Structural Requirements for Binding of an Immunodominant Myelin Basic Protein Peptide to DR2 Isotypes and for Its Recognition by Human T Cell Clones

By Kai W. Wucherpfennig,*† Alessandro Sette,§ Scott Southwood,§ Carla Oseroff,§ Makoto Matsui,† Jack L. Strominger,* and David A. Hafler†

From the *Department of Biochemistry and Molecular Biology, Harvard University, Cambridge, Massachusetts 02138; the †Center for Neurologic Diseases, Brigham and Women's Hospital, Harvard Medical School, Boston, Massachusetts 02115; and §Cytel, San Diego, California 92121

Summary
Immunodominant T cell epitopes of myelin basic protein (MBP) may be target antigens for major histocompatibility complex class II-restricted, autoreactive T cells in multiple sclerosis (MS). Since susceptibility to MS is associated with the DR2 haplotype, the binding and presentation of the immunodominant MBP(84–102) peptide by DR2 antigens were examined. The immunodominant MBP(84–102) peptide was found to bind with high affinity to DRB1*1501 and DRB5*0101 molecules of the disease-associated DR2 haplotype. Overlapping but distinct peptide segments were critical for binding to these molecules; hydrophobic residues (Val189 and Phe92) in the MBP(88–95) segment were critical for peptide binding to DRB1*1501 molecules, whereas hydrophobic and charged residues (Phe92, Lys93) in the MBP(89–101/102) sequence contributed to DRB5*0101 binding. The different registers for peptide binding made different peptide side chains available for interaction with the T cell receptor. Although the peptide was bound with high affinity by both DRB1 and DRB5 molecules, only DRB1 (DRB1*1501 and 1602) but not DRB5 molecules served as restriction elements for a panel of T cell clones generated from two MS patients suggesting that the complex of MBP(84–102) and DRB1 molecules is more immunogenic for MBP reactive T cells. The minimal MBP peptide epitope for several T cell clones and the residues important for binding to DRB1*1501 molecules and for T cell stimulation have been defined.

Susceptibility to a variety of human autoimmune diseases is associated with alleles of MHC class I or class II genes. Examples of autoimmune diseases associated with MHC class II haplotypes include insulin dependent diabetes (DR3 and DR4 haplotypes), myasthenia gravis (DR3) and multiple sclerosis (DR2) (1–3). The major hypothesis is that these allelic gene products define the specificity of an immune attack against self-antigens by presentation of tissue specific self-peptides to T cells.

In the case of multiple sclerosis (MS),1 this hypothesis implies that DR2 antigens (either DRB1*1501 or DRB5*0101 molecules encoded in the DR2 haplotype, or both) present self-peptide(s) from a myelin/oligodendrocyte antigen since the disease is restricted to the white matter of the central but not peripheral nervous system. Based on their encephalitogenicity in animal models (4–6), myelin basic protein (MBP) and proteolipid protein (PLP) have long been viewed as the principal candidate antigens. The role of MBP and PLP reactive T cells in the pathogenesis of MS has, however, been difficult to prove.

In animal models, encephalomyelitis is mediated by T cells reactive with immunodominant determinants of MBP or PLP (4–6). In humans, two dominant T cell epitopes are located in the center and in the COOH-terminal portion of the molecule [MBP(84–102) and MBP(143–168)] (7–10). These epitopes may be relevant to the pathogenesis of MS since both peptides can be presented by DRB1*1501 and DRB5*0101 molecules of the disease associated haplotype. DRB1*1501 molecules present MBP(80–99) and MBP(148–162) peptides; MBP(87–106), MBP(131–145), MBP(139–153), and MBP(76–91) are presented by the DRB5*0101 molecule (8, 9). A number of other DR and DQ antigens can also serve as restriction elements, in particular for T cells reactive with the MBP(87–106) peptide (11, 12).

The analysis of structural requirements for the interaction

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1 Abbreviations used in this paper: MBP, myelin basic protein; MNC, mononuclear cells; MS, multiple sclerosis; PLP, proteolipid protein.
of MBP peptides with MHC class II antigens, in particular with the MS associated DR2 isotypes (DRB1*1501 and DRB5*0101, sometimes also referred to as DR2b and DR2a, respectively), may help to define the biochemical basis for the association of DR2 with MS. The present data demonstrate that DRB1*1501 and DRB5*0101 molecules interact with different residues of the MBP(84-102) peptide. The MBP(84-102) peptide complexed with DRB1*1501 and DRB1*1602 molecules appears to generate a more vigorous immune response since MBP(84-102)-specific, DR-restricted T cell clones examined in the present study were restricted by DRB1*1501 or DRB1*1602 (a closely related DR2 allele) and not by DRB5*0101 molecules.

Materials and Methods

Cloning of MBP(84–102)-specific T Cells. MBP-specific T cell lines were generated from blood mononuclear cells as previously described (7). Briefly, mononuclear cells (MNC) from two MS patients were cultured with purified human MBP (100 /µg/ml) in RPMI 1640 supplemented with 10% pooled human serum, 2 mM l-glutamine, 10 mM Hepes, 100 U/100 /µl penicillin/streptomycin in 96-well plates at 2 x 10^5 cells/well. On day 3, IL-2 was added to 5% Human T-Stim (Becton Dickinson & Co., Mountain View, CA) and cells were fed with media containing 5% IL-2 every 3–4 d. On day 14, an aliquot of each cell line was assayed for reactivity to human MBP. 10^4 cells from each T cell line were cocultured with 10^4 irradiated autologous MNC in duplicate for 72 h; a [3H]thymidine pulse was performed during the first 18 h of culture. MNC were prepared by pulsing cells with or without 100 /µg/ml of MBP for 1 h at 37°C. MBP-specific T cell lines (stimulation index >3) were retested using a panel of 13 synthetic peptides encompassing the human MBP sequence. MBP-specific T cell lines were maintained by restimulation with MBP or peptide pulsed MNC at 10–14-d intervals. Following a third round of stimulation (generally two stimulations with MBP and one stimulation with peptide), T cell clones were cloned by limiting dilution using allogeneic feeder cells and phytohemagglutinin (1 µg/ml) (Murex Diagnostics, Dartford, UK) for stimulation. Allogeneic MNC were irradiated with 5,000 rad and co-cultured in 96-well plates (10^5 cells/well) with T cells (0.1–5 cell/well). IL-2 was added on day 3 and cells were fed every 3–4 d with media containing 5% IL-2. On days 12–14, growth positive wells were expanded by restimulation with PHA, IL-2, and allogeneic feeder cells. T cell clones were tested in proliferation assays for their reactivity to MBP and the MBP(84–102) peptide; an autologous EBV transformed cell line was used as APC for Hy clones while B cell line 9010 (DR2/DQ6) was used for T cell clones from patient Ob. Antigen reactive T cell clones were maintained by stimulation with PHA, IL-2, and irradiated allogeneic MNC in 7–10-d intervals.

Analysis of MHC Restriction of MBP-specific T Cell Clones. The MHC restriction of MBP-specific T cell clones was determined using blocking mAbs specific for DR (mAb L243), DQ (mAb S3/4), and DP (mAb B7/21) antigens. EBV transformed B cell lines were pulsed with the MBP(84–102) or the MBP(85–99) peptide at a concentration of 50 /µM for 2 h at 37°C. Washed and irradiated APC (2.5 x 10^5/well) were then co-cultured with 5 x 10^4 T cells in triplicates for 3 d. mAbs were added to cultures at a 1:100 dilution of ascites fluid. T cell proliferation was measured as [3H]thymidine incorporation after 18-h pulse. EBV-transformed B cell lines used were: an autologous EBV transformed B cell line from MS patient Hy, as well as B cell lines 9010 (DR2/DQ6, DRB1*1501), 9009 (DR2 Dw21/DQ1, DRB1*1601), and 9001 (DR1/DQ1, DRB1*0101).

To further define the MHC restriction of DR-restricted T cell clones, L cell transfectants expressing DRB1*1501, DRB5*0101, and DRB1*0401 molecules (a kind gift of Dr. R. Sekaly, Institut de Recherches Cliniques de Montreal, Canada) were used. L cell transfectants were grown in RPMI 1640 supplemented with 10% FCS, 2 mM l-glutamine, 10 mM Hepes, 100 U/100 /µl penicillin/streptomycin as well as mycophenolic acid (6 /µg/ml), xanthine (250 /µg/ml), and hypoxanthine (15 /µg/ml). Cell surface expression of DR molecules was verified by FACS® analysis (Becton Dickinson & Co.). For T cell stimulation assays, L cell transfectants were scrapped from plates, washed, irradiated (5,000 rad), and pulsed with the MBP(84–102) peptide at a concentration of 50 /µM for 2 h at 37°C. After the peptide pulse, cells were washed and co-cultured with T cells in triplicates for 3 d (2.5 x 10^4 L cells and 5 x 10^4 T cells/well of a 96-well plate). T cell proliferation was measured as [3H]thymidine incorporation after an 18-h pulse.

Analysis of T Cell Reactivity to MBP Peptides. The reactivity of T cell clones to a panel of truncated and substituted peptides of the MBP(84–102) epitope was determined in T cell proliferation assays. EBV transformed B cell lines [an autologous EBV transformed B cell line from patient Hy and B cell line 9010 (DR2/DQ6)] were pulsed with synthetic peptides at concentrations ranging from 5 nM to 50 /µM. After a 2-h pulse at 37°C, washed T cells were added (2.5 x 10^4 B cells and 5 x 10^4 T cells/well of a 96-well plate). T cells were co-cultured in triplicates with peptide pulsed B cells for 3 d followed by an 18-h pulse with [3H]thymidine. T cell proliferation was measured as the incorporation of [3H]thymidine. To determine the peptide concentration required for 50% maximum proliferation, data (cpm of incorporated [3H]thymidine) were plotted against the peptide concentration (µM) used for T cell stimulation. For each peptide, the point in the curve giving 50% of the maximum stimulation obtained with the MBP(84–102) reference peptide was determined; the corresponding peptide concentration (µM) was read off the x-axis as an indicator of the relative efficiency of peptides to activate MBP(84–102)-specific T cell clones.

Affinity Purification of DR Molecules. Transfected fibroblast cell lines L466.1 (DRB1*1501) and L466.3 (DRB5*0101) were used as source of HLA class II molecules. DR-transfected fibroblast cell lines were a kind gift of Dr. R. W. Karr (Monsanto, St. Louis, MO). Cell lines were cultured in RPMI 1640 supplemented with 10% heat-inactivated FCS or horse serum (Hazeltine Biologies, Inc., Lenexa, KS). Cells were lysed at a concentration of 10^6 cells/ml in 50 mM Tris-HCl, pH 8.5, containing 2% Renex, 150 mM NaCl, 5 mM EDTA, and 2 mM PMSF. The lysates were cleared of nuclei and debris by centrifugation at 10,000 g for 20 min.

DR molecules were purified essentially as described by Gorga et al. (13) using the mAb LB3.1 covalently coupled to protein A-Sepharose CL-4B. Aliquots of cell lysate equivalent to ~10 g of cells were passed sequentially through the following columns: Sepharose CL-4B (10 ml), and LB3.1-protein A-Sepharose (15 ml) using a flow rate of 15 ml/h. The columns were washed with 10-column volumes of 10 mM Tris-HCl, pH 8.0, 0.1% Renex (5 ml/h); 2-column volumes of PBS, and 2-column volumes of PBS, 1% octylglucoside. DR molecules were eluted from the LB3.1 column with 0.05 M diethylamine, in 0.15 M NaCl containing 1% octylglucoside (pH 11.5), immediately neutralized with 2 M glycine, pH 2.0, and concentrated by ultrafiltration through a membrane.
was evaluated by a BCA protein assay (Pierce Chemical Co., Rock-

Table 1.

Binding of hMBP Peptides to Purified HLA Class II Molecules.

| Molecule                | MBP(84-102) | + MBP(84-102) | DRB1*1501 transfectant | + MBP(84-102) | DRB5*0101 transfectant | + MBP(84-102) | DRB1*0401 transfectant | + MBP(84-102) |
|-------------------------|-------------|-------------|------------------------|-------------|------------------------|-------------|------------------------|-------------|
| BCL 9010 (DR2/DQ6)     | 378         | 183,894     | 177                    | 17,303      | 247                    | 333         | 417                    |
| + MBP(84-102)           | 2,303       | 149,050     | 373                    | 114,514     | 213                    | 497         | 371                    |
| DRB1*1501 transfectant | 2,253       | 31,956      | 303                    | 38,833      | 240                    | 302         | 266                    |
| + MBP(84-102)           | 2,549       | 93,008      | 1,561                  | 17,554      | 1,461                  | 1,563       | 1,025                  |
| DRB5*0101 transfectant | 2,055       | 30,206      | 215                    | 43,573      | 410                    | 659         | 865                    |
| + MBP(84-102)           | 613         | 28,851      | 509                    | 39,049      | 542                    | 569         | 489                    |
| DRB1*0401 transfectant | 1,258       | 48,884      | 598                    | 39,139      | 634                    | 1,110       | 995                    |
| + MBP(84-102)           | 1,139       | 48,884      | 888                    | 13,244      | 1,185                  | 1,110       | 1,438                  |

A homozygous B cell line (9010.DR2/DQ6) and stable L cell transfectants that express the DRB1*1501 or DRB5*0101 molecules of the DR2 haplotype were pulsed with the MBP(84-102) peptide (100 μg/ml for BCL 9010, 50 μM for DR transfectants), irradiated with 5,000 rad, and co-cultured with T cell clones for 3 d, followed by a [3H]thymidine pulse. Stable L cell transfectants expressing DR4 (DRB1*0401) served as control. Numbers represent cpm of [3H]thymidine incorporation as a measure of T cell proliferation.

Results

Presentation of the MBP(84-102) Peptide by DRB1*1501 Molecules of the DR2 Haplotype. MBP-specific T cell lines were generated from blood mononuclear cells of two patients with MS and tested for their epitope specificity using a panel of overlapping synthetic MBP peptides (7). After two rounds of stimulation with antigen and one stimulation with the MBP(84-102) peptide, lines were cloned by limiting dilution using PHA, IL-2, and allogeneic feeder cells. By serological typing patients were classified as DR2/DR4 (patient Ob) and DR2/DR7 (patient Hy); molecular subtyping of the DR2 allele by PCR demonstrated the common DRB1*1501 haplotype (patient Ob) as well as the infrequent DRB1*1602 haplotype (patient Hy). The MHC restriction of the resulting clones (8 clones from patient Ob, 16 clones from patient Hy) was established using blocking mAbs specific for DR, DQ, and DP antigens as well as homozygous B cell lines as APC. The majority of MBP(84-102) specific clones were DR restricted (22/24). All of these clones recognized the MBP(84-102) peptide when presented either by B cell line 9010 (DR2/DQ6; DRB1*1501 haplotype) or by B cell line 9016 (DR2/DQ1; DRB1*1602 haplotype) indicating that they were HLA-DR2 restricted. Two T cell clones (Ob.3D1 and Hy.2E11) proliferated to the MBP(84-102) peptide when presented by BCL 9010 or 9016 indicating that they could recognize the peptide presented by B cells with a DRB1*1501
or DRB1*1602 haplotype. All other clones from patient Ob and patient Hy had a DRB1*1501 or DRB1*1602 restriction, respectively (Wucherpfennig, K. W., J. L. Strominger, D. A. Hafer, manuscript in preparation).

The DRB1*1501 haplotype is associated with susceptibility to MS and carries two functional DRβ chain genes (DRB1*1501 and DRB5*0101) (14). Therefore, L cell transfectants expressing DRB1*1501 or DRB5*0101 molecules were used as antigen presenting cells for T cell clones that proliferated in response to peptide pulsed B cell line 9010 (homozygous for the DRB1*1501 haplotype). All MBP(84–102)-specific T cell clones tested proliferated in response to the MBP(84–102) peptide when presented by DRB1*1501 but not by DRB5*0101 molecules (Table 1).

Two other MBP(84–102)-specific T cell clones from patient Hy (DR2, DR7/DQ1, DQ3) were HLA-DQ restricted since the proliferative response was blocked by mAb 3/4. Both T cell clones are DQ1-restricted and recognize the peptide presented by BCL that express DQ1 (9001: DR1/DQ1, 9009: DR2/DQ1) but not by a BCL that expresses DQ6 (9010: DR2/DQ6) (Table 2). The DR1-associated DQ1 molecule (DQA1*0101-DQB1*0501) has only two amino acid differences (DQα34, DRβ57) from the DR2-associated DQ1 molecule (DQA1*0102-DQB1*0502) that apparently not affect presentation of the peptide (14).

A 14-Amino Acid Peptide, MBP(85–98) Is Required for Efficient Stimulation of the Majority of DRB1*1501-restricted T Cell Clones. Peptides recognized by MHC class I antigens have strict length requirements (8–10-mer) (15–18); truncation or addition of a single residue has a profound effect on peptide binding to class I molecules. In contrast, naturally processed peptides bound to class II antigens were found to be longer and more heterogeneous in size (13–25 residues in the case of HLA-DR1 bound peptides) (19–21). Whereas short peptides can bind with high affinity to MHC class II molecules (e.g., hen egg lysozyme, residues 52–61 to I-Ak) (22), longer peptides may be required for recognition by at least some T cell receptor molecules. Therefore, a set of synthetic peptides with successive truncations at either the NH2 or the COOH terminus of MBP(84–102) was used to define the minimal T cell epitope of MBP(84–102) among DRB1*1501-restricted T cell clones. To compare the relative efficiency of peptides with respect to T cell stimulation, a homozygous BCL (9010, DR2/DQ6) was pulsed with peptides at concentrations ranging from 5 nM to 50 μM. From these titration curves, the peptide concentration required for 50% max-

| Table 2. MBP(84–102) Is Also Presented by HLA-DQ1 |
|-----------|-----------|
| Experiment | 2Hy.1B11  | 2Hy.1F6  |
| A          |           |          |
| Autologous BCL (DR2Dw21,DR7/DQ1,DQ3) | 1,972     | 1,938    |
| + MBP(84–102) | 128,373   | 55,991   |
| BCL 9009 (DR2Dw21/DQ1) | 3,190     | 1,697    |
| + MBP(84–102) | 116,488   | 39,220   |
| BCL 9001 (DR1/DQ1) | 5,795     | 2,695    |
| + MBP(84–102) | 103,452   | 39,288   |
| BCL 9010 (DR2/DQ6) | 2,169     | 937      |
| + MBP(84–102) | 1,811     | 1,264    |
| B          |           |          |
| BCL 9001, no peptide | 1,612     | ND       |
| BCL 9001 + MBP(85–99) | 46,380    | ND       |
| + mAb L243 (αDR) | 145,924   | ND       |
| + mAb S3/4 (αDQ) | 1,401     | ND       |
| + mAb B7/21 (αDP) | 83,777    | ND       |

(Experiment A) EBV transformed B cell lines were pulsed with the MBP(84–102) peptide at 100 μg/ml, washed, irradiated, and co-cultured with T cells for 72 h. T cell proliferation was assessed by [3H]thymidine incorporation. (Experiment B) B cell line 9001 was pulsed with the MBP(85–99) peptide at 50 μM and co-cultured with T cells in the presence or absence of blocking mAbs specific for DR, DQ, and DP antigens. Antibodies were used at a 1:100 dilution of ascites. T cell proliferation was assessed by [3H]thymidine incorporation. Nomenclature of HLA-DR and DQ antigens expressed by these BCL: BCL 9001 (DR1/DQ1): DRBI*0101/DQA1*0101, DQB1*0501; BCL 9009 (DR2 Dw21/DQ1): DRBI*1601/DQA1*0102, DQB1*0502; BCL 9010 (DR2 Dw2/DQ6): DRBI*1501/DQA1*0102, DQB1*0602. The peptide is presented by both BCL 9001 and 9009 which differ by only one residue in the DQ α1 and β1 domain (residue 57 of β chain: Val in DQB1*0501, Ser in DQB1*0502; residue 34 in α chain: Glu in DQA1*0101, Gln in DQA1*0102). BCL 9001 (presents peptide) and 9010 (does not present peptide) share the same DQ α chain sequence; however, there are differences at 11 positions in the β1 domain.
\[ \text{maximum stimulation [reference peptide MBP(84-102)] was calculated (Table 3). T cell clones varied in their sensitivity to peptide antigen with clone Hy.2E11 being the most sensitive [50% maximum stimulation at 0.15 \mu M with the MBP(85-99) peptide] (Table 3). There was a substantial loss of T cell reactivity in five of eight clones with an NH\textsubscript{2}-terminal truncation of Glu85; three other clones tolerated truncation of Glu85 and Asn86 without a major loss of reactivity. In contrast, truncation at the COOH terminus to residue 97 greatly diminished the response by all clones. While two of the eight clones recognized peptides with further COOH-terminal truncations (truncation up to residue 95/96), high peptide concentrations were required for stimulation. Synthetic peptides truncated at both the NH\textsubscript{2} and the COOH terminus demonstrated that residue 99 was not required for seven of eight clones; however, clone Hy.2E11 required 53 times more peptide for 50% maximum stimulation when peptide (86-98) rather than (86-99) was used (Table 3). These results indicate that all MBP(84-102) specific clones share a MBP(87-97) core determinant but that they are heterogeneous with respect to peptide length requirements at the NH\textsubscript{2} or COOH terminus. For the majority of clones, a MBP(85-98) peptide (a 14-mer) gave optimal stimulation, however, shorter pep-}  

| Table 3. The Majority of MBP(84-102)-specific T Cell Clones Require a 14-aa Peptide for Optimal Stimulation |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| Peptide         | Ob.1H8 | Ob.1E10 | Ob.2G9 | Ob.1C3 | Ob.2F3 | Ob.1A12 | Ob.3D1 | Hy.2E11 |
| (84-102)        | 3.1*   | 4.0    | 3.8    | 2.9    | 2.1    | 1.6    | 2.0    | 0.26    |
| (85-99)         | 8.0    | 4.2    | ND     | 3.4    | 1.6    | 1.5    | 2.5    | 0.15    |
| NH\textsubscript{2}-terminal truncations |
| (85-102)Y       | 12     | 1.8    | 3.6    | 4.0    | 1.5    | 0.4    | 2.0    | 0.31    |
| (86-102)Y       | >50    | >50    | >50    | 50     | 17     | 4.8    | 2.0    | 0.45    |
| (87-102)Y       | >50    | >50    | 50     | >50    | >50    | >50    | 4.2    | 0.80    |
| (88-102)Y       | -      | -      | -      | -      | -      | >50    | 50     | -       |
| (89-102)Y       | -      | -      | -      | -      | -      | -      | >50    | -       |
| (90-102)Y       | -      | -      | -      | -      | -      | -      | -      | -       |
| COOH-terminal truncations |
| (84-100)        | 2.2    | 1.8    | 1.2    | 2.2    | 1.9    | 0.65   | 2.5    | 0.08    |
| (84-99)         | 3.1    | 4.0    | 2.1    | 2.3    | 2.1    | 1.6    | 2.2    | 0.26    |
| (84-97)         | >50    | >50    | >50    | >50    | 22     | 22     | 11     | 25      |
| (84-96)         | >50    | -      | -      | -      | -      | -      | 50     | >50     |
| (84-95)         | -      | -      | -      | -      | -      | -      | >50    | >50     |
| (84-94)         | -      | -      | -      | -      | -      | -      | >50    | >50     |
| (84-93)         | -      | -      | -      | -      | -      | -      | -      | -       |
| NH\textsubscript{2} and COOH-terminal truncations |
| (85-98)A        | 4.1    | 4.1    | 3.8    | 3.8    | 1.2    | 1.1    | 0.4    | 4.0     |
| (86-99)         | 13     | 25     | 9.1    | 17     | 4.1    | 3.9    | 0.6    | 0.052   |
| (86-98)         | >50    | >50    | >50    | >50    | >50    | >50    | 50     | 3.8     |
| (86-97)         | -      | >50    | -      | >50    | -      | >50    | 14     | 35      |
| (87-99)         | -      | >50    | >50    | >50    | >50    | >50    | 1.4    | 0.18    |
| (87-98)A        | -      | >50    | >50    | >50    | >50    | >50    | 1      | 9.5     |
| (87-97)         | -      | -      | -      | -      | -      | -      | 14     | 50      |
| Epitope         | 85-98  | 85-98  | 85-98  | 85-98  | 85-98  | 85-98  | 87-98  | 87-99   |
| Length          | 14     | 14     | 14     | 14     | 14     | 14     | 12     | 13      |

Peptides were tested in proliferation assays using an EBV transformed BCL (9010, DR2/DQ6) as an APC. B cells were pulsed with peptides (5 nM to 50 \mu M) for 2 h and co-cultured with T cell clones for 72 h (5 \times 10^4 T cells and 2.5 \times 10^4 B cells/well). T cell proliferation was determined by \[^{3}H\]thyidine incorporation.

\* Numbers represent peptide concentrations (\mu M) that give 50\% maximum proliferation [reference peptide: MBP(84-102)]. Peptides (85-98A) and (87-98A) have a substitution of Pro98 by alanine since synthesis of peptides with COOH-terminal proline is difficult. Truncations that result in a \textgreater 10-fold loss in activity [as compared with the MBP(84-102) peptide] are boldfaced.

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tides (12- and 13-mer) were sufficient for two other clones. Therefore, 12–14-amino acid peptides appear to be required for efficient recognition by class II restricted T cell receptors; the size distribution of naturally processed peptides from the class II pathway reflects this requirement (23).

A 25-Amino Acid Peptide Stimulates T Cells as Efficiently as a 14-Amino Acid Peptide. The isolation of nested sets of naturally processed peptides suggested that the peptide binding groove of class II molecules is open at both ends (19–21); this notion was confirmed by the crystal structure of HLA-DR1 (24). However, NH2- or COOH-terminal extensions beyond a certain size may interfere with binding to MHC class II molecules or with TCR recognition. Therefore, T cell stimulation by 14–25-amino acid peptides was compared. As demonstrated in Fig. 1, T cell stimulation curves over a wide range of peptide concentrations were identical for MBP(85–98), MBP(84–102), and MBP Y(80–103) peptides [the MBP Y(80–103) has a tyrosine residue NH2 terminal to the MBP(80–103) sequence]; these peptides also bound with a similar affinity to DRB1*1501 molecules. Therefore, even long peptides can bind to MHC class II molecules with high affinity and can be efficiently recognized by the TCR. Different Peptide Residues Are Critical for Binding to DRB1 and DRB5 Molecules of the DR2 Haplotype. The binding of the MBP(84–102) peptide to affinity purified DRB1*1501 and DRB5*0101 molecules was examined using radioiodinated MBP(Y80–103) and TT(830–843) peptides as probes for DRB1*1501 and DRB5*0101 molecules, respectively (Table 4); these peptides served as standards in the peptide competition assay. The MBP(84–102) peptide bound to DRB1*1501 and DRB5*0101 molecules with high affinity (IC50 for DRB1*1501: 4.2 nM; IC50 for DRB5*0101: 55 nM). Truncation of Val89 and Ile95 at the NH2 and COOH terminus, respectively, reduced the binding affinity to DRB1*1501 molecules 10.5- and 67-fold. In contrast, truncation of the two COOH-terminal proline residues (101 + 102) greatly diminished the affinity of the peptide binding to DRB5*0101 molecules; truncation of Ile95 abolished binding (Table 4 A). Thus, the peptide core sequences required for binding to DRB1*1501 and DRB5*0101 molecules overlap, but are not identical (residues 88–95 for binding to DRB1*1501 and a sequence within 89–101/102 for binding to DRB5*0101 molecules).

A set of analog peptides with single amino acid substitutions was used to further define peptide side chains critical for binding to DR2 molecules. Substitution of Val89 and Phe92 by alanine greatly diminished the peptide binding affinity to DRB1*1501 molecules while alanine substitution of Phe92, Lys93 and possibly Arg99 affected binding to DRB5*0101 molecules (Table 4 B). The side chain specificity requirements were further examined by introducing charged or aromatic residues at positions 88, 89, 92, or 95 (Fig. 2). A negatively charged residue (aspartic acid) was not tolerated at positions 88, 89, or 92 suggesting that the corresponding DR “pockets” represent a hydrophobic environment. A large aromatic residue (tryptophan) could be placed at position 92 while replacement of Val88 or Val89 by phenylalanine resulted in a complete or partial loss of T cell recognition, respectively (Fig. 2). The decreased binding affinity after truncation of Ile95 probably reflects an important contact of the peptide backbone rather than a peptide side chain with the DR molecule as substitutions by both aspartic acid and tryptophan were tolerated (Fig. 2).

Phenylalanine Is a Critical T Cell Receptor Contact Point for All MBP(84–102)-specific T Cell Clones. While only residues in the MBP(88–95) core sequence contributed significantly to binding of the peptide to DRB1*1501 molecules, TCR recognition of the peptide required residues 85–87. The presence of Pro87 was sufficient for stimulation of two T cell clones; all other clones, however, required also Glu85 + Asn86 or Asn86. Residues COOH-terminal to Ile95 were apparently not essential for binding of the peptide; however, Thr97 and Pro98 were critical for TCR recognition since a MBP(84–96) peptide had only minimal or no activity in the T cell proliferation assay. Even though residues 85–87 and 96–98 could not be truncated, substitution of the peptide side chains by alanine had no major effect on T cell recognition suggesting that the TCR makes essential contacts

![Figure 1](image-url)
Table 4. Identification of Peptide Residues Critical for Binding to DRB1*1501 and DRB5*0101 Molecules

| MBP peptide | Sequence | AA | DRB1*1501 Ratio to standard | DRB5*0101 Ratio to standard |
|-------------|----------|----|----------------------------|----------------------------|
| NH₂⁻ and COOH-terminal truncations |
| (84–102) | DENPVVHFFKNIVTPRTPP | 19 | 4.2 | 2.4 | 55 | 0.42 |
| (84–102)Y | DENPVVHFFKNIVTPRTPPY | 20 | 5.7 | 1.54 | ND | ND |
| (84–102)Y93R | DENPVVHFFRNIVTPRTPPY | 20 | 1.5 | 6.6 | 25 | 1.1 |
| (85–102)Y | ENPVVHFFKNIVTPRTPPY | 19 | 10 | 0.53 | 690 | 0.036 |
| (86–102)Y | NPPVFFHFFKNIVTPRTPPY | 18 | 8.6 | 1.3 | 140 | 0.17 |
| (87–102)Y | PVVHFFHFFKNIVTPRTPPY | 17 | 2.7 | 3.8 | 51 | 0.48 |
| (88–102)Y | VHHFFKNIVTPRTPPY | 16 | 7.5 | 2.0 | 51 | 0.47 |
| (89–102)Y | VHHFFKNIVTPRTPPY | 15 | 47 | 0.15 | 55 | 0.58 |
| (90–102)Y | HFFKNIVTPRTPPY | 14 | 405 | 0.020 | 230 | 0.056 |
| (91–102)Y | FFKNIVTPRTPPY | 13 | 19,500 | <0.001 | 3,900 | 0.003 |
| (84–100) | DENPVVHFFKNIVTPRT | 17 | 19 | 0.34 | 3,200 | 0.007 |
| (84–99) | DENPVVHFFKNIVTPR | 16 | 12 | 0.86 | 1,350 | 0.014 |
| (84–97) | DENPVVHFFKNIVT | 14 | 4.6 | 2.5 | 810 | 0.029 |
| (84–96) | DENPVVHFFKNIV | 13 | 7.0 | 1.4 | 2,300 | 0.011 |
| (84–95) | DENPVVHFFKN | 12 | 12 | 0.86 | 6,900 | 0.005 |
| (84–94) | DENPVVHFFKN | 11 | 707 | 0.013 | – | <0.001 |
| (84–93) | DENPVVHFFK | 10 | 12,000 | <0.001 | – | <0.001 |
| (85–99) | ENPVVHFFKNIVTPR | 15 | 3.6 | 1.8 | 800 | 0.045 |
| (85–98,98A) | ENPVVHFFKNIVTA | 14 | 12 | 1.3 | 20,000 | 0.001 |
| (86–99) | NPVHFFKNIVTPR | 14 | 6.8 | 2.6 | 1,200 | 0.011 |
| (86–98,98A) | NPVHFFKNIVTA | 13 | 7.8 | 1.5 | 30,000 | <0.001 |
| (86–97) | NPVHFFKNIVT | 12 | 26 | 0.39 | 8,000 | 0.002 |
| (87–99) | PVHFFKNIVTPR | 13 | 16 | 0.54 | 5,900 | 0.003 |
| (87–98,98A) | PVHFFKNIVTA | 12 | 14 | 0.62 | 31,000 | <0.001 |
| (87–97) | PVHFFKNIVT | 11 | 395 | 0.028 | – | <0.001 |
| (89–99) | VHHFFKNIVTPR | 11 | 1,300 | 0.007 | 1,200 | 0.012 |
| MBP Y(80–103) | YGRTQDENPVVHFFKNIVTPRTPPP | 25 | 8.8 | 1.0 | ND | ND |
| TT(830–843) | QYIKANSFIGITE | 14 | ND | ND | 21 | 1.0 |

Alanine analogs

| (85–99) | ENPVVHFFKNIVTPR | 15 | 3.6 | 1.8 | 800 | 0.045 |
| (85–99)85A | ANPVVHFFKNIVTPR | 15 | 0.89 | 7.2 | 420 | 0.069 |
| (85–99)86A | EAPVVHFFKNIVTPR | 15 | 2.2 | 3.3 | 940 | 0.032 |
| (85–99)87A | ENAVVHFFKNIVTPR | 15 | 3.1 | 2.0 | 610 | 0.048 |
| (85–99)88A | ENPAVHFFKNIVTPR | 15 | 1.1 | 5.7 | 945 | 0.030 |
| (85–99)89A | ENPVHFFKNIVTPR | 15 | 170 | 0.065 | 690 | 0.042 |
| (85–99)90A | ENPVVFKNIVTPR | 15 | 1.9 | 3.3 | 640 | 0.068 |
| (85–99)91A | ENPVVHAFKNIVTPR | 15 | 3.8 | 1.8 | 1,300 | 0.022 |
| (85–99)92A | ENPVVFKNIVTPR | 15 | 283 | 0.056 | 8,200 | 0.004 |
| (85–99)93A | ENPVVFKNIVTPR | 15 | 4.1 | 3.6 | 2,900 | 0.007 |
| (85–99)94A | ENPVVFKNIVTPR | 15 | 4.5 | 2.6 | 140 | 0.10 |
| (85–99)95A | ENPVVHFFKNNAVTPR | 15 | 2.1 | 3.8 | 1,100 | 0.014 |
| (85–99)96A | ENPVVHFFKNATPR | 15 | 2.7 | 2.3 | 730 | 0.022 |
| (85–99)97A | ENPVVHFFKNAPR | 15 | 0.49 | 13 | 49 | 0.31 |
| (85–99)98A | ENPVVHFFKNATAR | 15 | 0.66 | 9.5 | 350 | 0.046 |
| (85–99)99A | ENPVVHFFKNATPA | 15 | 2.3 | 4.3 | 2,300 | 0.007 |
| MBP Y(80–103) | YGRTQDENPVVHFFKNIVTPRTPPP | 25 | 8.8 | 1.0 | ND | ND |
| TT(830–843) | QYIKANSFIGITE | 14 | ND | ND | 21 | 1.0 |

Radiolabeled MBP Y(80–103) and TT(830–843) peptides were used as probes for affinity purified DRB1*1501 and DRB5*0101 molecules; unlabeled analogs of MBP(84–102) were used as competitors for radiolabeled peptide. Each peptide was tested in two to four independent experiments and results are represented as arithmetic mean of binding capacity expressed in nM.
with the peptide backbone rather than specific side chains in the regions flanking the MBP(88–95) core sequence (Tables 3 and 5).

The peptide side chain requirements for TCR recognition were further defined using a panel of analog peptides with single amino acid substitutions (Table 5). Substitution of Phe91 by alanine or tryptophan abolished TCR recognition. Phe91 presumably represents a critical TCR contact point since the peptide bound to DRB1*1501 molecules with a similar affinity as MBP(85–99). His90 and Lys93 were important TCR contact points for the majority of clones. Again, alanine analogs bound with a high affinity to DRB1*1501 molecules but failed to stimulate the T cell clones (Tables 5 and 6). Thus, the specificity of interaction with MHC and TCR molecules appears to reside in the MBP(88–95) segment with flanking residues (85–87 and 96–99) serving an accessory function in TCR recognition.

Other MBP Peptides Known to Be Presented by DRB1*1501 or DRB5*0101 Molecules Have Similar Structural Features That May Contribute to Peptide Binding. Interaction of the MBP(84–102) peptide with DRB1*1501 and DRB5*0101 molecules involves different core segments of the peptide; in the case of DRB1*1501, MHC contact residues are located in the MBP(88–95) segment; in the case of DRB5*0101 critical contact residues are present in the MBP(89–101/102) sequence. Alignment of four myelin basic protein peptides presented by DRB5*0101 molecules (8, 9) demonstrates that three of four peptides have structural similarities with DR contact residues of the MBP(84–102) peptide [aromatic residue (i), positively charged residue (i+1), aliphatic residue (i+3)] (Table 7). The fourth peptide, MBP(76–91) does not readily fit this description; however, as peptide binding to class II MHC molecules may be promiscuous, binding with intermediate or low affinity may not require strict adherence of every pe-

| Table 5. Effect of Single Amino Acid Substitutions on T Cell Recognition of the MBP(85–99) Peptide |
|--------------------------------------------------|
| MBP Peptide | Ob.1A12 | Ob.1C3 | Hy.2E11 |
|-------------|---------|--------|--------|
| A           | 85–99   | +++++  | +++++  | +++++  |
| B           | 85 E→A  | +++++  | ++++   | +++++  |
|             | 86 N→A  | +++++  | ++++   | +++++  |
|             | 87 P→A  | + ++   | + +    | + +    |
| C           | 90 H→A  | +      | –      | + +    |
|             | 91 F→A  | –      | –      | –      |
|             | 91 F→W  | –      | –      | –      |
|             | 93 K→A  | + + +  | –      | –      |
|             | 93 K→R  | + +    | + +    | + +    |
| D           | 94 N→A  | +++++  | +++++  | +++++  |
|             | 95 I→A  | +++++  | +++++  | +++++  |
|             | 96 V→A  | +++++  | +++++  | +++++  |
|             | 97 T→A  | +++++  | +++++  | +++++  |
|             | 98 P→A  | +++++  | +++++  | +++++  |
|             | 99 R→A  | +++++  | +++++  | +++++  |

Analog peptides were tested in T cell proliferation assays using homozygous B cell lines pulsed with peptides at concentrations ranging from 5 nM to 50 µM. T cell responses obtained are indicated as follows: + to + + + + which correspond to T cell responses at peptide concentrations of 50 µM (+), 5 µM (+ +), 500 nM (+ + +), and 50 nM (+ + + +), respectively. (A) MBP(85–99) peptide (reference peptide), (B) substitutions NH2 terminal to DR contact residues Val88 and Val89; (C) substitutions in the MBP(90–93) core segment, (D) substitutions COOH terminal to the MBP(90–93) core segment.

Figure 2. Substitution of DR contact residues Val88, Val89, and Phe92. A T cell proliferation assay (clone Ob.1A12) was performed using analog peptides presented by a DR2 homozygous B cell line (9010). A aliphatic residue is preferred at positions 88 and 89 of the peptide while a large aromatic residue (Trp) is allowed at position 92. Even though truncation of Ile95 greatly diminishes binding affinity, it can be replaced by a charged or an aromatic residue suggesting that the effect of Ile95 truncation is due to the loss of contacts with the peptide backbone rather than a specific side chain.
| Molecule | MHC binding | TCR recognition |
|----------|-------------|-----------------|
| DRB1*1501 | DRB5*0101 | DRB1*1501 | DRB5*0101 |
| 84 102 | DENPVHFFKN | VTPRTTPP | DENPVHFFKN | VTPRTTPP |
| -VV -F- | - -FK- | -HF - | -HF-K |
| -FF-KI- | -F-K | -N-V- | -R- |

| Clone | | |
|-------| | |
| Ob.1A12 | Ob.1C3 | Ob.3D1 | Hy.2E11 | MS18 (reference 28) |

MHC binding: Peptide residues that contribute to binding based on the analysis of alanine analogs in a direct binding assay are indicated in bold; an analysis of analog peptides (substitution of Val88 by a charged or aromatic residue) in a T cell stimulation assay also indicated that Val88 contributes to peptide binding to DRB1*1501 molecules. TCR recognition: Peptide segments required for optimal stimulation of T cell clones are indicated, residues that are putative TCR contacts are highlighted if an alanine analog gave minimal or no T cell stimulation. For secondary TCR contacts, a significant decrease in T cell reactivity was seen (in general, >100-fold higher peptide concentrations were required for a response equivalent to the MBP(85–99) peptide).

dide to a "motif" (25, 26). Alignment of the MBP(85–99) peptide with a second determinant, MBP(148–162), presented by DRB1*1501 molecules also reveals structural similarities in the center of the peptides. Both peptides have aliphatic residues (i) and a large hydrophobic residue with the same spacing [aliphatic (i), large hydrophobic (i + 3)]. In addition, valine and threonine at position i-1 are isosteric (Table 7).

Discussion

Immunodominant T cell epitopes of myelin basic protein and proteolipid protein may be target antigens for autogressive T cells in MS (27). The immunodominant MBP(84–102) peptide was found to bind with high affinity to DRB1*1501 and DRB5*0101 molecules of the disease associated DR2 haplotype. Overlapping but distinct peptide segments are critical for binding to these molecules: Hydrophobic residues (Val89 and Phe92) in the MBP(88–95) segment are critical for peptide binding to DRB1*1501 molecules while hydrophobic and charged residues (Phe92, Lys93) in the MBP(89–101/102) sequence contribute to DRB5*0101 binding.

The MBP(90–102) peptide was previously found to be presented by DRB5*0101, DRB1*0401, DRB1*0404, and DRB1*1302 molecules (DR2a, DR4 Dw4, DR4 Dw14, DR13 Dw19); the DRB5*0101-restricted T cell clone could also recognize a MBP(92–102) peptide (11). These T cell data are consistent with the peptide binding experiments that demonstrate that Phe92, Lys93, and Pro101/102 contribute to DRB5*0101 binding. Alanine substitutions of residues Asn94, Val97, and Arg99 (which do not appear to contribute to peptide binding) abolished T cell recognition of the MBP(89–100) peptide indicating that these residues are likely TCR contact residues (28). The different peptide reading frames in the two MHC molecules make different peptide residues available for contact with the TCR: Lys93 was found to be an important TCR contact residue for DRB1*1501 restricted T cell clones but a DR contact residue for DRBS*0101 molecules (Table 6). What is the structural basis for the different registers in which MBP(84–102) is bound to DRB1*1501 and DRB5*0101 molecules? The differential binding can in part be attributed to the Gly/Val dimorphism at position 86 of the DRβ chain that controls the size of a major hydrophobic pocket created by DRα chain residues Phe26 and Ile31 (24, 29, 30). As DR886 is a glycine in DRB5*0101 molecules sufficient space is available for an aromatic anchor residue (Phe92 of the MBP peptide). In contrast, the presence of valine at DR886 in DRB1*1501 molecules results in selection of a smaller aliphatic anchor residue, most likely Val89 of the MBP peptide. The adjacent residue (Val88) probably also resides in a hydrophobic environment as substitution by aspartic acid is not tolerated (Fig. 2). T cell stimulation data (Fig. 2) indicate, however, that an analog peptide with an aromatic side chain (Val89 substituted by Phe) can also be presented by DRB1*1501 molecules, albeit less efficiently. Therefore, other polymorphic residues in the cleft are likely to be involved in the selection of the peptide reading frame, possibly by not allowing particular residues (because of charge or size) at a given peptide position. In this respect, the most striking difference between
Table 7. Alignment of MBP Peptides Presented by DRB1*1501 and DRB5*0101 Molecules

| Peptides presented by:                      | DRB5*0101 molecules                 | DRB1*1501 molecules             |
|--------------------------------------------|-------------------------------------|---------------------------------|
| MBP(86-105)*                               | VVHFKN I VTPRTPPPPSQGK               | ENP VHFFKN I VTPR               |
| MBP(131-145)*                              | ASDYKSAHKGFVKGD                     | GTLSK I FKLGGDRSR              |
| MBP(139-153)*                              | KGFKGVDAQGTLKS I                    |                                 |
| MBP(76-91)*                                | SHGRTPQDENPVVHFFK                   |                                 |

Structural similarities

Aromatic (F/Y) (i), Positively charged (K) (i + 1), Aliphatic (I/A/V) (i + 3)

DRB1*1501 molecules

| MBP(85-99)                                | ENP VHFFKN I VTPR                   |
| MBP(148-162)*                             | GTLSK I FKLGGDRSR                   |

Structural similarities

V/T (i - 1)*, Aliphatic (V/L) (i), Large hydrophobic (F/I) (i + 3)

*P2 in Aw68 peptides is V or T (they are isosteric) (reference 18).

Peptides listed were found to stimulate MBP specific T cell lines when presented by L cell transfectants expressing DRB1*1501 or DRB5*0101 molecules (*Martin et al., reference 9, #Pette et al., reference 8). Peptide segments important for DRB5*0101 and DRB*1501 binding (based on truncation analysis) are underlined; residues critical for binding (Val89 and Phe92 for DRB1*1501 binding, Phe92 and Lys93 for DRB5*0101 binding) are bold. Numbering of MBP peptide sequences is as in references 8 and 9.

DRB1*1501 and DRB5*0101 molecules is the presence of a cluster of aspartic acid residues in DRB5*0101 molecules (DRβ residues Trp9, Pro11, Tyr30, Phe31, and Tyr32).

The MBP(86–105) peptide binds to DRB1*0101, DRB1*0401, DRB1*0404, DRB1*0701, DRB1*1101, and DRB1*1501 molecules but fails to interact with DRB1*0301 molecules (31). DRB1*0301 molecules may recognize a distinct peptide motif (aliphatic at position i, hydrogen bond donor at position i+3) (32, 33), criteria which are not met by the MBP(84–102) peptide as an aromatic residue (Phe) would be at i+3 if either Val88 or Val89 was placed at position i. Peptide binding motifs for DR1 specify an aromatic residue (predominantly Tyr and Phe but also Trp) at position i, with additional constraints (mostly hydrophobic amino acids) at positions i+3, i+5, and i+8 (34–38). Binding of the MBP(84–102) peptide to DR1 which also has a glycine residue at DRβ86 therefore probably involves Phe92 (i) and Ile95 (i+3), residues that are also involved in DRB5*0101 binding. In contrast to DRB5*0101, binding to DRB1*0101 molecules is unlikely to involve Lys93.

The detailed structural analysis of an immunodominant, encephalitogenic MBP peptide (Acl-9) presented by I-Au molecules allowed MHC and TCR contact points to be identified and nonstimulatory blocking peptides to be designed. Substitution of Gin and Lys (residues 3 and 4) resulted in a potent blocking peptide for Acl-11-specific T cells (39). The distinct requirements for peptide binding to DRB1*1501 and DRB5*0101 molecules may therefore allow blocking agents to be developed that selectively interfere with autoantigen presentation by either DRB1*1501 or DRB5*0101 molecules. Furthermore, an understanding of the structural features that result in high affinity binding of self-peptides by different DR2 molecules may aid in the identification of other encephalitogenic peptides involved in the immunopathogenesis of MS.

Clinical trials that demonstrate a therapeutic benefit from deletion/tolerization of MBP-specific T cells in MS patients are, however, required to prove the role of MBP peptides (or other self-peptides) in the immunopathogenesis of MS (27).

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Address correspondence to Dr. Kai W. Wucherpfennig, Department of Biochemistry and Molecular Biology, Harvard University, 7 Divinity Avenue, Cambridge, MA 02138-2092.

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