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Peptide Length Variants p2Ca and QL9 Present Distinct Conformations to Ld-Specific T Cells

Tara M. C. Hornell, Shiloh M. Martin, Nancy B. Myers, and Janet M. Connolly

Recent advances have provided insights into how the TCR interacts with MHC/peptide complexes and a rationale to predict optimal epitopes for MHC binding and T cell recognition. For example, peptides of nine residues are predicted to be optimal for binding to H2-Ld, although 8 mer epitopes have also been identified. It has been predicted that 8 mer and 9 mer length variant peptides bound to Ld present identical epitopes to T cells. However, in contrast to this prediction, we demonstrate here that the 8 mer peptide p2Ca and its 9 mer length variant QL9, extended by an N-terminal glutamine, assume distinct conformations when bound to Ld. We generated self-Ld-restricted CTL clones specific for p2Ca that recognize Ld/QL9 poorly if at all. This result is in sharp contrast to what has been observed with Ld-alloreactive T cells that possess a much higher affinity for Ld/QL9 than for Ld/p2Ca. Alanine substitutions of the N-terminal residues of the QL9 peptide rescue detection by these self-Ld/p2Ca-specific T cells, but decrease recognition by the Ld-alloreactive 2C T cell clone. In addition, 2C T cell recognition of the p2Ca peptide is affected by different alanine substitutions compared with 2C T cell recognition of the QL9 peptide. These data clearly demonstrate that the p2Ca and QL9 peptides assume distinct conformations when bound to Ld and, furthermore, demonstrate that there is flexibility in peptide binding within the MHC class I. The Journal of Immunology, 2001, 167: 4207–4214.

Recognition of specific MHC class I/peptide complexes by CD8+ T cells leads to the elimination of virally infected or cancerous cells. The peptide epitope bound within the MHC Ag-binding cleft is integral in determining the specificity of T cell recognition of Ag. Although each class I MHC allele binds a mixture of structurally diverse peptides, sequencing of eluted peptides has shown that they are generally 8–10 aa in length and possess allele-specific peptide binding motifs (1). Furthermore, in almost all cases the carboxyl-terminal peptide position is an anchor residue deeply buried in the F pocket. The crystal structure of several class I molecules has revealed that the peptide binding cleft is closed at both ends and the length of the cleft is identical for all class I molecules (2–8). These insights into peptide binding motifs and the structure of MHC/peptide complexes have provided a rationale to identify potential antigenic peptides within the native protein and also to improve the immunogenicity of known antigenic peptides by modifying specific amino acid residues (9, 10).

For the mouse class I molecule, H-2Ld, the optimal peptide sequence for T cell recognition, derived from the native protein, has been identified for several antigenic and self-epitopes (11–16). Self-peptides eluted from Ld as well as several antigenic epitopes have defined the Ld binding motif as a 9 mer with proline at position 2 and a carboxyl-terminal hydrophobic residue (11–13). However, Ld antigenic peptides such as tum− (QNNRALDL) and p2Ca (LSPFPFDL) are 8 mers that lack the proline at position 2 and thus use alternative amino-terminal anchors (14, 15). The p2Ca octamer was identified as the ligand that is naturally processed and presented to the Ld-alloreactive T cell clone, 2C (15, 16). 2C T cells also recognize the 9 mer length variant of p2Ca, QL9 (LSPFPFDL), derived from the same native protein, α-ketoglutarate dehydrogenase (15–17). The QL9 peptide has a higher affinity for Ld than does the p2Ca peptide (1.2–2 × 10^6 M−1 vs 4 × 10^5 M−1), and the TCR on 2C T cells has a higher affinity for Ld/QL9 (1–2 × 10^7 M−1) than for Ld/p2Ca (2 × 10^6 M−1) (17–19). Thus an N-terminal extension of p2Ca results in both increased affinity of peptide for MHC and increased 2C TCR affinity for MHC/peptide. Soluble 2C TCR has been reported to have a similar affinity for Ld/p2Ca and Ld/QL9 (20). However, the affinities measured using live 2C T cells are more applicable for functional recognition of Ld/QL9 vs Ld/p2Ca by 2C T cells.

The 2C clone and mice transgenic for the 2C TCR have been used as a model system to study the basis of allore cognition and thymic selection of alloreactive T cells (21–25). 2C T cells are alloreactive for Ld and positively selected on Kd. Therefore, the 2C system has been used as a model of how a given TCR can interact with two class I/peptide complexes: the agonist ligand, Ld complexed with the p2Ca peptide, and the positively selecting element, Kd. A peptide, dEV8, was identified that when bound to Kd activates 2C T cells in vitro and can positively select 2C T cells (23, 25). Cocrystallographic data of the 2C TCR interaction with Kd/dEV8 were obtained (26). A structure of Ld complexed with either the p2Ca 8 mer or its 9 mer length variant, QL9, has eluded crystallographers. However, the crystal structures of Ld complexed with the endogenous self-peptide P29 (27) and also with a mixture of peptides (21) were obtained. A model of how the 2C TCR might interact with Ld/p2Ca and Ld/QL9 was published based on the speculations that p2Ca and QL9 bind similarly to Ld, that the 2C TCR interacts similarly with Kd and Ld, and that the 2C TCR interacts similarly with Ld/QL9 and Ld/p2Ca (21). Furthermore, in vitro studies have used p2Ca and QL9 interchangeably, with the 8 mer considered a weak agonist and the 9 mer a strong agonist (28),
or assuming that the p2Ca and QL9 peptides are detected identically by T cells (21, 24, 29, 30). However, there has been no direct evidence to support these assumptions.

In this study, we generated self-Ld-restricted CTL clones specific for the endogenous self-peptide, p2Ca. Remarkably, we found that these self-Ld/p2Ca-specific T cell clones do not recognize Ld/QL9 or do so poorly. We assessed the ability of these clones and the 2C clone to functionally recognize alanine-substituted peptide variants of p2Ca and QL9. These analyses revealed that not only are there differences between 2C T cells and the self-Ld/p2Ca-specific clones in the ability to interact with Ld/QL9, but there are differences in how a single T cell, 2C, interacts with Ld/p2Ca vs Ld/QL9. Together, these findings provide strong evidence that these two peptides bind to Ld and are presented to T cells in a conformationally distinct manner.

Materials and Methods

Mice

BALB/c mice (H-2d) were obtained from The Jackson Laboratory (Bar Harbor, ME) or Charles River Breeding Laboratories (Wilmington, MA). Ld-transgenic, TAP−/− mice (H-2d) were generated by breeding TAPI−/− mice (The Jackson Laboratory) with Ld-transgenic C3H mice (H-2d), a gift from J. Forman (University of Texas Southwestern Medical Center, Dallas, TX). Progeny were typed by flow cytometry for the presence of Ld and absence of TAP, and therefore also the absence of H-2d, by testing PBMC for surface stabilization of Ld after incubation with Ld-binding peptides. The mice were fixed in the second generation and have been maintained by interbreeding. All mice were housed and bred in the barrier animal facility at Washington University School of Medicine (St. Louis, MO).

Peptides

The p2Ca (LSPFPFDL) and QL9 (QLSFPFDL) peptides (15–17) along with their alanine-substituted variants were synthesized using Merrifield's solid phase method (31) on a peptide synthesizer (model 431A; Applied Biosystems, Foster City, CA). Peptides were purified (>98%) by reverse phase HPLC and purity assessed (32).

Cell lines

LM1.8, a gift from Dr. P. Kourilsky (Institut National de la Santé et de la Recherche Médicale, Institut Pasteur, Paris, France), is an L cell (H-2d) fibroblast transfected with ICAM-1 selected with hypoxanthine/aminopterin/thymidine. LM1.8-Ld is LM1.8 transfected with the Ld cDNA selected with G418 (Geneticin; Life Technologies, Grand Island, NY). T2, a TAP-deficient human lymphoblastoid cell line, and T2-Ld, cells transfected with the Ld cDNA and selected with G418, were gifts from Dr. P. Cresswell (Yale University, New Haven, CT), P815 is an H-2d mouse mastocytoma. Cell lines were maintained at 37°C, 5% CO2 in RPMI 1640 medium (Life Technologies) supplemented with 2 mM L-glutamine, 10% (v/v) bovine calf serum (HyClone Laboratories, Logan, UT), 1 mM HEPES buffer, and 100 U/ml penicillin/streptomycin.

Ld mutants

Generation of the Ld mutants was reported previously (33). Briefly, the Ld mutants were made by PCR from a Ld cDNA template and sequenced to confirm the presence of the mutation and PCR fidelity. Each Ld mutant was cloned into the expression vector RSVc (34), and the constructs were transfected into LM1.8 cells using lipofectin (Life Technologies, Gaithersburg, MD).

Cytotoxic T cell lines and clones

The Ld-restricted, p2Ca-specific CTL were generated by incubating 7.5 × 105 BALB/c splenocytes with 3.5 × 105 irradiated (2000 rad) Ld− TAP−/− cells in a total volume of 2 ml of sensitization medium per well in 24-well plates. Sensitization medium consisted of RPMI 1640 medium supplemented with 2 mM L-glutamine, 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, 1 mM HEPES buffer, 100 U/ml penicillin/streptomycin, 50 µM 2-ME, and 10% (v/v) FBS (HyClone Laboratories). The cells were cultured at 37°C, 5% CO2 and after 5 days were tested for the ability to lyse Ld-expressing targets in the presence or absence of the p2Ca peptide. After 1 wk in culture, the lines were plated at 5 × 103 cells/well and stimulated with 5 × 105 irradiated (2000 rad) Ld− TAP−/− splenocytes per well in sensitization medium and 1 × 10−8 M peptide. After 2 wk, the lines were restimulated in the additional presence of 10 U/ml murine rIL-2 (Biosource International, Camarillo, CA).

The Ld-restricted, p2Ca-specific line was cloned by limiting dilution. Briefly, various concentrations of effector cells were plated into 96-well, round-bottom plates in the presence of 2.5 × 105 irradiated Ld− TAP−/− splenocytes per well, 10 U/ml rIL-2, and 10−8 M p2Ca. The clones were restimulated weekly by replacing 100 µl of the medium with 100 µl of fresh medium containing 5 × 105 irradiated Ld− TAP−/− splenocytes, 20 U/ml rIL-2 (10 U/ml final), and 2 × 10−8 M (1 × 10−7 M final) p2Ca. After 2 wk, a sample from each well was tested to determine the frequency of Ld− p2Ca. Wells with cells specific for Ld/p2Ca-expressing targets were expanded and maintained by weekly restimulation in 24-well plates with 0.5–1 × 105 T cells/well, 5 × 10−8 irradiated splenocytes per well, 10−8 M p2Ca, and 10 U/ml rIL-2. The Ld−alloreactive, p2Ca-specific clone 2C, generated from a BALB.B (H-2b) mouse that had been immunized with H-2d cells, was a gift from Dr. H. Eisen (Massachusetts Institute of Technology, Cambridge, MA). 2C was maintained in 24-well plates, plated at 5 × 103 cells/well, and stimulated weekly with 5 × 105 irradiated (2000 rad) BALB/c splenocytes per well in sensitization medium and 10 U/ml murine rIL-2.

51Cr-release assay

Target cells (1 × 106) were labeled for 1 h with 150–200 µCi of 51Cr (Na111CrO4; NEN, Boston, MA; 1 Ci = 37 GBq) in 200 µl of RPMI 1640 medium + 10% bovine calf serum at 37°C in 5% CO2. Effector cells were plated at various concentrations into 96-well microtiter plates, and 2.5 × 104 0.5 or 5 × 104 washed target cells per well were added. Various concentrations of peptide or medium alone were added to the wells. The plates were centrifuged at 50 × g for 1 min and incubated for 4 h at 37°C in 5% CO2. Radioactivity in 100 µl of supernatant was measured in an Isosopic gamma counter (ICN Biomedicals, Huntsville, AL). The mean of triplicate samples was calculated, and the percentage of 51Cr release was determined according to the following equation: percentage of 51Cr release = 100 × [(experimental 51Cr release − control 51Cr release)/(maximum 51Cr release − control 51Cr release)], where experimental represents target cells mixed with effector cells; control represents target cells in medium alone (spontaneous release); and maximum represents target cells lysed by 5% Triton X-100 (Sigma, St. Louis, MO). For assessments of alanine-substituted peptides, CTL assays were performed on day 4 after restimulation to determine the lowest E:T ratio that gave maximum lysis on LM1.8-Ld cells + native peptide. This optimal E:T ratio was used for CTL assays performed on day 5 after restimulation with the entire panel of target cell lines or alanine-substituted peptides. EC50 values were determined by fitting the specific lysis curves to the function: Bmax [(1 + (EC50/ [peptide])) + B0, where Bmax is the maximal lysis, EC50 is the peptide concentration that produces 50% of maximal lysis, n is the apparent cooperativity, and B0 is the lysis when [peptide] is 0.

Surface stabilization and flow cytometry analysis

LM1.8-Ld cells were cultured overnight at 37°C in cell culture medium in the presence or absence of various concentrations of peptide. The cells were washed and incubated at 4°C for 30 min in HBSS containing 0.2% BSA and 0.1% sodium azide in the presence of Ld-specific or control mAb. The cells were washed and incubated with a fluorescein-conjugated, Fc-specific, affinity-purified F(ab’), of goat anti-mouse IgG (Cappel, Organon-Teknika, West Chester, PA). Viable cells, gated by forward and side light scatter, were analyzed on a FACScan flow cytometer (BD Biosciences, Mountain View, CA). Mean fluorescence values were converted from log-logarithmic amplification by linear regression analysis using CellQuest 3.0 software (BD Biosciences). Cells incubated with the fluorescent Ab alone were included as controls. EC50 values were determined by fitting the peptide-induced Ld surface induction curves to the function: Bmax[(1 + (EC50/ [peptide])) + B0, where Bmax is the maximal surface induction of Ld, EC50 is the molar peptide concentration that produces 50% of maximal surface induction of Ld, n is the apparent cooperativity, and B0 is the surface expression of Ld when [peptide] is 0.

Results

CTL clones specific for self-Ld/p2Ca distinguish between the p2Ca and QL9 peptides

We generated self-Ld-restricted CTL specific for the self-peptide p2Ca to determine whether they react with the same MHC allele specific residues as do Ld−alloreactive T cells and self-Ld-restricted T cells specific for foreign antigenic peptides (33). Whereas using a high concentration of foreign antigenic peptide in vitro resulted
in the generation of L$^d$-restricted, peptide-specific CTL in primary cultures (35, 36), several weeks of in vitro culture were required to obtain activity against a self-peptide (37). However, using splenocytes from L$^d$ transgenic, TAP1$^{-/-}$ mice in the presence of 10$^{-4}$ M p2Ca peptide to stimulate BALB/c splenocytes in vitro, self-L$^d$/p2Ca-specific CTL are readily obtained in primary cultures (Fig. 1A). These conditions result in a 5- to 6-fold increase in L$^d$ surface expression such that >80% of surface L$^d$ is occupied with the exogenous peptide (38, 39). Therefore, using L$^d$$^{+/-}$ TAP$^{-/-}$ cells with a high concentration of peptide as APC permits the in vitro generation of primary CTL specific for self-peptide. As opposed to the alloreactive L$^d$/p2Ca-specific T cell clone, 2C, and other alloreactive L$^d$/p2Ca-specific T cell clones (40), the self-L$^d$/p2Ca-specific CTL do not recognize endogenous levels of p2Ca expressed by P815 (Fig. 1B). Therefore, although the self-L$^d$/p2Ca-specific CTL are specific for self-MHC with a self-peptide, they are not autoreactive.

Several L$^d$/p2Ca-specific clones were isolated from these self-L$^d$/p2Ca-specific CTL lines, some of which are shown in Fig. 2. These self-L$^d$/p2Ca-specific clones require 27–125 nM of exogenous p2Ca for half-maximal lysis of the TAP$^{-/-}$ cell line, T2-L$^d$ (Fig. 2 and Table I). This finding is in contrast to alloreactive L$^d$/p2Ca-specific T cell clones (40) that require <10 nM p2Ca (J. Connolly, unpublished observation) and 2C that requires 1.4 nM p2Ca for half-maximal lysis (Fig. 2d and Table I) and has an affinity for L$^d$/p2Ca of 2 × 10$^6$ M$^{-1}$ (17–19). Thus the self-L$^d$/p2Ca-specific clones possess lower avidity than L$^d$-alloreactive T cell clones specific for p2Ca. This is consistent with the failure of the primary self-L$^d$/p2Ca-specific CTL to recognize endogenous levels of p2Ca expressed by P815, and suggests that the failure to recognize P815 does not result from a different conformation induced by exogenous vs endogenous peptide.

To determine whether these self-L$^d$/p2Ca-specific CTL have similar peptide specificity to L$^d$-alloreactive T cells, we compared them with 2C T cells for recognition of p2Ca vs QL9. The QL9 peptide is a 9 mer variant of the p2Ca 8 mer, derived from the p2Ca-speciﬁc clones possess lower avidity than L$^d$-alloreactive T cell clones specific for p2Ca. Therefore, the self-L$^d$/p2Ca-specific CTL have [32] similar TCR contacts and thus interact in the same orientation as the self-L$^d$/p2Ca-specific clone, 2C. Therefore, the self-L$^d$/p2Ca-specific clones interact with similar MHC residues as primary TCR contacts and thus interact in the same orientation as other L$^d$-reactive T cells (33).

The self-L$^d$/p2Ca-specific clones interact with the same primary MHC residues as other L$^d$-reactive T cells

A possible explanation for the ability of these clones to distinguish between the p2Ca and QL9 peptides is that they interact with different L$^d$-α-helical residues than do 2C T cells. To determine whether this is the case, the self-L$^d$/p2Ca-specific clones 1D12 and 1H5 were tested for recognition of the 16 L$^d$ mutants used in our previous analysis to determine which residues are important for TCR interaction. We showed previously that the amino acid residues at positions 69, 72, 76, and 155/157 of L$^d$ are primary TCR contacts for both syngeneic and alloreactive L$^d$ T cell clones specific for L$^d$, including 2C (Ref. 33 and Fig. 3). Recognition of L$^d$/p2Ca by clone 1D12 is affected by mutation of the amino acid residues at positions 69, 72, 76, 155/157, and 158 of L$^d$ (Fig. 3). Recognition of L$^d$/p2Ca by clone 1H5 is affected by mutation of the amino acid residues at positions 58, 69, 72, 76, 82, 155/157, 158, 162, and 169 (Fig. 3). These results indicate that the L$^d$-restricted T cell clone 1D12 recognizes most, and 1H5 all, of the same key residues as other L$^d$-specific T cell clones, including 2C. Therefore, the self-L$^d$/p2Ca-specific clones interact with similar MHC residues as primary TCR contacts and thus interact in the same orientation as other L$^d$-reactive T cells (33).

The self-L$^d$/p2Ca-specific clones interact with the same p2Ca residues, but not the same QL9 residues, as the 2C clone

Because the self-L$^d$/p2Ca-specific T cells interact with similar MHC residues as other L$^d$-reactive T cells, a possible explanation for why they interact with p2Ca and not the QL9 peptide is that they interact with different p2Ca and/or QL9 residues than do 2C T cells. To determine whether this is the case, we compared the ability of the self-L$^d$/p2Ca-specific clones and the 2C clone to recognize alanine-substituted variants of p2Ca and QL9. To attribute differences in recognition to TCR interaction vs peptide binding to L$^d$, surface stabilization assays were performed with p2Ca, QL9, and the alanine-substituted variants. LM1.8-L$^d$ cells were cultured overnight in the presence of various concentrations of peptide or alanine-substituted variant, and the increase in L$^d$ expression was analyzed by flow cytometry (38, 39). The EC$_{50}$ values for binding of p2Ca, QL9, and the alanine-substituted peptide variants to L$^d$ are shown in Table I. Comparable to what has been previously reported for p2Ca, the C-terminal L8 has a significant effect on p2Ca peptide binding, as do F6 and D7 (Refs. 18 and 41, and Table I). For QL9, although the C-terminal residues are also involved in peptide binding, the contribution is less than for p2Ca. This is likely due to the participation of Q1 to binding. Substitution of Q1 considerably reduces peptide binding of QL9. It is also noteworthy that substitution of P5 of p2Ca and the equivalent P6 of QL9 results in reduced binding of peptide to L$^d$ and a parallel reduction in 2C T cell recognition. This result was reproduced several times. This is in contrast to previous studies that
FIGURE 2. Self-Ld/p2Ca-specific T cell clones possess the reciprocal peptide specificity as does the allorreactive Ld/p2Ca-specific 2C clone. Clones were generated from the BALB/c anti-Ld TAP +/− × p2Ca line by limiting dilution. Recognition of T2-Ld targets in the absence or presence of continuous p2Ca or QL9 is shown for three self-Ld/p2Ca-specific clones, 1A5, 1D12, and 1H5, at an E:T ratio of 20:1 (a−c) and for the 2C clone at an E:T ratio of 5:1 (d). For the data shown, the EC50 values (in nM) for 1A5, 1D12, 1H5, and 2C recognition of Ld/p2Ca are 125.18, 27.34, 55.07, and 1.43, respectively.

Table I. EC50 values for peptide induction of Ld surface expression and peptide-specific T cell recognition of Ld-expressing target cells by p2Ca, QL9, and the p2Ca or QL9 alanine-substituted variants

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Ld Binding EC50 (µM)a</th>
<th>2C T Cell Recognition EC50 (nM)a</th>
</tr>
</thead>
<tbody>
<tr>
<td>QL9</td>
<td>0.34</td>
<td>0.0059</td>
</tr>
<tr>
<td>Q1A</td>
<td>134.50</td>
<td>0.6317</td>
</tr>
<tr>
<td>L2A</td>
<td>8.09</td>
<td>0.0438</td>
</tr>
<tr>
<td>S3A</td>
<td>0.98</td>
<td>0.0021</td>
</tr>
<tr>
<td>P4A</td>
<td>6.70</td>
<td>0.0026</td>
</tr>
<tr>
<td>F5A</td>
<td>0.12</td>
<td>0.0065</td>
</tr>
<tr>
<td>P6A</td>
<td>231.10</td>
<td>56.9000</td>
</tr>
<tr>
<td>F7A</td>
<td>11.73</td>
<td>0.0394</td>
</tr>
<tr>
<td>D8A</td>
<td>52.92</td>
<td>0.0113</td>
</tr>
<tr>
<td>L9A</td>
<td>129.87</td>
<td>2.1304</td>
</tr>
<tr>
<td>p2Ca</td>
<td>45.58</td>
<td>1.4291</td>
</tr>
<tr>
<td>L1A</td>
<td>65.14</td>
<td>1.7712</td>
</tr>
<tr>
<td>S2A</td>
<td>45.38</td>
<td>2.1756</td>
</tr>
<tr>
<td>P3A</td>
<td>53.85</td>
<td>6.2256</td>
</tr>
<tr>
<td>F4A</td>
<td>24.79</td>
<td>203.5200</td>
</tr>
<tr>
<td>P5A</td>
<td>190.78</td>
<td>167.1000</td>
</tr>
<tr>
<td>F6A</td>
<td>283.66</td>
<td>196.2200</td>
</tr>
<tr>
<td>D7A</td>
<td>717.28</td>
<td>301.0100</td>
</tr>
<tr>
<td>L8A</td>
<td>1409.00</td>
<td>179.6900</td>
</tr>
</tbody>
</table>

*a EC50 values were determined by fitting the peptide-induced Ld surface expression data or peptide-specific T cell lysis, obtained over a range of peptide concentrations, as described in Materials and Methods.

Report that substitution of this residue results in reduced 2C recognition without an effect on Ld binding (18, 41). However, these previous analyses used soluble Ld as opposed to the results reported here that measure binding to Ld on intact cells.

To determine the peptide residues that are important for recognition by the self-Ld/p2Ca-specific T cells vs 2C T cells, we tested the ability of these T cell clones to recognize the alanine-substituted peptides. CTL assays were run using a broad range of peptide concentrations in the presence of continuous peptide to minimize minor effects on peptide binding. For these analyses the lowest E:T ratio that gave maximum lysis of T2-Ld/p2Ca or T2-Ld/QL9 was selected to test for recognition of T2-Ld in the continuous presence of various concentrations of peptide. The EC50 values for recognition of p2Ca, QL9, and the alanine-substituted peptide variants by 2C are shown in Table I. EC50 values for recognition of p2Ca by the self-Ld/p2Ca-specific T cells are shown in Fig. 2, whereas their recognition of the alanine-substituted peptide variants did not provide sufficient data points to calculate EC50 values. A comparison of the recognition of the p2Ca peptide variants by 2C vs the self-Ld/p2Ca-specific T cells is shown in Fig. 4. The p2Ca peptide and the eight alanine-substituted variants were tested at peptide concentrations from 10−6 to 10−12 M. The data shown represent the concentration of peptide that was limiting and thus best displays the differential recognition of the peptide variants by each T cell clone. For 2C T cells, this is 10−12 M peptide, whereas for 1A5 and 1D12 T cells it is 10−6 M peptide. As shown in Fig. 4, clones 1A5 and 1D12 recognize the A1, A2, and A3-substituted p2Ca peptides, but fail to recognize the peptide variants mutated at positions 4 through 8. Similarly, 2C T cells recognize the A1, A2, and A3 variants comparably to p2Ca and recognize the other alanine-substituted variants less well. Reduced peptide binding to Ld could contribute to the reduced recognition of variants substituted at positions 5 through 8. However the F4A variant binds well yet is not recognized by 2C or the self-Ld/p2Ca-specific T cells, indicating that F4 of p2Ca is a TCR contact for both types of clones.

FIGURE 3. Recognition of H-2Ld mutants by the self-Ld/p2Ca-specific clones demonstrates a pattern similar to other Ld-reactive T cells. Recognition of mutant Ld was compared with recognition of wild-type Ld by self-Ld/p2Ca-specific CTL clones. For each clone, the lowest E:T ratio that gave peak lysis on LM1.8-Ld in continuous 1 × 10−5 M peptide was used for recognition of the mutants. ■ Lysis of the mutant at levels <25% of wild-type Ld; □ lysis between 25 and 50% of wild-type Ld; □ lysis comparable to that of wild-type Ld (>50% of wild-type Ld). The consensus pattern was derived from a previous study performed with 20 different Ld-reactive T cells clones (33). For the consensus pattern, black boxes indicate a mutation that affected 75% or more of the clones, hatched boxes indicate a mutation that affected recognition by 50−75% of the clones, and white boxes indicate that the mutation affected <50% of the clones.
Thus, these self-L<sup>d</sup>/p2Ca-specific T cell clones are dependent upon interaction with the same amino acid residues of the p2Ca peptide as are 2C T cells.

We next tested T cell recognition of the alanine-substituted variants of QL9. We reasoned that failure of the self-L<sup>d</sup>/p2Ca-specific clones to recognize L<sup>d</sup>/QL9 could be due to Q1 directly interfering with TCR recognition or indirectly interfering through a conformational change induced in the TCR contacts elsewhere within the peptide. The QL9 peptide and its nine variants were tested at peptide concentrations from 10<sup>−8</sup> to 10<sup>−12</sup> M. A comparison similar to that performed for recognition of the alanine-substituted p2Ca variants was performed because recognition of QL9 by the self-L<sup>d</sup>/p2Ca-specific clones is very weak and requires both high peptide concentration and E:T ratios. The limiting peptide concentration that displays the differential recognition of the peptide variants is 10<sup>−11</sup> M peptide for 2C T cells and 10<sup>−6</sup> M peptide for 1A5 and 1D12 T cells. As shown in Fig. 5, recognition by the 2C T cell clone is reduced by alanine substitution at positions 1 and 2 of the QL9 peptide. In fact, peptide titration analysis revealed that substitution at these positions results in recognition similar to 2C T cell recognition of the p2Ca peptide (data not shown). In contrast, recognition of the QL9 peptide by the self-L<sup>d</sup>/p2Ca-specific T cell clones is rescued by alanine substitution at positions 1 and 2, as well as positions 3 and 4 (Fig. 5). The effect at position 1 is more dramatic for clone 1A5 at higher E:T ratios. Ten additional clones were tested and showed the identical pattern (data not shown). Peptide titration analysis revealed that the recognition of alanine-substituted QL9 peptide does not reach the level of recognition of the p2Ca peptide by these self-L<sup>d</sup>/p2Ca-specific T cell clones. This suggests that the amino-terminal glutamine exerts its effect indirectly by inducing a conformational change that affects the TCR contacts elsewhere in the peptide and/or MHC, and also suggests that p2Ca and QL9 are conformationally distinct when bound to L<sup>d</sup>. Whereas recognition of the alanine-substituted p2Ca peptide variants showed a similar pattern for 2C T cells and the self-L<sup>d</sup>/p2Ca T cell clones, recognition of the alanine-substituted variants of the QL9 peptide is quite different for the two types of clones.
For 2C T cells, substitution of A for Q1 or L2 of QL9 results in reduced recognition and substitution of A at S3 and P4 has little or no effect on recognition. For the self-L^d/p2Ca-specific T cell clones, all four of these substitutions rescued recognition of the QL9 peptide variant. The reduced binding of Q1A could explain the effect on recognition by 2C but the recognition by the self-L^d/p2Ca-specific clones is enhanced despite this reduced binding. Substitution of A at F5 has no effect on 2C T cell recognition but does not allow recognition by the self-L^d/p2Ca-specific T cell clones. Therefore, there are several residues that contribute to the distinct recognition of p2Ca and QL9 by the two types of clones; this observation suggests that there are differences in the conformations of the 8 mer and 9 mer peptides when bound to L^d and presented to T cells.

Recognition by 2C T cells is affected by substitution of distinct amino acid residues of the p2Ca vs QL9 peptides

If L^d/p2Ca and L^d/QL9 present identical conformations to T cells, then substitution of QL9 residues corresponding to positions of p2Ca known to affect T cell recognition would also interfere with T cell recognition of L^d/QL9 by the same T cell. To test this, we compared the ability of the 2C T cell clone to recognize alanine-substituted QL9 variants with the ability of 2C T cells to recognize p2Ca variants. Comparison of the top panels of Figs. 4 and 5 and the EC_{50} values in Table I demonstrates that there are distinct differences in recognition of these two peptides by 2C T cells. Strikingly, the F4 of p2Ca is critical for 2C recognition, whereas alanine substitution of the corresponding F5 of QL9 does not affect 2C recognition. This difference is not due to an effect on peptide binding (Ref. 41 and Table I) because the substituted peptides and the native peptide bind equivalently to L^d. In addition, mutation of Q1 and L2 of QL9 reduces 2C recognition substantially, whereas no effect is seen when the corresponding L1 of p2Ca is mutated. The Q1A variant was analyzed at higher concentrations for binding to L^d. Even at 200 μM, the Q1A variant was only able to increase L^d surface expression to 36% of maximum (data not shown) and the EC_{50} for Q1A binding to L^d is ~400-fold more than QL9 (Table I). The finding that alanine substitution of comparable residues in p2Ca vs QL9 has dramatically different effects on recognition by a single TCR provides compelling evidence that the 8 mer and 9 mer peptides present distinct epitopes to T cells when bound to L^d.

Discussion

The results reported here provide functional evidence that peptide variants that differ in sequence by a single N-terminal addition assume distinct conformations when bound to L^d and furthermore support a model in which there is flexibility in peptide binding within the MHC class I binding cleft. It has been predicted that the p2Ca and QL9 peptides bind to L^d in orientations that are conformationally similar to T cells (21). The affinity of the QL9 peptide for L^d has been found to be 30- to 50-fold higher than the affinity of p2Ca for L^d (17–19). However, recognition of L^d/QL9 by 2C T cells requires 2 to 3 logs less peptide than recognition of L^d/p2Ca by 2C T cells (Ref. 17 and present study), a difference not accounted for by peptide binding alone. Furthermore, the TCR affinity of 2C T cells is 5- to 10-fold higher for L^d complexed with QL9 vs p2Ca (17, 18). The results reported here show that differences in peptide conformation contribute to the affinity difference of the 2C TCR for the two peptides, and offer an explanation for the functional difference observed for 2C T cell recognition of L^d/p2Ca vs L^d/QL9. The self-L^d/p2Ca-specific clones generated in this study preferentially interact with L^d/p2Ca and not L^d/QL9. Thus they have the reciprocal peptide preference compared with 2C and other L^d-alloreactive CTL. This reciprocal pattern of reactivity cannot be explained solely by TCR affinity differences between 2C and the self-L^d/p2Ca-specific T cells because a similar recognition pattern would be predicted if the peptides present the same conformation. Thus it is likely that T cells can detect structural differences between L^d/p2Ca and L^d/QL9. In the absence of a crystal structure of either L^d/p2Ca or L^d/QL9 it is not possible to predict the precise differences that exist between the two structures. Therefore, it is not clear whether this difference in conformation involves peptide alone, whether there are differences in MHC conformation induced when the different length peptides bind, or whether conformational changes are induced in the peptide by TCR contacts as has been reported for the TCR interaction with HLA-A2/Tax peptide (42).

The ability to generate self-limited T cells that differentiate between L^d/p2Ca and L^d/QL9 may be a reflection of how alloreactive vs self-MHC-restricted T cells develop in the thymus. Only T cells with a low avidity for self-L^d/p2Ca escape negative selection and mature, whereas T cells with a high affinity for foreign MHC are not negatively selected and mature. This could allow the development of self-limited T cells with the ability to detect conformational differences that are overlooked by high affinity alloreactive T cells. Data examining recognition of alanine-substituted peptide variants demonstrate that 2C T cells and the self-L^d/p2Ca-specific T cell clones interact similarly with the p2Ca peptide but differently with the QL9 peptide. Substitution of the amino-terminal residues at positions 1 through 4 of QL9 permits recognition by the self-L^d/p2Ca-specific clones but reduces recognition by 2C. In addition to the differences observed in how the self-L^d/p2Ca-specific T cells compared with 2C interact with L^d/QL9, the data here demonstrate that the 2C T cell clone distinguishes between L^d/p2Ca and L^d/QL9. The F4A variant of p2Ca is not recognized by 2C, whereas the equivalent F5A variant of QL9 is recognized, indicating that the TCR contacts are not the same for p2Ca vs QL9. The conformation assumed by QL9 apparently results in increased interaction with the 2C TCR but reduced interaction with the self-L^d/p2Ca-specific TCR. Although we cannot exclude the possibility that alanine substitution induces different effects on p2Ca vs QL9 peptides, these data collectively demonstrate that QL9 binds L^d in a distinct manner from p2Ca and the overall conformation of the peptide/MHC complexes must be quite dissimilar.

The crystal structure of L^d bound to the endogenous 9 mer peptide, P29, has shown that L^d, like D^d, has a hydrophobic ridge in its peptide binding groove and 9 mers would be required to span the length of this groove (5, 27). It is also known that certain antigenic peptides bind to L^d as 8 mers (14, 15). Based on a putative structure derived from a model of L^d, it has been suggested that 8 mer and 9 mer peptides bind L^d in the same register, with the 8 mer unable to extend to the A pocket (21). These two complexes would appear identical with T cells. Alternatively, it has been proposed that both 8 mers and 9 mers could bind by spanning the entire L^d peptide-binding groove (27). This model suggests that the conformation of L^d/peptide differs depending on the length of the bound peptide. The 9 mer peptide used to obtain the L^d crystal structure possesses the L^d consensus peptide-binding motif. QL9 is a 9 mer but lacks the consensus peptide-binding motif, whereas p2Ca is neither a 9 mer nor does it possess the consensus motif. Thus predictions of how these peptides bind based on the known crystal structure are still speculative.

Several studies have examined how peptides longer than the predicted optimal length bind class I, and recently some studies have addressed how shorter peptides bind (4, 7, 43–48). Comparison of the structure of HLA-A2/Tax8 with HLA-A2/Tax9 found
them to be remarkably similar with water molecules substituting for some of the peptide interactions of the 8 mer in the binding site (46). However, loss of two hydrogen bonds to the N-terminally modified peptide resulted in the large decrease in stability of the A2/Tax8 complex. This, combined with reduced affinity of the TCR for the HLA-A2/Tax8 complex as compared with the HLA-A2/Tax9 complex, leads to decreased effectiveness of the A2/Tax8 complex as a ligand for HLA-A2/Tax9-specific TCR. These results are in contrast to our study in which we have identified T cells that preferentially recognize the 8 mer, p2Ca, that is less stable than the 9 mer, QL9, when bound to L$^d$. In addition, we have demonstrated that the 2C T cell clone that recognizes both 8 mer and 9 mer ligands recognizes distinct amino acid residues on the two peptides.

In another study the structures of variants of a 9 mer influenza-derived peptide bound to HLA-A2 demonstrated the importance of interactions at the peptide termini for MHC/peptide stability (49). In addition to affecting the stability of the complexes, substitution of the N- and C-terminal residues with a methyl group revealed some unanticipated conformational changes both in central peptide residues and in the MHC. Thus, modification of anchor residues can alter overall peptide conformation, providing additional importance to the hydrogen bonds at the peptide termini. These conformational modifications were not predicted by thermodynamic data and were only revealed by crystal structure analysis. By analogy, if the p2Ca 8 mer is lacking interaction at the N terminus, this could result in conformational arrangements different from the QL9 9 mer that possesses N-terminal hydrogen bond interactions (Table I).

A study comparing the crystal structure of HLA-B*3501 occupied with an 8 mer peptide to different class I alleles occupied with 9 mer and 10 mer peptides provides support for distinct conformations between class I/8 mer vs class I/9 mer complexes (7). These comparisons revealed differences in the N- and C-terminal regions of the peptide, a distinct conformation of the peptide in the groove, and a peptide-dependent alteration in the position of the N-terminal segment of the MHC α2 helix. Although the authors did not compare binding of 8 mer and 9 mer peptide variants to the same class I molecule, these results strongly support a model in which peptide length variants when bound to class I assume distinct conformations.

Earlier reports in class II systems suggested that a peptide could bind in more than one configuration to the same class II MHC molecule (50, 51). Recent evidence suggests that overlapping peptides within identified, longer peptide epitopes can bind MHC class II in different registers, presenting different conformations to T cells (52, 53). A similar observation has not been made for class I MHC, for which epitope variants have been identified as core 8–9 mer peptides. The structural differences between the class I vs class II MHC peptide-binding grooves likely preclude a similar phenomenon occurring for class I. The class II binding groove is open at both ends, and known peptide epitopes appear to possess degenerate anchor residues (10). Thus, the class II MHC peptide-binding groove likely permits flexibility in peptide binding, as has been demonstrated (52, 53). In contrast, the class I MHC peptide-binding cleft is closed at both ends and the length of the cleft is virtually identical in all class I molecules (2–8, 27). In all the MHC/peptide crystal structures solved thus far, including L$^d$, the carboxy-terminal peptide position is an anchor residue deeply buried in the F pocket. The alanine substitution data for p2Ca (Ref. 41 and present study) and QL9 (present study) demonstrate that the carboxy-terminal leucine serves as an anchor residue for both the 8 mer and the 9 mer, and thus both peptides are anchored in the F pocket.

Therefore, the two peptides are not bound in different registers but rather assume distinct conformations within the MHC cleft.

In conclusion, the results described here provide the first functional evidence that the two related peptides, p2Ca and QL9, assume different conformations when bound to L$^d$. Furthermore, these results suggest that there is flexibility in how the peptide binds within the groove such that a slight variation in peptide, such as length, can have a profound impact on TCR recognition.

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References


