC II) molecules. To be of clinical use, APL must be capable of resisting hostile environments including the proteolytic machinery of antigen presenting cells (APC). The current design of APL relies on cost- and labour-intensive strategies. To overcome these major drawbacks, we used a deductive approach which involved modifying proteolytic cleavage sites in APL. Cleavage site-directed amino acid substitution of the autoantigen myelin basic protein (MBP) resulted in lysosomal protease-resistant, high-affinity binding peptides. In addition, these peptides mitigated T cell activation in a similar fashion as conventional APL. The strategy outlined allows the development of protease-resistant APL and provides a universal design strategy to improve peptide-based immunotherapeutics.

* *Corresponding author at*: Division of Endocrinology and Diabetes, Department of Internal Medicine I, Ulm University Robert Koch Str. 8, 89081 Ulm, Germany. Tel.: +49 731 500 44521.
** *Corresponding author at*: Department of Medicine II, University of Tübingen, Otfried Müller Strasse 10, 72076 Tübingen, Germany. Tel.: +49 7071 2985210; fax: +49 7121 204325.
E-mail addresses: tburster@stanford.edu (T. Burster), christoph.driessen@med.uni-tuebingen.de (C. Driessen).
[1] Present address: Kantonsspital St. Gallen, Klinik für Onkologie und Hämatologie, St. Gallen, Switzerland.

Abbreviations: AEP, asparagine-specific endopeptidase; APC, antigen presenting cells; APL, altered peptide ligand; BLC, B-lymphoblastoid cell; Cat, cathepsin; EAE, experimental allergic encephalomyelitis; HPSEC, high performance size exclusion chromatography; HRP, horseradish-peroxidase; HLA, human leukocyte antigen; Ii, MHC class II invariant chain; MBP, myelin basic protein; MS, multiple sclerosis.

0006-2952/$ – see front matter © 2007 Elsevier Inc. All rights reserved.
doi:10.1016/j.bcp.2007.07.037

1. Introduction

Multiple Sclerosis (MS) is one of the most common autoimmune disorders in humans and is generally considered to be a T cell-mediated autoimmune disease. The activation of pathogenic self-reactive T cells ultimately results in destruction of the myelin sheath in MS patients. It is believed that MS is caused by a complex genetic trait to which a large number of genes contribute. For instance, human leukocyte antigens (HLA)-DRB1*1501, HLA-DRB5*0101 and HLA-DQB1*0602 have consistently been identified as conferring MS susceptibility [1]. Several indications point towards a role of myelin basic protein (MBP), a cytoplasmic protein of the myelin sheath, as a potential autoantigen in MS [2,3]. The immunodominant epitope has been localized as a minimal T cell epitope to MBP residue 85–99 (MBP85-99) for HLA-DRB1*1501 and to MBP residue 116–123 (MBP116-123) for HLA-DRB1*0401 [4]. MBP is also expressed in the thymus, and should lead to the elimination of MBP-specific T cells [5,6]. However, not all autoreactive T cells are deleted during thymic selection [7], so that T cells with the potential to recognize autoantigens, including myelin proteins, escape into the periphery [8]. Under certain conditions, such as viral infections [9], these T cells may become activated and cause autoimmune demyelination in the CNS.

One particularly attractive treatment strategy for autoimmune diseases is to generate peptides, which can prevent T cell activation by forming a MHC II-peptide-T cell receptor (TCR)-tricomplex [10–12]. Such altered peptide ligands (APLs), not only can mediate T cell receptor antagonism, they can also force T cells to secrete anti-inflammatory cytokines, and thereby reduce autoaggressive T cells (bystander suppression) [13]. MBP-derived APLs have already been tested in clinical trials [14–16].

The caveat of peptide-based APL treatment is that the peptides can be degraded by proteases from blood serum or by the cathepsins (Cats) located in the MHC-class II antigen processing machinery. Cathepsins are divided into three classes according to the amino acid in their active center, the aspartate- (CatD and E), cysteine- (C1: CatB, C, F, H, L, S, V, X, and C13: asparagine specific endoprotease (AEP)) and serine proteases (CatG). It was shown that the immunodominant MBP epitope (MBP85-99) is efficiently degraded by several proteases, such as CatS, D, L, G and AEP [17–20]. Thus, protease-resistant APLs may be better suited to interfere with T cell activation, because as compared to naturally occurring ligands, protease-resistant APLs are likely to have a substantially greater half-life in the MHC II antigen processing compartment. Also, due to the protease-sensitivity of the natural ligands/natural peptides, it is difficult to transform such peptides into efficient APL. To overcome these major drawbacks, we applied a strategy to design a protease-resistant APL, by exchanging the amino acids at the targets of the major proteases, the result being a more stable APL. High-affinity binding to MHC molecules was maintained by keeping the anchor residues constant, and by avoiding the use of D-amino acids and instead incorporating methylated amino acids. This strategy shows promise of being used to prolong the biological half-life of peptide-based immunotherapeutics resulting in a rational treatment of diseases.

2. Materials and methods

2.1. Peptide synthesis and purification

Peptides were synthesized by the solid phase Fmoc-strategy on the multiple peptide synthesizer Syro II (MultiSynTech, Witten, Germany) by using a six-fold molar excess of amino acids. Activation and coupling were performed with TBTU, HOBT and NMM (Merck, Darmstadt, Germany). The crude peptides were purified by HPLC using a C18 column 125 × 8 (Grom, Herrenberg, Germany) and analyzed by MALDI-TOF (G2025A, Hewlett-Packard, Waldbronn, Germany).

2.2. Cells

The human B-lymphoblastoid cell lines LD2B (HLA-DRB1*1501), WT-51 (HLA-DRB1*1501), MGAR (HLA-DRB1*1501) and myelomonocytoid cells THP-1 were cultured in complete RPMI 1640 medium (10% FCS, 70 μg/ml antibiotics: gentamycin). The HLA-DR2-restricted MBP86-98 chimeric T cell hybridoma 08073 (provides by L. Fugger) was cultured in DMEM medium supplemented with 5% FCS.

2.3. Cathepsins and generation of lysosomal extracts and in vitro processing

Lysosomal extracts were generated from B-lymphoblastoid cells (WT-51) and myelomonocytoid cells (THP-1) by differential centrifugation and characterized as published before [21]. In brief, crude endosomal compartments were enriched by differential centrifugation from postnuclear supernatants, followed by a short exposure of the membrane pellet to water, 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. For *in vitro* processing, substrate solution (0.2 μg/μl peptide, 0.1 M citrate pH 5.0, 2.5 mM DTT) was incubated with lysosomal fractions at 37 °C (2.6 μg of total protein). CatG was purchased from Sigma (Taufkirchen, Germany) and CatS, L, B, H, C, X and D from R and D Systems (Wiesbaden, Germany).

2.4. Identification of processing products from MBP85-99

Separation of *in vitro* processing products was achieved by microbore reverse phase HPLC using a C8 150 mm × 2 mm column (Wicom, Heppenheim, Germany) and mass spectrometry (MALDI-TOF G2025A, Hewlett-Packard, Waldbronn, Germany).

2.5. Isolation of HLA-DRB1*1501

Pellets of the B-lymphoblastoid cell line LD2B were disrupted by using lysis buffer (10 mM Tris, 140 mM NaCl, 2% Triton, pH 7.8). After centrifugation the supernatant was filtered with a 45 μm-filter, the probe was bound to a L243-sepharose-column, washed (50 mM Na-phosphate, 150 mM NaCl, 0.1%

zwittergent, pH 8) and eluted (100 mM Na-phosphate pH 8, 0.1% zwittergent). The identity and purity of the isolated complexes was tested by SDS-PAGE and coomassie-staining under boiled/non-boiled conditions.

2.6. Peptide-binding assay

High performance size exclusion chromatography (HPSEC) was used for competition studies to reveal peptide binding to purified HLA-DRB1*1501 essentially as described [22]. Briefly, solubilized HLA-DRB1*1501 molecules were incubated with N-terminally labeled (7-amino-4-methylcoumarin-3-acetic acid, AMCA), allele specific peptide (AMCA-MBP85-97) at pH 6.0 in the presence of detergent. Peptides (MBP85-99 or protease-resistant peptides 0.1 μg/μl) were added as competitors and samples were analyzed by HPSEC. Both the fluorescence and UV signals were recorded, comparing the fluorescence intensity co-eluting with HLA-DRB1*1501 dimers in the presence/absence of competitor peptide. The fraction of competitor peptide that was bound to HLA-DRB1*1501 complexes was determined.

2.7. T cell proliferation assay with MBP86-98-specific T cells

Human B-lymphoblastoid MGAR cells (5×10^4/ml; HLA-DRB1*1501) were incubated with wt-peptide (10 μg/ml) and protease-resistant peptides (4 μg/ml, 20 μg/ml and 100 μg/ml) in the presence of the T cell hybridoma 08073 (5×10^4 cells) in a final volume of 200 μl medium DMEM, 5% FCS for 24 h. Supernatants were then harvested and the released IL-2 was determined by measuring the 3H-thymidine incorporation of the CTLL-2 cell line (5×10^4 ml^{-1}) incubated in the supernatants for 12 h.

2.8. Western blot

Anti-human cathepsin L antiserum was generated against affinity-purified human cathepsin L [23]. Cells were lysed in lysis buffer (10 mM Tris pH 7.5, 150 mM NaCl, 0.5% NP-40), adjusted for equal total protein quantified by Bradford, resolved by SDS-PAGE and blotted using published conditions [24].

2.9. Determination of cathepsin-activity with fluorogenic substrate

Hydrolysis of 200 μM of the CatG-sensitive fluorogenic substrate Z-AAPF-AMC (Bachem, Weil am Rhein, Germany) was measured with or without preincubation with the CatG-specific inhibitor I (Calbiochem, Schwalbach, Germany) after incubation with 5 ng of CatG (Sigma, Germany), or 2.6 μg lysosomal extracts from WT51, THP-1 and CD14-cells in 100 μl assay buffer (0.1 M Tri/HCl pH 8.0, 0.5 M $MgCl_2$). The fluorogenic substrate MOCA-GKPILFFRLK-(Dnp)-D-R-NH2 (Bachem, Weil am Rhein, Germany) (3 μM) was used to determine CatD/E activity in reaction buffer (150 mM sodium-acetate buffer pH 4.0) with and without pepstatin A (Bachem, Weil am Rhein, Germany). The substrate turnover was determined by measuring the emission at 480 nm (excitation at 360 nm) (Tecan SpectraFluor, Crailsheim, Germany).

2.10. Affinity-labeling of active cysteine proteases

Lysosomal extracts (1.3 μg) were incubated with reaction buffer (0.1 M citrate pH 5.0, 50 mM DTT) in the presence of DCG-04 [25] for 1 h at room temperature. Reactions were terminated by addition of SDS reducing sample buffer and immediate boiling. Samples were resolved by 12% SDS-PAGE, blotted on a PVDF-membrane and active polypeptides visualized using streptavidin HRP and the ECL-detection kit [23].

3. Results

3.1. In vitro processing of MBP85-99 with isolated cathepsins

In Fig. 1A we compared two possible approaches for generating a protease-resistant peptide: the conventional random amino acid substitution method versus to a novel systematic cleavage site-directed amino acid substitution approach. The conventional approach generates considerably more peptide permutations than the systematic substitution. For this systematic site-directed strategy, it was essential to distinguish the exact cleavage site of a given cathepsin in order to substitute different amino acids until the cathepsin cleavage site was eliminated. We simultaneously preserved the HLA-DRB1*1501 binding anchor while increasing the peptide's protease resistance. These peptide alterations lead to reduce T cell activation. Fig. 1B (left panel), illustrates the binding of HLA-DRB1*1501 to the immunodominant T cell epitope MBP85-99 (designated wild type (wt)-peptide in the remainder) with the binding anchors valine at position 87 (V87), phenylalanine at position 90 (F90), and isoleucine at position 93 (I93). In order to determine the positions at which isolated cathepsins (Cats), such as CatB, C, D, G, H, L, S and X, can digest soluble wt-peptide, we incubated wt-peptide with individual proteases, followed by identification of the resulting fragments by HPLC and mass spectrometry. We found that the serine protease CatG digested wt-peptide between phenylalanine 90 and lysine 91 (90FK91) as summarized in Fig. 1B (right panel). The endoprotease CatS was proteolytically active between histidine 88 and phenylalanine 89 (88HF89), as well as between lysine 91 and asparagine 92 (91KN92). Likewise the endoprotease CatL showed major cleavage sites between lysine 91 and asparagine 92 (91KN92). CatB, which is known to have carboxyexopeptidase activity, removed two amino acids at the carboxy terminus of wt-peptide between arginine 97 and threonine 98 (97RT98). While the aminopeptidase CatH removed individual amino acids from the N-terminus of the wt-peptide, CatC with its dipeptidyl-peptidase activity cleaved N-terminal dipeptides. The carboxypeptidase CatX did not cause C-terminal hydrolysis of wt-peptide, possibly due to the presence of the C-terminal proline. It is perhaps noteworthy that when we incubated peptide Mu12, which contains a lysine at position 99 (K99), with CatX, this amino acid was removed (data not shown). The aspartate protease CatD with

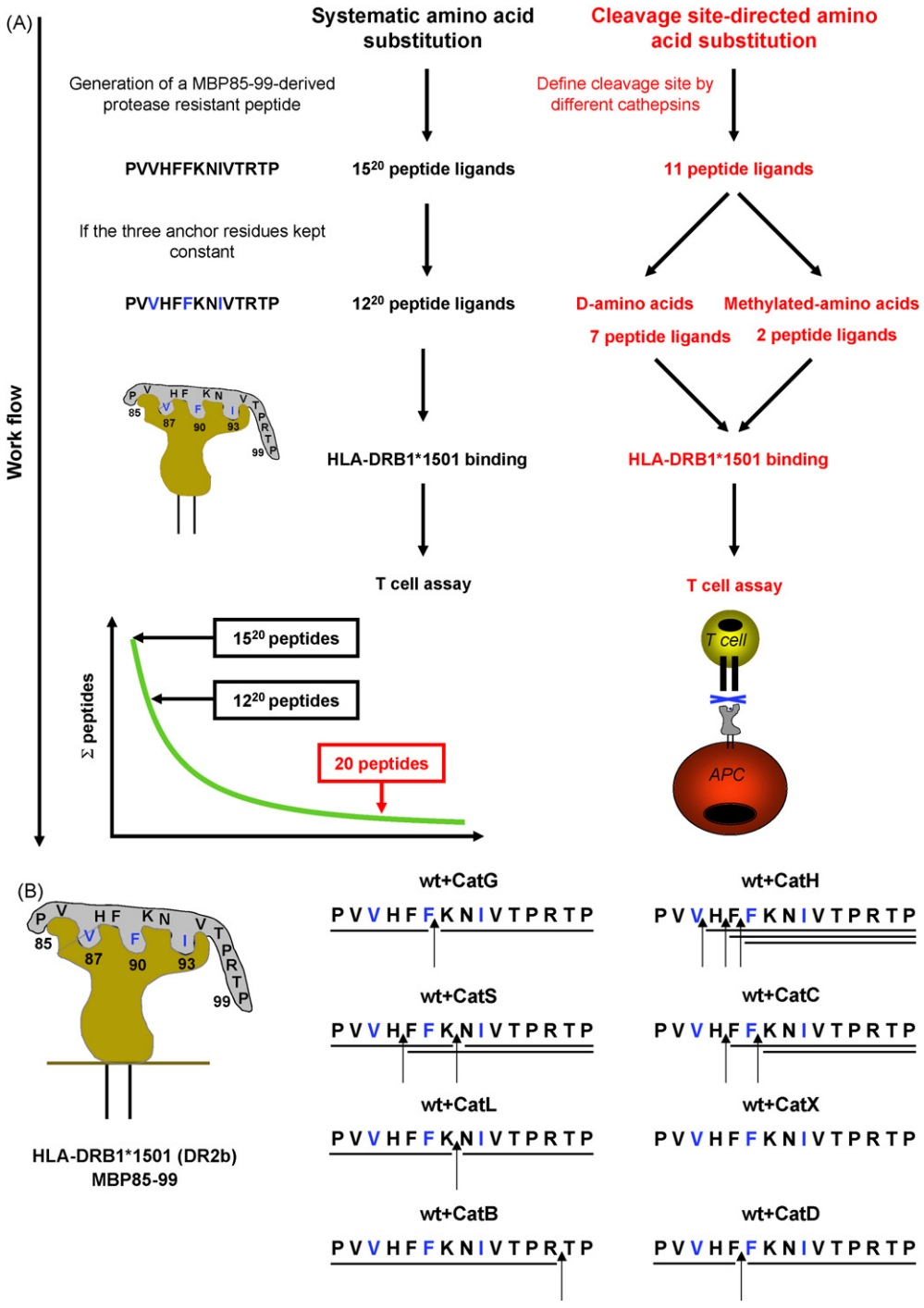

Fig. 1 – Generation of MBP85-99-derived peptides resistant to the proteolytic activity of proteases. (A) Comparison of a classical random amino acid substitution method to the here presented cleavage site-directed amino acid substitution strategy, for the generation of protease-resistant peptides. The first approach will require the synthesis of 15^{20} or 12^{20} peptides (using only natural amino acids) to identify those protease-resistant whereas the second approach we designed only 20 peptides: 11 only with natural amino acids and nine with modified amino acids, to generate MBP85-99-derived peptides highly resistant to lysosomal proteases. (B) *In vitro* processing of MBP85-99-peptide (wt-peptide) with isolated cathepsins. Left panel shows a cartoon depicting the anchor residues of MBP85-99 to HLA-DRB1*1501: valine at position 87 (V87), phenylalanine at position 90 (F90), and isoleucine at position 93 (I93). The right panel shows a summary of digestion pattern using wt-peptides that were incubated with different isolated cathepsins and the resulting fragments were identified by HPLC and mass spectrometry.

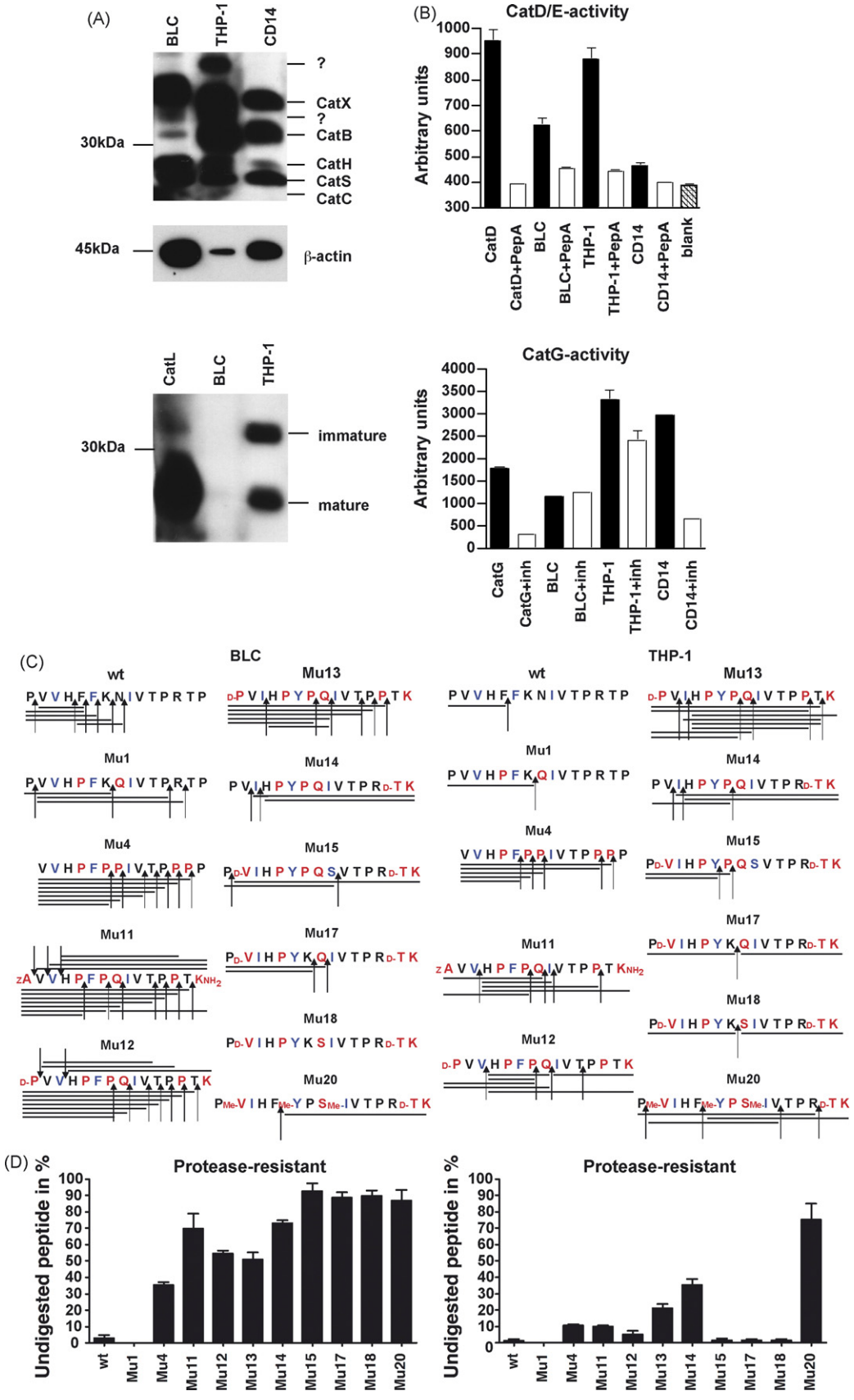

Fig. 2 – Generating MBP85-99-derived protease-resistant peptides. (A) Lysosomal proteases from purified B lymphoblastoid cells (BLC), myelomonocytoid cells THP-1 and CD14-derived lysosomal proteases were normalized for total protein content, labeled with the biotinylated activity-based probe DCG-04, resolved by SDS-PAGE and papain-like cysteine proteases were

its specificity preference between hydrophobic residues, digested the wt-peptide between phenylalanine 89 and phenylalanine 90 (89FF90).

3.2. Determination of cathepsin distribution of BLC and THP-1

Since different types of APC contain distinct sets of proteases, we generated lysosomal fractions from the B lymphoblastoid cells (BLC) and myelomonocytoid cell line THP-1 obtained by differential centrifugation and hypotonic lysis [21]. To compare the sets of active papain family cysteine proteases between both types of cells (BLC and THP-1), respective lysosomal proteases were incubated with the biotinylated protease activity-restricted probe DCG-04, which binds covalently to active cysteine proteases of the papain family, allowing the direct visualization of active cathepsin polypeptides via a biotin moiety. We detected active CatX (37 kDa), CatB (33 kDa), CatH (29 kDa) and CatS (28 kDa) in THP-1, BLC as well as $CD14^+$ cells (Fig. 2A, upper panel), in agreement with published data [23]. THP-1-derived lysosomal proteases contained more CatB, as well as additional active polypeptides with a molecular mass around 35 and 40 kDa. THP-1 cells also contained sizable amounts of mature CatL. In contrast to THP-1, DCG-04 labeled human CatL in BLC-derived lysosomal proteases only poorly, as assessed by Western blot (Fig. 2A, lower panel). CatL was detected abundantly as pro- (32 kDa) and mature form (25 kDa) in THP-1-derived lysosomal proteases.

Aspartate protease activity in THP-1, BLC and CD14 cells (presumably combined CatD/E-activity) was assessed by measuring the turnover of the fluorescent substrate MOCA-GKPILFFRLK-(Dnp)-D-R-NH2, in the presence or absence of the respective inhibitor pepstatin A (PepA). Activity was markedly higher in THP-1-derived lysosomal proteases, compared to BLC- and CD14-derived controls (Fig. 2B, upper panel). CatG activity was assessed in a similar fashion using the substrate Z-AAPF-AMC, and verified by specific inhibition. While measurable CatG activity was absent from BLC cells, it was present in THP-1, as well as in CD14 controls (Fig. 2B, lower panel). Thus, compared to BLC, lysosomal extracts from THP-1 cells contain markedly higher amounts of cysteine-type endo- and exopeptidases (CatB, CatL), serine proteases (CatG) as well as aspartate proteases (presumably CatD and CatE), representing an extremely hostile environment for the integrity of short linear peptides.

3.3. Generation of a protease-resistant peptide based on MBP85-99

Subsequently, we synthesized MBP85-99-based protease-resistant peptides, which withstand proteolytic activity for at least 24 h, using natural and non-natural amino acids and eliminated protease cleavage sites in a stepwise fashion. To this end, the amino acid positions that are expected to determine the cleavages of wt-peptide by isolated cathepsins as observed above (e.g. 88HF89 for CatS, 90FK91 for CatG, C-and N-terminal amino acids for CatC, H and B) were subsequently replaced by amino acids that were less likely to result in protease attack at its dominant cleavage site. At the same time, we maintained HLA-DRB1*1501 anchors. Altered peptides (Mu1-Mu20) were incubated with lysosomal proteases from both human BLC and THP-1 cells, to address differences in the action of the two protease mixes. We used mass spectrometry to identify the resulting proteolytic fragments, and quantified the ratio between the amounts of intact peptides at the start of the incubation period with that after 24 h of incubation with lysosomal proteases by HPLC. Fig. 2C (left panel) summarizes the proteolytic patterns and protease stability of 10 selected altered peptides (Mu1-Mu20) incubated with BLC-derived lysosomal proteases.

We eliminated the cleavage site of AEP and CatD in Mu1, however, these changes did not influence the amounts of intact peptide retrieved after 24 h of incubation with BLC-derived lysosomal proteases, compared to the wt-peptide. We then substituted amino acids in position K91 and I92 by P91 and P92, and added two prolines at the C-terminus (Mu4) or we synthesized a peptide with protecting groups (Mu11) such as Z-alanine (Z-A) and amide-bound lysine ($K\text{-}NH_2$ 99) for exoprotease protection. Not until we used D-amino acids (D-P85, D-V86), was complete protection of the amino-terminus observed (Mu14, 15, 18, 20) and D-T98 prevented degradation from the carboxy-terminus (Mu12, 13, 15, 17, 20).

Fig. 2D (left panel) shows relative amounts of the intact original peptide after 24 h of incubation with lysosomal proteases from BLC, as quantified by HPLC. We detected only traces of residual full-length peptide from the wt-peptide, as well as from Mu1. In contrast to wt-peptide, Mu4, 11, 12, 13, 14 showed an increase in protease resistance (30–70% intact peptide after 24 h of digest, compared to

visualized by streptavidin HRP-blot. CatX, B, H, S and C were detected as active cysteine proteases (upper panel). In addition, CatL-levels were assessed by performing CatL-specific Western blot (lower panel). (B) CatD/E-activity was determined at pH 4.0 by measuring the turnover of the fluorogenic substrate MOCA-GKPILFFRLK-(Dnp)-D-R-NH2 in lysosomal proteases from BLC, THP-1 and CD14 (upper panel). CatG-activity was measured by using Z-AAPF-AMC at pH 8.0 and specified with the specific CatG-inhibitor I (lower panel). Data are representative of two independent experiments and each was done in duplicates. PepA: pepstatin A and inh: CatG-specific inhibitor I. (C) Altered peptides (Mu1-Mu20) were incubated for 24 h at 37 °C with BLC (left panel) or THP-1 (right panel)-derived lysosomal proteases. To eliminate AEP cleavage site asparagine to glutamine in position 92 (N92 to Q92) was exchanged and phenylalanine to proline 89 (F89 to P89) to abolish proteolytic activity by CatD (Mu1). Proline amino acids at the C-terminus (Mu4) and using protecting groups (Mu11) such as Z-alanine (Z-A) and amide-bound lysine ($K\text{-}NH_2$ 99) were used to reduce exoprotease activity. To protect proteolytic attack at the N-terminus or C-terminus, respectively, D- (Mu12, 13, 14, 15, 17, 18, 20) and methylated amino acids (Mu20) were synthesized. (D) The relative amounts of intact peptides remaining after lysosomal-derived protease digestion were quantified by HPLC in percent. Three independent experiments are shown. Blue amino acid letters indicate anchor residues, in red substituted amino acids and arrows indicate protease cleavage sites.

~3% with the wt-peptide). Significant protease protection was achieved (>90%) when both N and C-terminal exoprotease cleavage was blocked by the introduction of either D- or methylated amino acids at both preterminal positions (Mu15, 17, 18, and 20).

Fig. 2C and D (right panel) summarizes the different cleavage sites when we incubated protease-resistant peptides with THP-1-derived-lysosomal proteases as compared to BLC. Mu11, 12, 15, 17 and 18 were not resistant against the lysosomal protease pool present in THP-1 cells. The protease-lability of Mu17 and 18 in this setting was found to be most likely due to one or two single proteases not present in BLC. To address which proteases are capable of digesting Mu18, we incubated Mu18 with different isolated cathepsins and found that only CatL was still able to cleave Mu18 between K91 and S92 (data not shown). Based on this information we synthesized Mu20 which incorporates methylated amino acids, such as isoleucine at position 93 (Me-I93), valine at 86 (Me-V86) and thyrosine at 90 (Me-Y90) to both eliminate the CatL cleavage site and increase binding to HLA-DRB*1501. Mu20 resisted degradation by lysosomal proteases from both BLC and THP-1 cells *in vitro* for at least 24 h by more > 80%.

3.4. *Amino acids next to anchor residues interfere with binding of MBP85-99 to HLA-DRB1*1501*

Peptide binding to MHC class II molecules is mediated by the interaction of the anchor residues of the peptide with the binding groove of MHC class II molecules. To quantify peptide binding we performed a competition assay [22]. Fluorescent labeled MBP85-97 peptide (AMCA-MBP85-97) was loaded onto HLA-DRB1*1501. Subsequently, protease-resistant peptides were added at an equimolar ratio and binding was analyzed by high performance size exclusion chromatography with simultaneous fluorescent and UV-detection to determine which portion of the fluorescent peptide co-eluted with the MHC class II complex. Using this assay, different peptide permutations were compared in order to define which amino acid-charges would allow competition of wt-peptide from binding to HLA-DRB1*1501. We found that the exchange of asparagine 91 (N91) to glutamine 91 (Q91) (MuQ) reduced HLA-DRB1*1501 peptide competition capacity to 50%, aspartic acid 91 (D91, MuD) to ~60%, whereas serine 91 (S91, MuS) had no negative effect on competition of the immunogenic peptide (Fig. 3A). In contrast to D-valine 86 (D-V86, Mu15D-V) methylated valine at 86 (Me-V86, Mu15Me-V) also did not

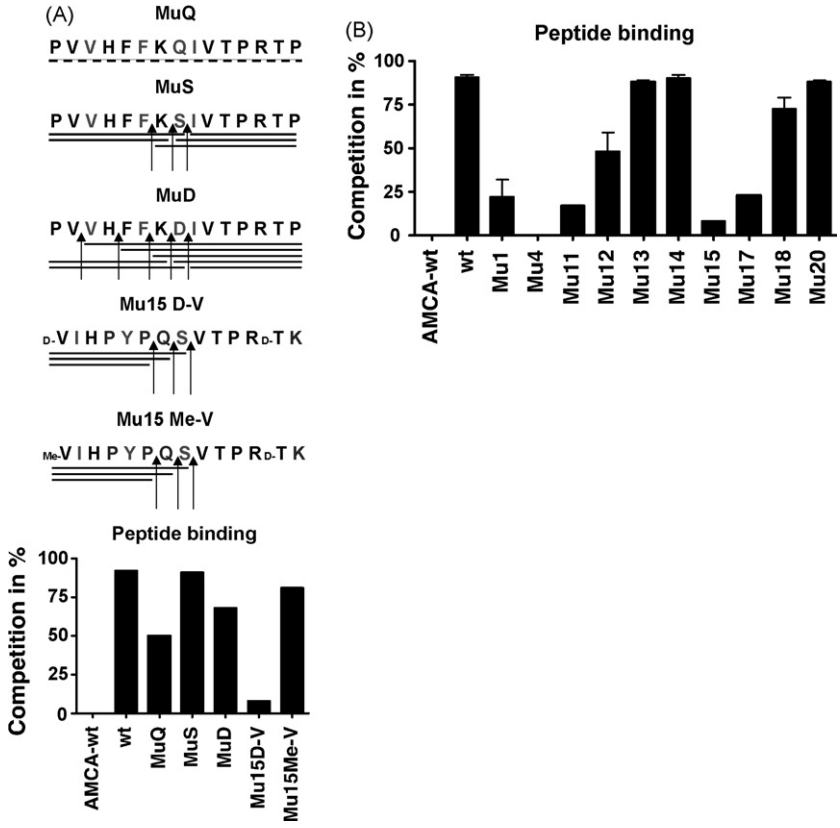

Fig. 3 – Protease-resistant peptides bind to HLA-DRB1*1501 with affinity similar to MBP85-99. (A) Peptides with single exchanged-, D- or methylated amino acids were incubated for 24 h at 37 °C with BLC-derived lysosomal proteases and the proteolytic intermediates were analyzed by mass spectrometry (arrows show the cleavage sites of lysosomal proteases). Peptide-binding to HLA-DRB1*1501 were performed by a competition assay. Fluorescent labeled MBP85-97 peptide (AMCA-MBP85-97) was preloaded onto HLA-DRB1*1501 molecules and was competed by altered peptide ligands at equimolar ratio and analyzed by high performance size exclusion chromatography (HPSEC). (B) Protease-resistant peptides (Mu1-Mu20) were added to AMCA-MBP85-97-HLA-DRB1*1501 complexes at equimolar ratio and analyzed by HPSEC.

grossly interfere with binding to HLA-DRB1*1501. All of this suggested that amino acids adjacent to the anchor residues are also important for binding to HLA-DRB*1501.

Having generated protease-resistant peptides, we wanted to know whether these peptides would also bind to HLA-DRB1*1501. Mu1, Mu4, Mu11, Mu12 as well as Mu15 and Mu17 competed poorly with the AMCA-MBP85-97 for binding to HLA-DRB1*1501, compared to Mu13, Mu14, Mu18 and Mu20, which efficiently replaced the AMCA-MBP85-97 from the HLA-DRB1*1501 binding groove (as shown in Fig. 3B).

3.5. *Protease-resistant peptides inhibit MBP86-98-specific T cell activation*

Self-reactive T cells lead to autoimmunity. Therefore, we tested the effect of the protease-resistant peptides on the MBP-specific T cell response *in vitro*. We incubated a HLA-DRB1*1501-positive B cell line MGAR simultaneously with 10 μg/ml wt-peptide and serially increasing amounts of protease-resistant peptides, followed by the addition of MBP86-98 specific T cells. Secreted IL-2 was quantified by assessing the incorporation of [^{3}H] thymidine (cpm) by CTLL-2 cells grown in the respective culture supernatants. As expected, in the absence of competing protease-resistant peptides, significant T cell IL-2 secretion was detected (Fig. 4). Addition of Mu1, which was not protease-resistant and had a low affinity to HLA-DRB1*1501, resulted in only modest reduction of T cell IL-2 secretion at equimolar concentrations. Mu13 and Mu14 both showed a binding affinity similar to the wt-peptide, however, they were significantly more protease resistant. As a result, they reduced T cell IL-2 secretion already at sub-equimolar concentrations, indicating that introducing protease-resistance is indeed a method to augment the effect of APL. In contrast, Mu18, which was resistant to BLC-derived lysosomal proteases, but bound poorly, showed significant inhibition of T cell IL-2 secretion only when added at high concentrations. Mu20, the most protease-resistant peptide with strong HLA-DRB1*1501-binding also inhibited T cell proliferation when cultured simultaneously with wt-peptide and is therefore an attractive prototype candidate to block MBP-specific T cell proliferation.

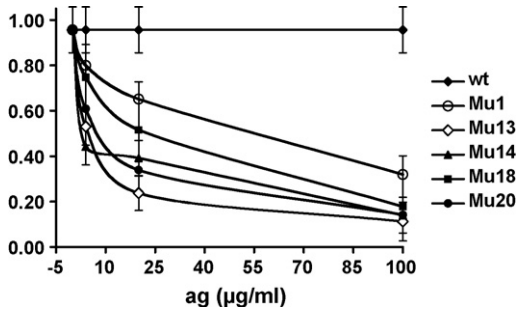

Fig. 4 – Protease-resistant peptides inhibit MBP86-98-specific T cell proliferation. HLA-DRB1*1501-positive B lymphoblastoid cells MGAR was incubated with 10 μg/ml MBP85-99 and increasing amounts of protease-resistant peptides (4, 20 and 100 μg/ml), followed by the addition of MBP85-99-specific T cells. IL-2 U/ml levels were measured by CTLL-2 cells grown in the culture supernatants. One of three experiments with similar results is shown. Ag: antigen.

4. Discussion

We show that the design of protease-resistant peptides by cleavage site directed amino acid substitution results in virtually complete resistance to lysosomal proteases derived from both B lymphoblastoid cells and myelomonocytoid cells THP-1. Altered peptide ligands are prone to degradation in a proteolytic environment. We address this major obstacle by generating protease-resistant peptides based on a multiple sclerosis- (MBP85-99) autoantigen, which antagonized MBP85-99-specific T cell activation *in vitro*.

We systematically eliminated endoprotease cleavage sites based on the proteolytic patterns obtained from isolated proteases. The subset of cathepsins used in this digest represents the major endocytic proteases known to date. Although this list is not necessarily complete, the proteases analysed are responsible for the majority of cleavage sites found when peptides are incubated with lysosomal proteases, arguing that functionally, the most important proteases are covered by this selection. We protected the N- and C-terminal ends of the MBP85-99 peptide by adding non-natural amino acids in the preterminal position, and at the same time optimized binding to HLA-DRB1*1501 by changing amino acids in the respective anchor positions (Mu13, 14, 17, 18 and 20). Interestingly, at the TCR contact sites (F89, K91, P96), the exchange of one amino acid (K91 to P91), as shown for Mu13, 14, 15 and 20, was sufficient to antagonize T cell proliferation. While D-amino acids increased the stability of the peptide in a proteolytic environment, binding affinity to HLA-DRB1*1501 appeared to be reduced. However, when we used methylated amino acids at the same position, the resulting peptides were not only protease-resistant, which is in agreement with published data [10], but also bound to HLA-DRB1*1501 with affinity comparable to natural amino acids. Thus, the use of methylated amino acids represents a method for generating a protease-resistant peptide without losing binding affinity to MHC class II molecules.

Although bioinformatic tools can be used to generate peptides that are capable of resisting proteolytic activity with results that are comparable to our approach. The strategy that we used involves substantially lower amounts of time and resources. This is especially important, because the approach has to be tailored to different HLA haplotypes and autoantigens, which would require extensive computer modelling resources.

Even if an APL is not loaded on MHC class II within the endocytic compartment, but rather at the cell surface, as may be the case for peptide competition of the MBP immunogenic peptide, such an APL needs protection from proteolysis, as shown by resistance to the lysosomal protease contents of THP-1 cells. The Mu20 peptide generated by our approach can withstand 24 h of incubation time

in a concentrated mixture of cysteine-, aspartate- and serine-proteases of the endoprotease and exoprotease type. Several proteases of the bloodstream, such as serine proteases (for instance CatG or the coagulation factor thrombin), limit the potential use of APL *in vivo*. Therefore, any APLs used must also be able to withstand the proteolytic components of the blood. We incubated Mu20 with human blood serum for 24 h and found that Mu20 was stable from degradation by protease activity, suggesting a prolonged stability during *in vivo* use (data not shown).

Tselios et al. have reported an improved protease stability for cyclic analogous of the immunodominant epitope MBP87-99 (cyclo (87–99) [Arg91 Ala96] MBP87-99) that translated into better T cell inhibition. Cyclo (87–99) [Arg91 Ala96] MBP87-99 bound to HLA-DR4 molecules, inhibited EAE in Lewis rats as well as suppressed $CD4^+$ T cells from MS patients and was therefore suggested as a potential therapeutic treatment for MS [26]. These cyclic analogues demonstrated less protease-resistance when lysosomal proteases from THP-1 were used as compared to the linear protease-resistant peptides synthesized by the method described here (data not shown).

Glatiramer acetate (copaxone), a random synthetic amino acid (poly [Y, E, A, K]), which potently binds to HLA-DRB1*1501, was originally designed to induce EAE, but was later found to block the induction of EAE [27,28]. It was subsequently tested in Multiple Sclerosis patients and was found to be a successful therapeutic in clinical trials [29]. Although copaxone is a peptide-based therapeutic, it is very likely to suffer from proteolytic destruction when administered systemically or even internalized into endocytic compartments of APC, with or without prior binding to MHC II on the cell surface. To demonstrate this, we incubated copaxone with lysosomal extracts from THP-1. This resulted in its complete digestion after eight hours (data not shown). Introduction of appropriate non-natural methylated amino acids might prolong the biological half live of copaxone, improve its efficacy, or at least reduce the 20 mg daily injection recommended for the treatment of MS patients.

Cleavage site-directed amino acid substitution strategy entails a relatively few number of steps, and incorporates published knowledge regarding the major immunogenic peptide and its binding preferences to the most relevant MHC II. Such information is now available for a number of MHC II-associated autoimmune diseases. Thus, it should be possible to transfer this strategy to other autoimmune diseases, bypassing the need for technically challenging, time-consuming and expensive molecular modelling strategies that aim for a similar goal.

Low stability and protease destruction are a common problem for virtually all small, peptide-based types of drugs, including APL and those used for peptide vaccinations. We demonstrated that virtually entirely protease-resistant peptides can be designed and synthesized, using a relatively simple deductive approach. These peptides retain the ability of binding HLA-DRB1*1501 and can be used to suppress MBP-specific T cell activation. The transfer of the knowledge of the approach presented here is promising to other peptide-based immunotherapies currently limited by proteolytic destruction.

Acknowledgments

This work was supported by grants from the Deutsche Forschungsgemeinschaft (BU 1822/3-1, DR378.2-1, DR378.2-3, SFB 685, and 518), Federal Ministry of Education and Research (Fö.01KS9602), Else Kröner-Fresensius-Stiftung to BOB, GRK 1041 Molecular Diabetology and Endocrinology in Clinical Medicine, the Interdisciplinary Centre of Clinical Research Tübingen (IZKF) and the Marie Curie research training network ''drugs for therapy''. We gratefully acknowledge L. Fugger for providing the MBP-specific T cell hybridoma and N. Zaidi for her help with CatD/E-activity test. We thank also M. Deeg for initial experiments with ESI and S. Stevanovic, for his help with MALDI-TOF and K. Belisle for critical reading of the manuscript.

REFERENCES

[1] Martin R, McFarland HF, McFarlin DE. Immunological aspects of demyelinating diseases. Annu Rev Immunol 1992;10:153–87.
[2] Whitaker JN. Myelin basic protein and multiple sclerosis. Ital J Neurol Sci 1983;4:153–7.
[3] Kamholz J, Toffenetti J, Lazzarini RA. Organization and expression of the human myelin basic protein gene. J Neurosci Res 1988;21:62–70.
[4] Muraro PA, Vergelli M, Kalbus M, Banks DE, Nagle JW, Tranquill LR, et al. Immunodominance of a low-affinity major histocompatibility complex-binding myelin basic protein epitope (residues 111-129) in HLA-DR4 (B1*0401) subjects is associated with a restricted T cell receptor repertoire. J Clin Invest 1997;100:339–49.
[5] Heath VL, Moore NC, Parnell SM, Mason DW. Intrathymic expression of genes involved in organ specific autoimmune disease. J Autoimmun 1998;11:309–18.
[6] Heath VL, Saoudi A, Seddon BP, Moore NC, Fowell DJ, Mason DW. The role of the thymus in the control of autoimmunity. J Autoimmun 1996;9:241–6.
[7] Anderson MS, Venanzi ES, Klein L, Chen Z, Berzins SP, Turley SJ, et al. Projection of an immunological self shadow within the thymus by the aire protein. Science 2002;298:1395–401.
[8] Ota K, Matsui M, Milford EL, Mackin GA, Weiner HL, Hafler DA. T-cell recognition of an immunodominant myelin basic protein epitope in multiple sclerosis. Nature 1990;346:183–7.
[9] Regner M, Lambert PH. Autoimmunity through infection or immunization? Nat Immunol 2001;2:185–8.
[10] Falcioni F, Ito K, Vidovic D, Belunis C, Campbell R, Berthel SJ, et al. Peptidomimetic compounds that inhibit antigen presentation by autoimmune disease-associated class II major histocompatibility molecules. Nat Biotechnol 1999;17:562–7.
[11] Lehmann PV. Selective peptidomimetic blockers of autoantigen presentation: a novel therapeutic approach to autoimmune disease. Trends Pharmacol Sci 2000;21:79–80.
[12] Sakai K, Zamvil SS, Mitchell DJ, Hodgkinson S, Rothbard JB, Steinman L. Prevention of experimental encephalomyelitis with peptides that block interaction of T cells with major histocompatibility complex proteins. Proc Natl Acad Sci USA 1989;86:9470–4.
[13] Aharoni R, Teitelbaum D, Sela M, Arnon R. Bystander suppression of experimental autoimmune

encephalomyelitis by T cell lines and clones of the Th2 type induced by copolymer 1. J Neuroimmunol 1998;91:135–46.
[14] Vergelli M, Hemmer B, Utz U, Vogt A, Kalbus M, Tranquill L, et al. Differential activation of human autoreactive T cell clones by altered peptide ligands derived from myelin basic protein peptide (87–99). Eur J Immunol 1996;26:2624–34.
[15] Kappos L, Comi G, Panitch H, Oger J, Antel J, Conlon P, et al. Induction of a non-encephalitogenic type 2 T helper-cell autoimmune response in multiple sclerosis after administration of an altered peptide ligand in a placebo-controlled, randomized phase II trial. The Altered Peptide Ligand in Relapsing MS Study Group. Nat Med 2000;6: 1176–82.
[16] Bielekova B, Goodwin B, Richert N, Cortese I, Kondo T, Afshar G, et al. Encephalitogenic potential of the myelin basic protein peptide (amino acids 83–99) in multiple sclerosis: results of a phase II clinical trial with an altered peptide ligand. Nat Med 2000;6:1167–75.
[17] Manoury B, Mazzeo D, Fugger L, Viner N, Ponsford M, Streeter H, et al. Destructive processing by asparagine endopeptidase limits presentation of a dominant T cell epitope in MBP. Nat Immunol 2002.
[18] Beck H, Schwarz G, Schroter CJ, Deeg M, Baier D, Stevanovic S, et al. Cathepsin S and an asparagine-specific endoprotease dominate the proteolytic processing of human myelin basic protein in vitro. Eur J Immunol 2001;31:3726–36.
[19] Burster T, Beck A, Tolosa E, Marin-Esteban V, Rotzschke O, Falk K, et al. Cathepsin G, and not the asparagine-specific endoprotease, controls the processing of myelin basic protein in lysosomes from human B lymphocytes. J Immunol 2004;172:5495–503.
[20] Burster T, Beck A, Poeschel S, Oren A, Baechle D, Reich M, et al. Interferon-gamma regulates cathepsin G activity in microglia-derived lysosomes and controls the proteolytic processing of myelin basic protein in vitro. Immunology 2007;121:82–93.
[21] Schroter CJ, Braun M, Englert J, Beck H, Schmid H, Kalbacher H. A rapid method to separate endosomes from lysosomal contents using differential centrifugation and hypotonic lysis of lysosomes. J Immunol Methods 1999;227:161–8.
[22] Max H, Halder T, Kalbus M, Gnau V, Jung G, Kalbacher H. A 16mer peptide of the human autoantigen calreticulin is a most prominent HLA-DR4Dw4-associated self-peptide. Hum Immunol 1994;41:39–45.
[23] Lautwein A, Kraus M, Reich M, Burster T, Brandenburg J, Overkleeft HS, et al. Human B lymphoblastoid cells contain distinct patterns of cathepsin activity in endocytic compartments and regulate MHC class II transport in a cathepsin S-independent manner. J Leukoc Biol 2004;75:844–55.
[24] Burster T, Beck A, Tolosa E, Schnorrer P, Weissert R, Reich M, et al. Differential processing of autoantigens in lysosomes from human monocyte-derived and peripheral blood dendritic cells. J Immunol 2005;175: 5940–9.
[25] Greenbaum D, Medzihradszky KF, Burlingame A, Bogyo M. Epoxide electrophiles as activity-dependent cysteine protease profiling and discovery tools. Chem Biol 2000;7:569–81.
[26] Tselios T, Apostolopoulos V, Daliani I, Deraos S, Grdadolnik S, Mavromoustakos T, et al. Antagonistic effects of human cyclic MBP(87–99) altered peptide ligands in experimental allergic encephalomyelitis and human T-cell proliferation. J Med Chem 2002;45:275–83.
[27] Arnon R, Aharoni R. Mechanism of action of glatiramer acetate in multiple sclerosis and its potential for the development of new applications. Proc Natl Acad Sci USA 2004;101:14593–8.
[28] Sela M, Mozes E. Therapeutic vaccines in autoimmunity. Proc Natl Acad Sci USA 2004;101:14586–92.
[29] Johnson KP, Brooks BR, Cohen JA, Ford CC, Goldstein J, Lisak RP, et al. Copolymer 1 reduces relapse rate and improves disability in relapsing-remitting multiple sclerosis: results of a phase III multicenter, double-blind placebo-controlled trial. The Copolymer 1 Multiple Sclerosis Study Group. Neurology 1995;45:1268–76.