T Cell Recognition of Distinct Peptide: I-A^u Conformers in Murine Experimental Autoimmune Encephalomyelitis

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T cell receptors (αβ) expressed by CD4+ cells recognize complexes of antigenic peptides bound to MHC class II proteins on the surface of APCs. A complex series of events is involved in the loading of antigenic peptides derived from exogenous proteins onto MHC class II molecules. Newly synthesized MHC class II proteins egress the endoplasmic reticulum (ER) in association with invariant chain (Ii) (1). These complexes traffic into specialized compartments called multivesicular late endosomes (MIIC) or class II vesicles (CIIV) (2, 3), where Ii is proteolysed to leave peptides encompassing Ii residues 81–104 (class II-associated Ii peptide (CLIP)) in the Ag binding groove of the MHC class II molecule (4, 5). CLIP occupancy of the groove prevents loading by peptides in the ER or Golgi before trafficking of the class II molecule into MIIC compartments (6, 7). In MIIC compartments CLIP is displaced by antigenic peptides derived from exogenous Ag, in concert with the peptide editing functions of H2-M (8–10). Peptide editing by H2-M ensures that stably bound peptides are selectively bound to the MHC class II groove. These “mature” peptide-MHC (pMHC) complexes are subsequently transported to the cell surface by either vesicular transport (11) or secreted as exosomes (12). Alternatively, MHC class II presentation of peptide or partially denatured Ags can occur via an Ii independent route, which involves either direct loading on the cell surface or recycling of MHC class II molecules from the cell surface into endocytic vesicles (13–16).

Multiple studies have demonstrated that MHC class II molecules are highly flexible and can exist in distinct conformational states which may have different stabilities and propensities to bind peptide (17–23). For example, Ii coexpression affects the conformation of surface MHC class II molecules (24–26) and variations in conditions for in vitro loading of peptide can affect the proportions and stabilities of pMHC isomers present (18–22, 27, 28). Studies have also shown that distinct pMHC complexes can be formed depending on the route of Ag loading (29–32): intracellular processing of Ag results in complexes that have been designated type A by Unanue and colleagues (29, 30, 32), whereas peptide pulsing generates type B complexes. Subsets of Ag-specific T cells can distinguish between these complexes (29–32). The generation of type A complexes in intracellular acidic vesicles in the presence of H2-M, in contrast to type B complexes which are produced by loading on the cell surface or in less acidic recycling vesicles, has led to the suggestion that type A complexes may be less flexible than those of the type B class (32, 33). In addition, Janeway and colleagues (34) have shown that pMHC complexes can exist in at least two distinct conformations on the surface of
APCs that can be distinguished by reactivity with the anti-I-A\(^\beta\) Ab, 25-9-17. The properties of the bound peptide appear to play a central role in inducing these two conformers which can be distinguished by T cells (34). Thus, both the route of presentation and the properties of the antigenic peptide can affect T cell recognition, and this has obvious implications for fundamental aspects of T cell biology such as tolerance induction (30, 33–35).

In the current study, we have used T cells bearing TCRs that are closely related in sequence as probes to detect conformational variants of pMHC complexes. Although recent structural studies have given valuable insight into the molecular nature of TCR-pMHC interactions (36–40), there is a paucity of data concerning the functional contribution of individual TCR residues to Ag recognition. Knowledge of this is of central relevance to understanding T cell activation. In this study, we have investigated the contribution of exposed complementarity determining region (CDR) residues of the 1934.4 TCR V\(^\alpha\) domain to the recognition of Ag. The V\(^\alpha\) domain of this TCR was chosen for two reasons: first, the x-ray structure of this V\(^\alpha\) domain has been solved (41), allowing mutations to be chosen that would minimize perturbation of the V\(^\alpha\) domain fold. Second, in other TCR-pMHC systems, both structural and functional studies have demonstrated a dominant role for the TCR V\(^\alpha\) domain in Ag recognition (37–40, 42, 43).

The 1934.4 TCR is derived from a T cell clone that is associated with the mouse model of multiple sclerosis, experimental autoimmune encephalomyelitis (EAE) (44, 45). The relevant Ag in this system is the immunodominant N-terminal (acycetoxy) epitope of myelin basic protein (MBP), MBP1–9, associated with the MHC class II protein, I-A\(^\beta\) (46). We have systematically mutated the 1934.4 CDR\(\alpha\) residues. The effect of the mutations on T cell responsiveness has been assessed and a map of functionally important residues generated. Significantly, our analyses reveal that mutation of residue 95 of CDR3\(\alpha\) results in a TCR that can distinguish between pMHC complexes expressed in unconventional (T cells, insect cells) vs professional APCs. We show that a complex resembling the pMHC conformer on the surface of professional APCs that can be distinguished by reactivity with the I-A\(^\beta\) molecule during biosynthesis followed by displacement with antigenic peptide in vitro. These studies have led to a detailed characterization of the types of pMHC conformers that can be produced under distinct conditions of Ag loading and how these might be used to distinguish subsets of Ag-specific T cells in murine EAE. Our observations also have obvious relevance to the analysis of Ag-specific T cells using recombinant, multimeric pMHC complexes.

Materials and Methods
Mice, cell lines, Abs, and peptides
Mice transgenic for the \(\beta\)-chain of the 172.10 TCR (47) were generously provided by Dr. J. Goverman (University of Washington, Seattle, WA). The I-A\(^\alpha\)-expressing B cell line, PL-8 (48) was kindly provided by Dr. D. Wraith (University of Bristol, Bristol, U.K.). The I-A\(^\alpha\)-transfected derivative of BW5147, Utm6.15 (49), was a generous gift of Dr. H. McConnell (Stanford University, Stanford, CA). Cells lines expressing the 1934.4 TCR were made by transfecting T cells into a CD4\(^-\) T cell line 58x \(\beta\). This TCR-negative derivative of BW5147 was kindly provided by Dr. S. Hedrick (University of California, San Diego, CA) with permission from Dr. B. Malissen (Institut National de la Santé et de la Recherche Médicale-Centre National de la Recherche Scientifique, Marseille-Luminy, France). The T cell hybridomas HRC.1 and HCQ.4 (50) recognize type II collagen residues 256–270 associated with I-A\(^\alpha\) and were a generous gift of Dr. R. Holmdahl (Lund University, Lund, Sweden). PE/FITC-labeled anti-TCR C\(\beta\) (H57-597), PE-labeled anti-TCR V\(\beta\)8 (F23.1), PerCP-labeled anti-CD4 (RM4-5), allophycocyanin-labeled anti-CD62 ligand (CD62L) (MEL-14), PE/FITC-labeled anti-CD2 (B2.1), and ahamster isotype-matched control (for H57-597 staining) were purchased from BD Pharmingen (San Diego, CA). The N-terminal peptide of murine MBP (MBP1–9, ASQKRPSQR, acetylated at residue 1) and (acylated) analogues MBP1–9[4Y], MBP1–9[3F,4Y], MBP1–9[3M,4Y], and MBP1–9[3M,4Y,9] were synthesized at the Peptide Synthesis Unit of the Howard Hughes Medical Institute (University of Texas Southwestern Medical Center, Dallas, TX).

Generation of T cell transfectants
The 1934.4 TCR \(\alpha\) and \(\beta\)-chain expression vectors used for transfections were constructed using \(\alpha\) and \(\beta\) shuttle vectors (generously provided by Dr. M. Davis, Stanford University) and have been described previously (51). Site-directed mutagenesis to mutate residues 29 (CDR1), 51, 52, 53 (CDR2), and 94, 95 (CDR3) of the V\(^\alpha\) domain was conducted using splicing by overlap extension and mutated PCR products were recombined as described (51). Tyr\(^\alpha\) was mutated to alanine or phenylalanine (Y29A, Y29F). Arg\(^\alpha\) was mutated to alanine or lysine (R51A or R51K). Asp\(^\alpha\) was mutated to alanine or glutamic acid (D52A, D52E), and Lys\(^\alpha\), Asn\(^\alpha\), and Tyr\(^\alpha\) were mutated to alanine (K53A, N94A, and Y95A). Sequences of oligonucleotides are available upon request. All constructs were cloned into the \(\alpha\) shuttle vector and sequenced before transfusions in transfections. 58x \(\beta\) cells were transfected with full-length TCR constructs by electroporation and stable transfectants selected as described (51). Clones were assayed for surface expression using PE-labeled H57-597 (anti-\(\beta\)) and PE-labeled F23.1 (anti-\(\beta\)).

Production of soluble pMHC complexes
Soluble, recombinant MBP1–111–A\(^\alpha\) (4K3-A\(^\alpha\)) and MBP1–111–[4Y]:A\(^\alpha\) (4Y1-A\(^\alpha\)) complexes were generated and purified using baculovirus-infected High-Five cells (52). Codons encoding the wild-type (WT) MBP1–11 peptide (lysine at position 4, “4K”) or a position 4 analog (lysine to tyrosine; “4Y”) of MBP1–11 were covalently tethered to I-A\(^\beta\) via flexible peptide linkers (53). The immunodominant MBP epitope requires acetylation for recognition (46). The N terminus of the peptides were therefore extended with a glycine which mimics the acetyl group (54). The 4Y analog was used for the majority of the studies as this peptide binds more tightly to I-A\(^\alpha\) than the WT peptide (49, 55, 56) and recombinant complexes made with the WT peptide are significantly less stable (44).

To generate CLIP-I-A\(^\alpha\) complexes, codons encoding CLIP84–101 (57) followed by a rhinovirus 3C protease cleavage site (Leu-Glu-Val-Leu-Phe-Glu-Gly-Pro-Glu) were engineered into a unique KpnI site in the I-A\(^\alpha\) expression construct (53). The CLIP peptide–protease cleavage site codons were inserted on the 5′ side of a sequence encoding a flexible linker, Gly-Ser-Gly-Ser-Gly-Gly-Ser, which is appended to the N terminus of the \(\beta\) polypeptide (53). This resulted in tethering of the CLIP sequence to the N terminus of the \(\beta\) chain via a rhinovirus 3C cleavage site, using an analogous strategy to that used for 4Y1-A\(^\alpha\) complexes (52). CLIP-I-A\(^\alpha\) complexes were purified from baculovirus-infected High-Five cells using Talon Superflow resin (Clontech Laboratories, Palo Alto, CA) followed by 3Y3 Sepharose as described (53). Purified material was digested with PreScission Protease (rhinovirus 3C protease; Amersham Biosciences, Piscataway, NJ) as recommended by the manufacturer. During the digestion, either MBP68–90 [4Y] or OVA232–339 were added in a 10-fold molar excess over CLIP-I-A\(^\alpha\). OVA232–339 binds tightly to I-A\(^\alpha\) (56). Protease was removed by addition of glutathione-Sepharose (20 μl per 2 μg of protease). Pefabloc (Roche Applied Science, Indianapolis, IN) was added to a final concentration of 4 mM to the proteolysed peptide-I-A\(^\alpha\) complexes. Empty I-A\(^\alpha\) molecules, or molecules with only weakly bound peptide, are unstable (49, 53). Therefore, proteolysed complexes were washed to the presence of 50 μg/ml MBP1–9[4Y] or OVA232–339 (as appropriate) to minimize the generation of empty molecules by peptide dissociation. MBP68–801-I-A\(^\alpha\) and rabies-derived peptide-I (RDP-1)-I-A\(^\alpha\) complexes were generated using an analogous approach to that used for CLIP-I-A\(^\alpha\).

The amino acid sequences of the two peptides are MBP68–80 (Try-Gly-Ser-Pro-Gln-Lys-Ser-Arg-Thr-Glu-Pro) or Ova232–339 (Gln-Val-Ala-Leu-Lys-Pro-Ala-Ile-Ala-Ala-Ala-Ala) (56).

T cell activation assays
T cell transfectants or hybridomas (5 × 10⁵ cells/well) were incubated with APCs (5 × 10⁴ cells/well) pulsed with MBP1–9 or analogues. PL-8, Utm6.15 cells, or I-A\(^\alpha\)-expressing splenocytes were used as APCs in these assays. T cell transfectants (5 × 10⁴ cells/well) were also stimulated with plate-bound anti-CD3ε (145-2C11, 4Y1-A\(^\alpha\), 4K1-A\(^\alpha\), CLIP-I-A\(^\alpha\) (or modified variants), MBP68–801-I-A\(^\alpha\) (or modified variants), and RDP-1-I-A\(^\alpha\) (or modified variants) complexes as described (52, 58). Additional peptide (MBP1–9[4Y] or OVA323–339) was added at 10 μg/ml both during and following coating with proteolysed, peptide-loaded CLIP-I-A\(^\alpha\), MBP68–801-I-A\(^\alpha\), and RDP-1-I-A\(^\alpha\) complexes as appropriate. Cells were stimulated in duplicates or triplicates for 24 h and IL-2 levels in culture supernatants were
quantitated by IL-2 ELISA (58). For each experiment shown, averages of duplicate or triplicate data points are plotted. For duplicates and triplicates, 85% of the SDs were <20% and 15% of the average values, respectively. The numbers of repeats for each experiment are shown in the figure legends.

Analysis of tetrameric staining of transfecteds and hybridomas

4Y-J-A\textsuperscript{\alpha} complexes were generated as described (53). Tetramer staining was conducted at 37°C. PE-labeled anti-TCR \(\beta\)-chain Ab, H57-597, and anti-TCR V\(\beta\)8/Ab, F23.1, were used to normalize tetramer staining levels for TCR expression levels (53, 59).

Generation of T cell hybridomas

Male 172.10 TCR \(\beta\)-chain transgenic mice (47) were immunized s.c. (footpad and tail base) with 200 \(\mu\)g of MBP1–9[4Y] in CFA supplemented with 4 mg/ml Mycobacterium tuberculosis strain H37RA (Difco, Detroit, MI). Ten days following immunization, splenocytes and lymph node (LN) cells were extracted and expanded using 50 \(\mu\)g/ml MBP1–9. Following 4 days of expansion, cells were fused with TCR-negative BW5147 cells (American Type Culture Collection, Manassas, VA) and hybridomas were selected as described (60). Sequences of TCR V\(\alpha\) and V\(\beta\) domain genes were analyzed using RT-PCR and standard methods.

Ex vivo analysis of Ag-specific T cells

172.10 TCR \(\beta\)-chain transgenic mice were immunized as above. Expression of CD62L by Ag (MBP1–9[4Y])-specific CD4\(^+\) T cells in draining LNs was monitored by immunofluorescence and on days 3, 5, 7, and 11 postimmunization. Four color staining of 4Y-I-A\textsuperscript{\alpha} complexes to the WT and mutant transfectants bearing detectable levels of Y29F could not be obtained. This lack of expression precluded analysis of the effect of mutating this CDR1\(\alpha\) residue. Transfectants expressing TCRs with mutations in CDRs 2 and 3 that expressed similar levels of surface TCR (data not shown) were used in further analyses.

The responsiveness of the TCR transfectants to a position 4 analog of the N-terminal epitope of MBP, MBP1–9[4Y], complexed with I-A\textsuperscript{\alpha}, was analyzed. The MBP1–9[4Y] analog was used with a position 4 substitution of lysine by tyrosine; this results in a peptide that binds to I-A\textsuperscript{\alpha} with \(10^{-6}\) to \(10^{-2}\)-fold higher affinity than the WT peptide, MBP1–9 (49, 55, 56). The position 4 substitution does not affect the disposition of T cell contacts (48, 61). As APCs, a line (PL-8) made by fusing I-A\textsuperscript{\alpha} splenocytes with the I-A-negative B lymphoma M12.C3 was used (48). For each mutation, several transfecteds were made and representative data for one transfectant for each TCR type is shown (Fig. 1). The analyses indicated that the mutations in CDR2 and CDR3 affect Ag recognition to varying degrees: K53A has an intermediate effect, whereas the activity of R51K, D52E, and Y95A are markedly decreased relative to transfectants expressing the WT 1934.4 TCR. N94A does not produce detectable amounts of IL-2 at any Ag dose. Significantly, all transfectants show similar responses to plate-bound anti-CD3\(\varepsilon\) (145-2C11), indicating that the machinery for signaling is intact (Fig. 1).

Analysis of the avidity of the TCR-pMHC interactions

Binding of multimeric pMHC ligands to Ag-specific T cells can be used to assess the avidity of the corresponding TCR-pMHC interaction (59, 62–64). Therefore, we analyzed the binding of multimeric (tetrameric) 4Y-J-A\textsuperscript{\alpha} complexes to the WT and mutant transfectants (Fig. 2). The 4Y-J-A\textsuperscript{\alpha} complexes comprise a position 4...
analog (Lys to Tyr) of the N-terminal epitope of MBP covalently tethered to I-A<sub>u</sub>, in association with I-A<sub>u</sub> (53). To account for slight differences in TCR expression, tetramer staining was normalized for expression levels. Generally, a good correlation between Ag responsiveness (Figs. 1 and 2) and staining intensity was obtained. However, the Y95A transfectant is an exception. Despite the low Ag responsiveness of this transfectant (Fig. 1), it consistently stained to slightly higher (normalized) levels than WT transfectants with the multimeric Ag complexes (Fig. 2B). This suggested that the Y95A TCR might sense conformational differences between I-A<sub>u</sub> expressed in B cells and in insect cells (the host used to make our recombinant 4Y:I-A<sub>u</sub> complexes) or other unconventional APCs. Alternatively, the Y95A TCR might bind relatively strongly to recombinant 4Y:I-A<sub>u</sub> complexes due to the presence of additional favorable interactions with the linker peptide tethering the MBP peptide to I-A<sub>u</sub> (53) (including two extra MBP residues in the MBP11-4Y sequence used to make the recombinant complexes). To distinguish between these possibilities, the responsiveness of the transfectants to both MBP1-9[4Y]-pulsed Utm6.15 cells (unconventional APCs comprising a T cell line transfected with I-A<sub>u</sub> (49)) and plate-bound 4Y:I-A<sub>u</sub> complexes was assessed. For both Utm6.15-presented peptide and recombinant pMHC complexes, a relationship between multimer staining and responsiveness was seen (Fig. 3). Notably, for all transfectants except Y95A, responsiveness in these assays showed a similar pattern to that seen for Ag-pulsed PL-8 cells (Fig. 1). In an earlier study, we have also shown that the conformational-dependent Ab Y3P stains I-A<sub>u</sub> on the surface of PL-8 and Utm6.15 cells to similar levels (65), demonstrating that at least in the vicinity of the Y3P interaction site there are no large conformational differences between the I-A<sub>u</sub> molecules expressed on these distinct cell types.

The responses of the transfectants to MBP1-9 (WT MBP peptide, 4K)-pulsed Utm6.15 cells were also analyzed (Fig. 3). The lower affinity of this peptide for I-A<sub>u</sub> results in shifts of the dose response curves to higher Ag doses (48). Again, Y95A transfecteds were more responsive to Ag than WT cells. Taken together, the data show that the linker peptide in the recombinant 4Y:I-A<sub>u</sub> complexes of peptide:I-A<sub>u</sub> complexes.

**FIGURE 2.** Staining of the TCR transfectants with fluorescently labeled 4Y:I-A<sub>u</sub> tetramers. A, Transfectants were incubated with extravidin-PE-labeled 4Y:I-A<sub>u</sub> complexes or PE-labeled F23.1 (anti-V<sub>/H9252</sub>8). Histogram plots for WT and Y95A transfectants are shown. Filled histograms represent fluorescence levels for cells treated with extravidin-PE only (for tetramer staining) or isotype control (for F23.1). B, Normalized tetramer staining for WT and mutant transfectants. Cells were stained with extravidin-PE labeled 4Y:I-A<sub>u</sub> tetramers or PE-labeled F23.1 as in A. The ratios of mean fluorescence intensities for staining with tetramers to F23.1 are shown. Data are representative of four independent experiments.

**FIGURE 3.** Responses of the TCR transfectants to Ag generated in professional vs unconventional APCs. IL-2 levels following stimulation of transfectants expressing WT or mutated TCRs with: A, MBP1-9[4Y]-pulsed Utm6.15 cells; B, plate-bound, recombinant 4Y:I-A<sub>u</sub> complexes; C, MBP1-9 (WT peptide with lysine at position 4)-pulsed Utm6.15 cells; D, MBP1-9[4Y]-pulsed I-A<sub>u</sub> splenocytes. Data are representative of at least four independent experiments.
completes does not contribute to the enhanced recognition of recombinant 4Y:I-A<sup>A</sup> by the Y95A TCR. In addition, analysis of the responses of WT and Y95A cells to Ag-pulsed, I-A<sup>A</sup> splenocytes showed that WT cells are more responsive to this form of Ag, recapitulating the pattern seen with the lymphoblastoid line, PL-8 (Figs. 1 and 3).

**Analysis of the differences between Ag generated in professional vs unconventional APCs**

The differences in patterns of responsiveness of WT and Y95A transfectants to MBP1–9 (or MBP1–9[4Y]) with I-A<sup>A</sup> from professional vs unconventional APCs prompted us to further investigate possible reasons for conformational variation of the pMHC ligand. Treatment of APCs with the lysosomotropic agent, chloroquine, had minor effects on Ag presentation to WT or Y95A transfectants, and the effect was similar for both cell types (data not shown). This indicates that for both PL-8 and Utm6.15 cells, peptide loading occurs primarily on the cell surface or in early (neutral) endosomes rather than in acidified endosomal compartments. Therefore, the two conformers of I-A<sup>A</sup> are not a secondary consequence of peptide loading in different intracellular environments.

An obvious difference between professional and unconventional APCs is that the latter do not express Ii chain (verified for Utm6.15 cells by immunoblotting; data not shown). To investigate a possible role for Ii-derived CLIP in inducing the different I-A<sup>A</sup> conformers, we first transfected Utm6.15 cells with an Ii expression construct with the aim that they would become PL-8 like. However, despite the analysis of multiple Utm6.15 transfectants, the expression of Ii was always significantly less than that in PL-8 cells, precluding the validity of their use in such studies. Therefore, we took an alternative, more direct approach of expressing recombinant I-A<sup>A</sup> with covalently tethered CLIP. This approach would have the advantage that it would also exclude any contribution from the known trafficking functions of Ii through, for example, its transmembrane domain. In the expression construct, the linker peptide connecting CLIP to the β<sup>e</sup> polypeptide contained a recognition site for rhinovirus 3C protease, allowing site-specific cleavage of the tether following purification. CLIP/I-A<sup>A</sup> complexes were treated with protease in the presence of an excess of MBP1–9[4Y] with the aim of displacing CLIP by the MBP peptide. Simultaneous proteolysis and peptide loading were conducted to minimize loss of activity of empty I-A<sup>A</sup> molecules which are known to be unstable (49). Cleavage and peptide loading were conducted at pH 7.0 as this pH is optimal for the protease activity. Furthermore, the lack of a significant effect of chloroquine on MBP1–9[4Y] presentation indicates that peptide loading onto APCs occurs at near neutral pH, and we aimed to mimic these conditions. Proteolysis appeared to be complete, as assessed by a mobility shift using SDS-PAGE (Fig. 4).

Recombinant, cleaved CLIP/I-A<sup>A</sup> loaded with MBP1–9[4Y] (designated CLIP/4Y:I-A<sup>A</sup>) was immobilized on plastic and used to stimulate WT and Y95A transfectants. The pattern of stimulation was essentially the same as that induced by Ag-pulsed PL-8 cells, with Y95A cells being significantly less responsive than WT transfectants (Figs. 1 and 4). This pattern contrasts with the hierarchy of responsiveness induced by plate-bound 4Y:I-A<sup>A</sup> complexes expressed in the absence of CLIP preassociation (Fig. 4). In addition, consistent with the inability of K53A transfectants to distinguish pMHC on PL-8 vs Utm6.15 cells (Figs. 1 and 3), these cells showed similar responses to 4Y:I-A<sup>A</sup> complexes made with and without CLIP prebinding (data not shown).

To exclude the possibility that the difference in stimulatory properties between 4Y:I-A<sup>A</sup> and CLIP/4Y: I-A<sup>A</sup> were due to the incubation conditions used during protease cleavage, 4Y:I-A<sup>A</sup> complexes were incubated in protease digestion buffer in the presence and absence of added protease. These pretreatments did not affect their stimulatory properties (data not shown). Undigested CLIP/I-A<sup>A</sup> complexes showed no stimulatory activity (Fig. 4). Furthermore, CLIP/ova/I-A<sup>A</sup> complexes were generated by cleavage of CLIP followed by loading with OVA peptide residues 323–339 (OVA323–339) which are known to bind to I-A<sup>A</sup> (56). CLIP/OVA: I-A<sup>A</sup> complexes did not stimulate either WT or Y95A transfectants, demonstrating that the response is specific for peptide (Fig. 4). Finally, in the absence of protease cleavage, CLIP could not be displaced by exogenously added MBP1–9[4Y] to generate complexes that could be recognized by the WT and Y95A transfectants (data not shown). The data show that CLIP binding to I-A<sup>A</sup>, followed by displacement by MBP1–9[4Y], results in a pMHC

**FIGURE 4.** Properties of CLIP/I-A<sup>A</sup> complexes and their peptide (MBP1–9[4Y] or OVA323–339) loaded derivatives. A, Fifteen-percent PAGE analysis of undigested CLIP/I-A<sup>A</sup> complexes and complexes generated by rhinovirus 3C protease digestion followed by loading with OVA323–339 or MBP1–9[4Y] (CLIP/OVA: I-A<sup>A</sup> and CLIP/4Y: I-A<sup>A</sup>, respectively). Molecular masses (in kilodaltons) are indicated on the right margin. B–E, IL-2 levels were quantitated following stimulation of transfectants expressing WT or Y95A TCR with: B, plate-bound, recombinant 4Y:I-A<sup>A</sup> complexes; C, plate-bound CLIP/4Y: I-A<sup>A</sup> complexes; D, plate-bound, undigested CLIP/I-A<sup>A</sup> complexes; E, plate-bound CLIP/OVA: I-A<sup>A</sup> complexes. The HRC.1 hybridoma (50) was used as a negative control. Data are representative of five independent experiments for B, C, and E and two for D.
plexes, CLIP/4Y:I-A<sup>u</sup> complexes, MBP1<sup>9[4Y]</sup>.

The presence of the transgenic β-chain in these mice restricts the diversity of the TCRs borne by responding T cells to receptors comprising the 172.10 β-chain paired with different endogenous α chains, with a strong bias toward Vα2 usage (43) which is the Vα-chain of the 172.10 TCR (66). We compared the stimulation of the hybridomas with plate-bound 4Y:I-A<sup>u</sup> complexes, CLIP/4Y:I-A<sup>u</sup> complexes, MBP1<sup>9[4Y]</sup>-pulsed Utm6.15 cells (unconventional APCs), and MBP1<sup>9[4Y]</sup>-pulsed splenocytes (professional APCs) (Fig. 5). The majority of hybridomas responded similarly to both types of pMHC complexes (Fig. 5 and not shown). Consistent with their responses to plate-bound, recombinant 4Y:I-A<sup>u</sup> complexes or MBP1<sup>9[4Y]</sup>-pulsed splenocytes relative to plate-bound 4Y:I-A<sup>u</sup> complexes or MBP1<sup>9[4Y]</sup>-pulsed Utm6.15 cells. Therefore, the behavior of hybridoma 20 resembles that of Y95A cells.

The “CLIP/4Y:I-A<sup>u</sup> responsive” hybridomas are Vα2<sup>+</sup> and, as expected from the studies of others of Vα2<sup>+</sup> cells in 172.10 β tg mice (43), expressed TCRs that are either the same or very similar in sequence to that of the 172.10 αβ TCR (Fig. 5 and not shown). In contrast, hybridoma 20 is Vα2<sup>+</sup> and expresses a TCR comprising Vα4.2-Jα39. This Vα domain is closely related (one residue difference: Pro to Ala in CDR3) to the 214.12 hybridoma isolated from MBP-immunized B10.PL mice (66) and shares the same Vα usage with the 1934.4 TCR Vα. The junctional region of the α-chain of hybridoma 20 is also closely related to that of the 172.10 hybridoma (Fig. 5).

We further investigated the properties of 4Y:I-A<sup>u</sup>-specific T cells in 172.10 β tg mice. In naive mice, two similarly sized populations (Vα2<sup>+</sup> and Vα2−) of CD4<sup>+</sup> cells which stain with 4Y:I-A<sup>u</sup> tetramers can be detected (Fig. 6). A comparative analysis of both the expansion and activation (assessed by CD62L down-regulation) of these two populations following immunization of 172.10 β tg mice with MBP1<sup>9[4Y]</sup> indicates that Vα2<sup>+</sup> cells are significantly less responsive to MBP1<sup>9[4Y]</sup> in vivo than Vα2− cells. Thus, the properties of the Vα2<sup>+</sup> and Vα2− populations appear to be recapitulated by the hybridomas. Despite the relatively poor activation/expansion of the Vα2<sup>+</sup> population in 172.10 β tg mice, Ag-specific Vα2− cells can apparently be rescued following in vitro expansion and fusion to make hybridomas. However, consistent with the low Ag-specificity of the Vα2− T cells, the isolation of such hybridomas is a relatively rare event (1 in ~20 hybridomas).

**FIGURE 5.** Analysis of representative hybridomas from 172.10 TCR β-chain tg mice immunized with MBP1<sup>9[4Y]</sup>. A, Flow cytometric analysis of TCR expression of representative hybridomas (2, 10, and 20) using PE-labeled anti-TCR Vα2 and FITC-labeled anti-TCR Cβ (H57-597). B–E, IL-2 levels following stimulation of hybridomas with: B, plate-bound 4Y:I-A<sup>u</sup> complexes; C, plate-bound CLIP/4Y:I-A<sup>u</sup> complexes generated by rhinovirus 3C protease cleavage of CLIP/I-A<sup>u</sup> complexes and replacement of CLIP by MBP1<sup>9[4Y]</sup>; D, MBP1<sup>9[4Y]</sup>-pulsed Utm6.15 cells; E, MBP1<sup>9[4Y]</sup>-pulsed I-A<sup>u</sup> splenocytes. The HCQ4 hybridoma (50) was used as a negative control. F, Amino acid sequences of the junctional regions of the Vα domains of hybridomas used in A–E. Jα usage for all hybridomas shown is Jα39. Data shown in B–E are representative of at least three independent experiments.
relative affinities that are similar (RDP-1) and ~50 fold lower (MBP68–80) than the binding of MBP1–9[4Y] (56). The recombinant proteins were expressed, purified, and cleaved by protease in the presence of MBP1–9[4Y] or OVA323–339, in an analogous way to that described for the CLIP:I-Au complexes. Plate-bound complexes were used to stimulate hybridomas 2, 10, and 20 or the WT and Y95A transfectants (Fig. 7 and data not shown). Both RDP-1/4Y:I-Au and MBP68–80/4Y:I-Au complexes had similar properties to those seen for CLIP/4Y:I-Au complexes. Thus, the distinct conformers appear to be induced by the binding of relatively low affinity peptides during biosynthesis followed by their displacement in vitro. However, low affinity binding of peptide during biosynthesis is alone insufficient to induce this conformer, as recombinant 4K:I-Au complexes containing the low affinity WT N-terminal MBP peptide (position 4 is lysine; this peptide has an at least 1000-fold lower relative affinity than RDP-1 and MBP1–9[4Y]) for I-Au (49, 55, 56)) have stimulatory properties that are qualitatively similar to those of 4Y:I-Au complexes (Fig. 7). The higher doses of recombinant protein that are needed to achieve T cell stimulation by 4K:I-Au complexes are consistent with our earlier observations describing the instability of peptide:I-Au complexes made with the WT N-terminal epitope of MBP (53).

**Location of the site of the conformational difference**

The 1934.4 TCR has been previously shown to cross-react with MBP1–9 or MBP1–9[4Y] analogues with substitutions of the T cell contact residue at position 3 (Gln) by phenylalanine, tyrosine, histidine, and methionine (48). Therefore, comparison of the fine specificity of Ag recognition by WT and Y95A transfectants might allow us to map the contact site of the region encompassing Y95 (WT) or A95 (Y95A mutant) of the TCR on MBP1–9[4Y]:I-Au complexes. MBP1–9[4Y] analogues with position 3 substitutions were used to pulse Utm6.15 cells and IL-2 secretion by WT and Y95A transfectants were quantitated (Fig. 8). The analyses show that compared with WT, Y95A transfectants are less responsive to all of the position 3 substituted analogues. In particular, they are significantly less responsive to MBP1–9[3Y,4Y]. The x-ray crystallographic structure of the recombinant 4Y:I-Au complex (54) forms a TCR complex with a position 3 substituent in the correct orientation to mimic the WT contact residue.

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**FIGURE 6.** Activation of Ag-specific T cells in 172.10 TCR \( \beta \)-chain tg mice following immunization with MBP1–9[4Y]. LN cells were stained with PerCP-labeled anti-CD4, FITC-labeled anti-Vo2, PE-labeled 4Y:I-Au tetramers, and allophycocyanin-labeled anti-CD62L before immunization (naive) or after 3, 5, 7, and 11 days of immunization with 200 \( \mu \)g of MBP1–9[4Y]. A, Upper panels, tetramer staining of CD4+ cells; lower panels, CD62L expression on CD4+, tetramer-positive populations (on naive cells and day 5 postimmunization). B, CD62L down-regulation, represented as the percentage of CD62Llow cells, for CD4+ tetramer+Vo2+ populations and CD4+ tetramer+Vo2+ populations at day 0 (naive), days 3, 5, 7, and 11 postimmunization. For each day of analysis, LN cells from two mice were pooled. Data are representative of three independent experiments.

**FIGURE 7.** Effects of prebinding of irrelevant peptides to I-Au followed by their displacement by MBP1–9[4Y] on T cell recognition and stimulatory properties of 4K:I-Au complexes. IL-2 levels were quantitated following stimulation of T cell hybridomas with: A, plate-bound RDP-1/4Y:I-Au complexes generated by rhinovirus 3C protease cleavage of RDP-1:I-Au complexes and replacement of RDP-1 by MBP1–9[4Y]; B, plate-bound RDP-1/OVA:I-Au complexes generated as in A but with replacement of RDP-1 by OVA323–339; C, plate-bound 4Y:I-Au complexes; D, plate-bound 4K:I-Au complexes. The HRC.1 hybridoma (50) was used as a negative control. Data shown are representative of at least three independent experiments.
FIGURE 8. Recognition of position 3 analogues of MBP1–9[4Y] by transfectants expressing WT and Y95A TCRs. A, WT or Y95A transfectants were incubated with U3m6.15 cells pulsed with MBP1–9[4Y] or position 3 analogues (2 or 0.2 μg/ml) and IL-2 levels were quantitated. Position 3 analogues of MBP1–9[4Y] are abbreviated as follows: 3Q = WT residue; 3F = MBP1–9[3F,4Y] etc. B, α-Carbon trace of 4Y:I-A* complex (54). The location of residue 3 of MBP (arrow) and flanking residues of α1 (61–68) and β1 helices (66–74) are indicated in black. The figure was drawn using RASMOL (courtesy of R. Saile, Bioinformatics Research Institute, University of Edinburgh). Data are representative of four independent experiments.

Discussion

In the current study, an analysis of the functional effects of mutations of CDR residues of the 1934.4 TCR α domain has led to the analysis of two possible conformers of the MHC class II molecule, I-A*. The 1934.4 TCR is representative of the receptors borne by encephalitogenic T cells in H-2* mice and recognizes the N-terminal epitope of MBP associated with the class II molecule I-A* (44, 46). Mutated TCRs have been expressed in transfectants and responsiveness to Ag analyzed. “Functional avidity” of the transfectants has been compared with TCR-pMHC (“intrinsic”) avidity by assessing the binding of tetramer 4Y:I-A* complexes comprising MBP1–11[4Y] tethered to I-A*. Generally, the correlation between functional and intrinsic avidity is high. However, mutation of the CDR3α residue, Y95, to alanine (Y95A) results in an outlier. This mutation generates a TCR that binds tetramer slightly better than the WT TCR and yet is significantly less responsive to professional APCs pulsed with peptide Ag. Unexpectedly, Y95A transfectants are slightly more responsive than WT cells to Ag-pulsed, unconventional APCs expressing I-A*. Therefore, the Y95A TCR senses conformational differences between pMHC ligands generated on the surface of professional vs unconventional APCs. Significantly, these two pMHC conformers can also be distinguished by Ag-specific T cells isolated from immunized H-2* mice.

An obvious distinction between professional and unconventional APCs is that they differ in the expression of Ii. Initially, we therefore investigated a role for the Ii-derived peptide, CLIP, which binds to the MHC class II groove (4, 5) in shaping the conformation of I-A*. The CLIP:I-A* interaction has been extensively investigated in earlier studies (67–69) and a model for CLIP:I-A* built (70). CLIP dissociates more rapidly from I-A* than OVA323–339 (68), and competes with biontitulated OVA323–339 for binding to I-A* slightly less well than unlabeled OVA323–339 (67). Taken together with the report that the relative affinity of OVA323–339 is 3- to 4-fold higher (3- to 4-fold lower Kd) for binding to I-A* than that of MBP1–9[4Y] (56), this suggests that the affinity of MBP1–9[4Y] and CLIP for I-A* fall in a similar range. By generating recombinant CLIP:I-A* complexes in which CLIP can be replaced by MBP1–9[4Y], we have obtained support for the concept that CLIP prebinding can affect the conformation of the peptide:I-A* complex that is subsequently formed by peptide exchange. However, the effect of CLIP is not specific: expression of recombinant peptide:I-A* complexes containing either MBP68–80 or RDP-1 followed by their displacement by MBP1–9[4Y] also generates complexes which resemble those on the surface of peptide-pulsed, professional APCs. Thus, preloading by any (relatively weakly bound) peptide followed by displacement in vitro results in these types of complexes. This mode of peptide loading most likely involves a peptide receptive state of I-A*, which is similar to that described by others for I-E and DR molecules (21, 22).

Analogous to the CLIP, RDP-1, or MBP68–80:I-A* complexes that are subsequently loaded in vitro with MBP1–9[4Y], peptide loading onto I-A* on the surface of APCs presumably occurs via displacement of weakly bound peptides or interaction with empty, unstable I-A* molecules which would be expected to be transient (21, 22). These MHC molecules would be predicted to have relatively weakly bound peptides and, as a result, are targets for binding by exogenous peptides. During the “second round” of peptide loading, such complexes may evade H2-M editing through either Ag loading on the cell surface or the endosomal recycling pathway (14), although recent data support the possibility that editing can also occur during these Ag-loading pathways (16). We would expect complexes generated by peptide pulsing to be more open, in contrast to tighter, less flexible complexes that might form when...
recombinant (4Y-I-A\textsuperscript{a}) protein traverses the secretory pathway with antigenic peptide covalently tethered. In this context, although tighter 4Y-I-A\textsuperscript{a} complexes do not form SDS-resistant compact dimers (52), they are more stable upon long-term storage (>2 mo) at 4°C than CLIP/4Y-I-A\textsuperscript{a} complexes (our unpublished observations). However, the question still remains as to how peptide loading onto I-A\textsuperscript{a} expressed by unconventional APCs (Utm6.15) results in what appears to be the tighter conformer, and we currently do not have an explanation for this.

The presumptive tight and open conformers that we have described are reminiscent of the type A and B complexes, respectively, described by Unanue and colleagues (29, 30, 32). However, there is a notable difference. The T cells that we have analyzed all recognize tight (type A) complexes, but only a subset respond well to the more open (type B) complexes, whereas the reverse appears to be the case for the studies of Unanue and colleagues (29, 30, 32). The reasons for this are currently unclear, but may relate to differences in the nature of Ag which in turn could affect repertoire selection. In two recent studies it has been shown that I-A\textsuperscript{a}-restricted type B cells specific for dominant epitopes derived from transgenically expressed hen egg lysozyme (residues 46–61) or autologous \(\alpha\)- \(\beta\) (residues 37–53) are not tolerized and enter the periphery, whereas type A cells are negatively selected (30, 33). In contrast, due to the low affinity of MBP1–9 for I-A\textsuperscript{a} (49, 55, 56), Ag-specific T cells escape negative selection and immunodominance to this epitope is seen following MBP immunization (71, 72). Thus, for this epitope, both type A and B cells could contribute to the peripheral T cell repertoire. A second, but not mutually exclusive, reason for the difference is that distinct I-A haplotypes are being compared which most likely have different intrinsic stabilities and biochemical properties (49, 73). It is also possible that the conformers we have described bear some similarity to the short- and long-lived isomers of MBP1–14 (and analogues) bound to I-A\textsuperscript{a} that can be distinguished by specific T cells (19). Further studies will be necessary to characterize the different pMHC conformers and to evaluate their interrelatedness in distinct Ag recognition systems.

Toward identifying the conformationally distinct region in the I-A\textsuperscript{a} conformers, we have mapped the interaction site for residue 95 of the 1934.4 \(\alpha\)-chain of I-A\textsuperscript{b} domain on cognate ligand. This has been conducted by comparing the recognition of position 3 analogues of MBP1–9[4Y] by transfectants expressing the WT 1934.4 TCR and Y95A mutant. Y95 appears to contact ligand in the region encompassing Gln3 of MBP1–9. From the recent x-ray structure of recombinant 4Y-I-A\textsuperscript{a} complexes (54) this residue is in the vicinity of residues 60–68 of the \(\alpha1\) helix and 65–74 of the \(\beta1\) helix (Fig. 8). The shift in register of the MBP peptide in the I-A\textsuperscript{a} groove results in a more central location for MBP-Gln3 than would otherwise be seen (54, 74). Interaction of Y95 with ligand in this region would be predicted from the diagonal orientation of the TCR on peptide recognition has been shown in other Ag systems in both structural (36–40) and functional studies (81, 82). CDR3\(\alpha\) residues of the 1934.4 TCR play a similarly important contribution: alteration of N94 to alanine completely ablates Ag recognition whereas the Y95A mutation has variable effects. Furthermore, our data suggest that the CDR3\(\alpha\) loop is in proximity to residue 3 of MBP1–9 which has a central location in the I-A\textsuperscript{a} groove (54, 74) (Fig. 8).

In summary, our analyses indicate that two conformers of MBP1–9-I-A\textsuperscript{a} complexes can be distinguished by closely related TCRs. The analysis of the fine specificities of two TCRs which differ by only one CDR3\(\alpha\) residue (Y95) has allowed us to map the region of conformational variation on the pMHC complex. We have also shown that the two distinct conformers can be produced in recombinant form in insect cells by manipulating the conditions of expression and antigenic peptide loading. Future work will be directed toward investigating how recognition of these distinct conformers might contribute toward autoimmunity in murine EAE.

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References


