Tetramer-Guided Epitope Mapping: Rapid Identification and Characterization of Immunodominant CD4+ T Cell Epitopes from Complex Antigens


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Tetramer-Guided Epitope Mapping: Rapid Identification and Characterization of Immunodominant CD4+ T Cell Epitopes from Complex Antigens


T cell responses to Ags involve recognition of selected peptide epitopes contained within the antigenic protein. In this report, we describe a new approach for direct identification of CD4+ T cell epitopes of complex Ags that uses human class II tetramers to identify reactive cells. With a panel of 60 overlapping peptides covering the entire sequence of the VP16 protein, a major Ag for HSV-2, we generated a panel of class II MHC tetramers loaded with peptide pools that were used to stain peripheral lymphocytes of an HSV-2 infected individual. With this approach, we identified four new DRA1*0101/DRB1*0401- and two DRA1*0101/DRB1*0404-restricted epitopes. By using tetramers to sort individual cells, we easily obtained a large number of clones specific to these epitopes. Although DRA1*0101/DRB1*0401 and DRA1*0101/DRB1*0404 are structurally very similar, nonoverlapping VP16 epitopes were identified, illustrating high selectivity of individual allele polymorphisms within common MHC variants. This rapid approach to detecting CD4+ T cell epitopes from complex Ags can be applied to any known Ag that gives a T cell response. *The Journal of Immunology, 2001, 166: 6665–6670.

The T cell-mediated response to complex Ags involves recognition of selected peptide epitopes presented in the context of MHC molecules expressed on APCs. The choice of these immunogenic epitopes from among the often hundreds or thousands of amino acids comprising an antigenic protein depends significantly on the binding properties of a given MHC type and the interactions of specific amino acids with the TCR. Understanding which peptide epitopes participate in T cell-mediated immunity provides a basis for directed modulation of the immune response, including development of peptide vaccines and therapies against allergens, autoimmune diseases and tumors (1–5). However, elucidation of specific epitopes from complex Ags can be a cumbersome and difficult process, as it generally involves extensive phenotype screening of T cell clones isolated from whole Ag-stimulated cells.

A number of recent studies have used soluble MHC multimers to directly identify T cells restricted to specific peptide epitopes. This technology has been used to track T cells specific for both viral Ags (6–9) and tumor Ags (10–13) in both animal models and in humans when the peptide epitope is known. The majority of these studies have focused on class I-restricted T cells, as initial efforts in producing class II molecules were hampered by difficulties in generating stable soluble forms of the molecules and inefficiency in biotinylation of these molecules. One approach to stabilizing the soluble class II molecules has involved covalently tethering the peptide to the β-chain of the class II molecule (14, 15). However, tethering of peptide to class II molecules does not appear to be essential for the stability of soluble class II molecules, as demonstrated by the successful detection of Ag-specific T cells in mice and humans by using soluble MHC molecules with exogenously loaded peptide (16–19).

Efficient production of class II MHC tetramers containing soluble peptide offers the potential to detect and isolate T cells specific to Ags in which the T cell epitopes are not previously known. In this approach, tetramers are loaded with a mixture of overlapping peptides that cover the entire Ag. These tetramers then are used to identify and isolate epitope-specific T cells from PBMC that have been stimulated with the whole Ag. With this approach, we have identified four DRA1*0101/DRB1*0401 (DR0401)3-restricted T cell epitopes and two DRA1*0101/DRB1*0404 (DR0404)-restricted T cell epitopes of the HSV-2 tegument protein VP16. Earlier studies have demonstrated that VP16 epitopes are recognized by CD4+ T cells that infiltrate genital lesions in individuals with HSV-2 infection, suggesting that the protein serves as an important Ag in the natural immune response to HSV-2 virus (20, 21).

Materials and Methods

Generation of DR0401 and DR0404 tetramers

The construction of the expression vectors for generation of soluble DR0401 has been described previously (18). A similar approach using the same primers was used to generate the DRB1*0404-leucine zipper-biotinylation site expression vector from DRB1*0404 cDNA. DRB1*0404 cDNA was isolated from the EBV-transformed B lymphoblastoid cell line Bin-40, and it was a gift from Dr. P. Gregersen (North Shore University

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Hospital, Manhasset, NY). Briefly, a chimeric cassette containing the extracellular coding region for the DRB1*0404 chain appended to the acidic leucine zipper motif was generated from DRB1*0404 and leucine zipper cDNA by using the PCR-mediated splicing overlap technique (22–23). A site-specific biotinylation sequence then was added to the 3’ end of the DRB1*0404/leucine zipper cassette. The chimeric cDNA was subcloned into the Cu-inducible pBmHa-3 Drosophila expression vector. For class II production, DRe and DRβ expression vectors were cotransfected into Schneider S-2 cells. Soluble class II molecules produced after addition of CuSO4 were purified by affinity chromatography, concentrated, and subsequently biotinylated with the Bir A enzyme (Avidity, Denver, CO; Ref. 24).

For generation of tetramer pools, a panel of 60 overlapping VP16 peptides, p3 to p62, were used. These peptides, each 20 aa in length, corresponded to the entire VP16 protein with a 12-aa overlap between adjacent peptides. Peptides were synthesized on polyethylene pins with 9-fluorenylmethoxycarbonyl chemistry by Chiron Technologies (Clayton, CA). Individual peptides were weighed out and dissolved in DMSO to achieve the appropriate concentration. The peptides were divided into 12 pools, each containing 5 different overlapping peptides. Five peptides per pool was found to preserve sensitivity in identifying individual peptide epitopes. A similar range of peptides per pool has been used in earlier epitope mapping studies (25, 26). The biotinylated class II molecules DR0401 and DR0404 at a concentration of 1 mg/ml were each loaded with the 12 different peptide pools by incubation for 48 h at 37°C with 25-fold molar excess of peptides (total) in 100 mM sodium phosphate, pH 6.0, and 0.2% n-octyl-D-glucopyranoside. Tetramers were formed by incubating class II molecules with PE-labeled streptavidin (BioSource International, Camarillo, CA) for 6 h at room temperature at a molar ratio of 8 to 1. For single peptide tetramers, the peptide was loaded at a concentration of 5-fold molar excess over the class II concentration.

Staining and isolation of VP-16-specific T cells

PBMC from a DRB1*0401, DRB1*0404 HSV-2-positive individual were stimulated with VP16 protein at 2 μg/ml (a gift from Chiron Corporation). IL-2 was added (10 U/ml final concentration) every other day starting on day 5. T cells were stained with tetramer pools on day 11 or 12. For each pool, 2 × 106 cells were incubated with 0.5 μg of PE-labeled tetramer in 50 μl of culture medium (10 μg/ml) at 37°C for 1 to 2 h, and then stained with anti-CD4-FITC (BD PharMingen, San Diego, CA) for 15 min at room temperature. Near maximal staining of T cell clones was observed with 0.05 μg of tetramer reagent, indicating that 10 μg/ml provides an excess of MHC molecules for the staining reaction. Cells were washed and analyzed with a Becton Dickinson FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA). Tetramers loaded with the corresponding single peptide were stained for those pools that gave positive staining, and analysis was done on day 14 or 15. Levels of background staining, generally around 0.1%, were determined by using tetramers loaded with an irrelevant peptide, HA307–319 (influenza A hemagglutinin protein, residues 307–319). Cells that were positive for a particular tetramer were single-cell sorted into 96-well U-bottom plates by using a Becton Dickinson FACSVantage (San Jose, CA) on the same or following day. Sorted cells were expanded with 1.5 ml of RPMI supplemented, irradiated (5000 rad) PBMC per well as feeders with 2.5 μg/ml PHA and 10 U/ml IL-2 added 24 h later. Specificity of cloned T cells was confirmed by staining with tetramers (loaded with cognate peptide or control peptide, HA307–319) and T cell proliferation assays with 10 μg/ml of specific peptide and DR-transfected bare lymphocyte syndrome (BLS) line BLS-DRA1*0101/DRB1*0401 (BLS-DR0401) or BLS-DRA1*0101/DRB1*0404 (BLS-DR0404) as APCs (18, 27). Competition binding assays of identified peptides were performed with purified DR0401 and DR0404 protein as previously described (28).

Measurement of cytokine secretion

In experiments that used IFN-γ secretion as an indicator of T cell reactivity, PBMC were stimulated with VP16 protein as described above and assayed on day 11 or 12. PBMC (1 × 106) were incubated together with an equal number of BLS-DR0401 or BLS-DR0404 APCs that had been pulsed with 50 μg/ml of each peptide pool. After 3 h of incubation, IFN-γ secretion was determined by use of a cytokine secretion capture assay following the protocol supplied by the manufacturer (Milenyi Biotec, Auburn, CA; Ref. 29). Briefly, cells were washed once in PBS and incubated in 100 μl of medium on ice for 5 min with an Ab-Ab conjugate directed against both CD45 and IFN-γ. Prewarmed medium was added to a final volume of 2 ml, and cells were incubated at 37°C for 45 min under gentle rotation to allow cell-surface capture of secreted IFN-γ. Cells were washed once in PBS and then stained for 15 min on ice with a second PE-conjugated Ab directed against IFN-γ as well as a FITC-conjugated Ab directed against CD4. Cells were washed once in PBS and analyzed by flow cytometry as described above. For pools that showed significant IFN-γ staining, reactivities of individual peptides were assayed by the same approach.

Results

Epitope mapping with class II tetramer pools

to determine the class II-restricted epitopes of the HSV-2 VP16 protein, peptides of 20 aa in length spanning the entire sequence of the VP16 protein, with a 12-aa overlap between consecutive peptides, were used to generate two tetramer panels, one for the DR0401 molecules and the other for the DR0404 molecules. Each panel included 12 different tetramer pools, designated as pool 1 to pool 12. Each pool contained the soluble class II molecules together with five different peptides of the VP16 protein.

With these two tetramer panels, we analyzed PBMC from a DRB1*0401, DRB1*0404 HSV-2-positive individual. Cells were stimulated with 2 μg/ml of VP16 protein and 11 or 12 days following stimulation were stained with each tetramer panel. Four pools, 2, 5, 6 and 12, gave significant staining above background with the DR0401 tetramer panel (Fig. 1A). Staining with the DR0404 tetramer panel identified two pools, 6 and 12, as having significant staining (Fig. 1B). We then generated tetramers loaded with the individual peptides of the positive pools and used these to determine which peptide epitopes in the pool were recognized by responding T cells. For DR0401/pool 2, peptides p9, p10, and p12
gave positive staining (Fig. 2A); for DR0401/pool 6, peptide p32 gave positive staining; and for DR0401/pool 12, peptides p61 and p62 gave positive staining. We were unable to identify any individual peptides for pool 5 despite repeated attempts. We speculate that this may reflect T cells of low avidity, which are difficult to consistently stain using tetramers. For DR0404/pool 6, peptides p31 and p32 gave positive staining (Fig. 2B); and for DR0404/pool 12, peptide p58 showed positive staining.

To assess the efficiency of pooled-peptide tetramers in identifying T cell epitopes, we compared our approach with that of Kern and colleagues (25) who used IFN-γ production as an indicator for epitope-specific cells. Cells were stimulated with 2 μg/ml of VP16 protein and 11 or 12 days following stimulation were restimulated for 3 h with BLS-DR0401 or BLS-DR0404 cell lines pulsed with 50 μg/ml of each peptide pool. For PBMC stimulated with the BLS-DR0401 cell line, pools 2, 5, and 12 were positive for IFN-γ secretion as measured by flow cytometry (Fig. 3). As seen in Fig. 1A, both pools 2 and 12 were positive in the DR0401 tetramer analysis. Pool 5 was positive in the tetramer screen; however, as noted, we were unable to see tetramer staining with the individual peptides of pool 5. Interestingly, pool 6, which was positive in the tetramer screen, did not show appreciable IFN-γ production. This suggests that although the two methods generally coincide, differences in what each approach measures, TCR/MHC avidity with tetramers and specific effector function with IFN-γ production, allows each method to identify occasional cells that would be undetectable by the alternate approach.

Characterization of tetramer-positive T cell clones

T cells that were specific for peptides p10 and p61 for DR0401 and peptide p58 for DR0404 were isolated and tested for specificity. Tetramer-positive T cell clones were single-cell sorted and expanded, and >20 clones specific for each DR/peptide complex were identified. All of the clones obtained demonstrated Ag specificity as shown by proliferation assays and tetramer staining. Tetramer staining and proliferation data are shown for three different clones for each specificity in Fig. 4. Although strong staining and Ag-specific proliferation were observed for most clones, there existed variations in staining intensity and degree of proliferation for a number of clones. Some of these atypical patterns are illustrated in Fig. 4C with DR0401-restricted clones specific for p10. Both clones p10–1 and p10–2 consistently showed a broader range of staining intensity compared with the majority of clones, which showed more focal staining. In contrast, clone p10–3 consistently showed poor tetramer staining despite vigorous proliferation to specific peptide. We speculate that these different staining patterns reflect different avidities of the TCR for the MHC/peptide complex in the different clones. These atypical staining patterns were not unique to clones restricted to p10; a few clones specific for p58 and p61 also showed weak staining, whereas clones specific for p10 with strong staining were also observed.

To more precisely define the epitopes recognized by the T cell clones, we evaluated probable DR0401 and DR0404 peptide binding motifs by using truncation analyses. A number of studies have extensively characterized the peptide-binding motifs for DR4 (30–32). Examination of peptide p10 (VP1657–76) revealed that the 12-mer peptide VP1658–69 possesses good DR0401 binding motifs. Experiments with the VP1658–69 peptide demonstrated that the peptide could indeed stimulate the DR0401-restricted T cell clones specific for the p10 epitope (data not shown). These data suggest that the positive staining observed in Fig. 2A with the DR0401/p9 tetramers was likely attributable to the overlapping of

FIGURE 2. Defining individual VP16 epitopes from peptide pools. The individual peptide constituents from those pools that gave positive staining were singularly loaded into tetramers of the same DR type. These single peptide tetramers were then used to stain the same PBMC previously stained with pooled peptide tetramers. Each row shows the tetramer staining for the constituent peptides of (A) pool 2, pool 6, and pool 12 for DR0401, and (B) pool 6 and pool 12 for DR0404. Each panel shows analysis of at least 3 × 10⁶ cells, and percentages represent the percent of total cells in each quadrant.
peptide p9 (VP16_{49-69}) and peptide p10 (VP16_{57-70}), as both peptides contain the shorter epitope.

Similar examination of peptide p61 (VP16_{465-484}) identified a 13-mer peptide, VP16_{472-484}, which stimulated DR0401-restricted T cell clones directed against the p61 epitope (data not shown). Therefore, the positive staining seen with the DR0401/p62 tetramer in Fig. 2A was likely attributable to the overlapping of peptides p61 and p62. These results also demonstrate that tetramer staining with overlapping peptides provides an alternative approach to directly mapping minimal epitopes. Truncation studies with peptide p58 refined the DR0404-restricted epitope to the 13-mer peptide, VP16_{472-484}, which stimulated DR041-restricted APCs pulsed with 50 μg/ml of total peptide. IFN-γ secretion was measured by flow cytometry. Each panel shows analysis of at least 3 × 10⁶ cells, and percentages represent the percent of total cells in each quadrant.

Discussion

The tetramer-guided epitope mapping (TGEM) technique described here provides a rapid approach for identifying T cell epitopes of known Ags, taking advantage of the fact that empty class II molecules can be loaded easily with peptides of interest and subsequently tetramerized. Knowing only the HLA class II genotype of an individual, it is feasible to identify major epitopes of complex Ags by a straightforward tetramer staining analysis. As seen in this study, the TGEM protocol discriminates both between peptides and between closely related MHC molecules. Indeed, for both the VP16_{58-69} and VP16_{472-484} epitopes presented by DR0401, TGEM analysis revealed minimal epitopes that were subsequently confirmed by direct synthesis and testing of the minimal peptide. There was a general concordance when comparing epitopes identified by TGEM with those epitopes identified by IFN-γ secretion; however, some epitopes were identified only by one or the other method, likely reflecting the different T cell characteristics each approach measures. Earlier studies examining Ag-specific CD8⁺ T cells with class I tetramers have likewise demonstrated this incomplete concordance between Ag-specific cytokine production and tetramer staining (10, 35).

The MHC class II tetramers in this study were generated by using a peptide mixture. Notably, the current results demonstrate that tetramers generated with peptide mixtures are capable of staining Ag-specific T cells. We speculate that within a given peptide mixture, only one or two peptides can bind to the MHC with high affinity. These high-affinity peptide complexes likely ensure the formation of sufficient numbers of tetramers containing identical MHC/peptide complexes. Data from our laboratory and others suggest that 10–50% of the soluble MHC molecules are loaded when incubated with exogenous peptide (Ref. 36 and W. Kwok, B. McFarland, C. Beeson, unpublished observations). Applying these observations to tetrameric molecules suggests that with exogenous peptide loading, less than 6% of the tetramers have all four MHC peptide loading, less than 6% of the tetramers have all four MHC molecules loaded with peptide, assuming a lack of cooperative interactions. Staining with dimeric or trimeric MHC/peptide complexes previously has been reported (37). Therefore, the T cell staining observed in our study is likely at least partly attributable to binding of MHC tetramer complexes with only two or three sites occupied by the cognate peptide.

One of the concerns of the TGEM approach is that the relevant peptides will be outcompeted by irrelevant peptides present in the pool. For this reason, we purposefully loaded DR0401 molecules with different molar ratios of cognate peptide (VP16 p61) to competitor peptide (HA_{307-319}), and these mixed tetramers were used...
to stain a DR0401/p61 clone. In competition experiments, these two peptides possess similar affinities for the DR0401 molecule (data not shown). There was no significant difference in staining pattern when the molar ratio of p61 to HA307–319 was 1:1. We observed an appreciable decrease in staining intensity of the DR0401/p61 clone with a 1:10 molar ratio; nonetheless, staining of clones with these mixed tetramers was still 10-fold above background (data not shown). Likewise, we were able to obtain similar staining above background by using cognate tetramer diluted 100-fold, suggesting that an excess of class II molecules are present during the staining reaction. These findings suggest that loading of peptide mixtures onto class II within the parameters described should not preclude the TGEM approach from detecting Ag-specific T cells of interest. Earlier studies that used pooled peptides to stimulate cells have shown similar results (25, 26, 38), and studies that used combinatorial peptide libraries have demonstrated that HLA competition only becomes a contributing factor in T cell stimulation when competitor peptides are present in several orders of magnitude excess (39). Nonetheless, it is conceivable that the pooled peptide approach may have difficulty in detecting peptide epitopes with very low MHC binding affinity in the presence of other peptides in the same peptide pool with high MHC binding affinity.

The standard approach for cloning T cells and mapping out antigenic epitopes involves Ag challenge of PBMC followed by plating individual cells into 96-well plates. Cells then are expanded and assayed for MHC restriction and peptide specificities by screening clones with overlapping peptides that cover the Ag, a labor-intensive and time-consuming process. Alternatively, epitopes can be identified by using a recently described flow cytometry-based approach that uses IFN-γ production as a marker of reactivity. Although this approach simplifies isolation of epitope-specific clones, the task of identifying individual MHC restriction elements remains. With TGEM, the HLA restriction and peptide specificity of the T cells are known because of the specificity of tetramer staining. Tetramer staining also offers the opportunity for straightforward cloning of the Ag-specific T cells through single-cell sorting. As a consequence, large numbers of T cells clones with known Ag specificities and MHC restrictions can be obtained easily. In addition, we have found that the Ag-dependent expansion of T cells in the TGEM procedure can be performed without purified Ag. For assay of viral antigenic epitopes, we have previously shown that stimulation with UV-inactivated virus is effective

Table I. Relative binding affinity of DR0401- and DR0404-restricted VP16 epitopes

<table>
<thead>
<tr>
<th>VP16 epitopes</th>
<th>IC50 0401 (μM)</th>
<th>IC50 0404 (μM)</th>
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<tr>
<td>DR0401-restricted VP16 epitopes:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>VP16555-60 ALENRLDDLG</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>VP16472-484 DFEFEQMFDTAMG</td>
<td>0.5</td>
<td>0.5</td>
</tr>
<tr>
<td>DR0404-restricted VP16 epitopes:</td>
<td></td>
<td></td>
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<tr>
<td>VP16443-450 FDLEMLGDPVESPS</td>
<td>&gt;5</td>
<td>0.005</td>
</tr>
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* Underlined residue is the proposed first anchor for binding to the respective allele as determined by peptide truncation studies (data not shown).
(19). Similarly, peptide pools can be used for initial stimulation in the event that whole Ag is not available. Knowledge of specific Ag epitopes and their associated MHC restrictions provides a valuable foundation for directed immunotherapies in a number of different areas including autoimmune and infectious diseases and cancer (1–5). TGEM allows rapid identification of CD4+ T cell epitopes and can easily be applied to a variety of Ags, especially as tetramer reagents become more widely available.

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References


