**Contribution of Individual Amino Acids Within MHC Molecule or Antigenic Peptide to TCR Ligand Potency**

Bernhard Hemmer, Clemencia Pinilla, Bruno Gran, Marco Vergelli, Nick Ling, Paul Conlon, Henry F. McFarland, Richard Houghten and Roland Martin

The TCR recognition of peptides bound to MHC class II molecules is highly flexible in some T cells. Although progress has been made in understanding the interactions within the trimolecular complex, to what extent the individual components and their amino acid composition contribute to ligand recognition by individual T cells is not completely understood. We investigated how single amino acid residues influence Ag recognition of T cells by combining several experimental approaches. We defined TCR motifs for CD4\(^+\) T cells using peptide synthetic combinatorial libraries in the positional scanning format (PS-SCL) and single amino acid-modified peptide analogues. The similarity of the TCR motifs defined by both methods and the identification of stimulatory antigenic peptides by the PS-SCL approach argue for a contribution of each amino acid residue to the overall potency of the antigenic peptide ligand. In some instances, however, motifs are formed by adjacent amino acids, and their combined influence is superimposed on the overall contribution of each amino acid within the peptide epitope. In contrast to the flexibility of the TCR to interact with different peptides, recognition was very sensitive toward modifications of the MHC-restriction element. Exchanges of just one amino acid of the MHC molecule drastically reduced the number of peptides recognized. The results indicate that a specific MHC molecule not only selects certain peptides, but also is crucial for setting an affinity threshold for TCR recognition, which determines the flexibility in peptide recognition for a given TCR. *The Journal of Immunology, 2000, 164: 861–871.*

D4\(^+\) T cells recognize short peptides bound to MHC class II molecules. This interaction was initially considered highly specific and limited to a few peptides with similar or closely related sequences. Recent observations have challenged this view and demonstrated that recognition of MHC-peptide complexes by the TCR is highly flexible (1, 2). Accordingly, it is assumed that degeneracy in Ag recognition is important for thymic selection of the T cell repertoire, since a broad spectrum of T cell specificities can be selected on a limited number of self-MHC-self-peptide complexes (3). It is also possible that flexibility of the TCR is crucial for peripheral survival of mature T cells. The observation that T cells require a continuous signal through their Ag receptor to stay alive (4) has promoted the idea that individual TCRs may interact with a wide range of self-MHC-self-peptide complexes and that the latter provide a survival signal. Therefore, positive selection in the thymus and survival of T cells in the periphery may be the result of low-affinity interactions of the TCR with self-MHC-self-peptide complexes on the basis of degenerate T cell recognition (1, 2). Although low potency stimulation may serve to support survival of peripheral T cells, high potency ligands permit full agonist responses at low Ag concentrations, e.g., during immune responses against invading pathogens.

In addition to the implications of this concept for physiological immune responses, degeneracy in T cell Ag recognition has allowed the application of novel techniques such as peptide positional scanning combinatorial libraries (PS-SCL) to decrypt the interaction of the TCR with its MHC-peptide ligand. A few laboratories, including the authors’, have successfully employed PS-SCL to define interactions within the trimolecular complex. This approach has allowed the identification of binding motifs for several MHC molecules (5, 6) as well as ligands for CD8\(^+\) and CD4\(^+\) T cells (7–15). However, it is not clear to what extent T cell clones (TCC) can be studied by the current technique, or exactly how PS-SCL allow the determination of T cell epitopes as compared with conventional epitope mapping approaches. In addition, the importance of residues within the MHC molecule for recognition has been neglected in the above studies, although PS-SCL provide the ideal tool to investigate these interactions.

Herein, we define in detail the effect of amino acid substitutions to ligand potency in either antigenic peptides or MHC molecules. Human CD4\(^+\) TCC were tested for their response to 1) a decapeptide PS-SCL, 2) a set of single amino acid-modified peptides based on the immunodominant myelin basic protein (MBP) peptide (87–99), and 3) a panel of synthetic peptides identified using the decapeptide PS-SCL. To assess the impact of MHC amino acid residues on T cell recognition by a defined peptide sequence without bias, we measured the response of these TCC to PS-SCL presented by closely related MHC molecules. These studies demonstrate the

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**Abbreviations used in this paper:** PS-SCL, positional scanning synthetic combinatorial library(ies); T cell clone(s), TCC; MBP, myelin basic protein; Ac, acetylated; non-Ac, nonacetylated.
stringency of the TCR in its interaction with the MHC, its flexibility for the peptide ligand, and the possibility to predict peptide ligands for T cells on the basis of their response to PS-SCL. The potency of these Ags is defined by the contribution of each amino acid within the peptide sequence combined with the influence of particular motifs formed by adjacent amino acids.

Materials and Methods

Library and peptide synthesis

A decapeptide PS-SCL was prepared as first presented elsewhere (16, 17) using the simultaneous multiple peptide synthesis approach (18), methylbenzhydrylamidine polystyrene resin, and t-Boc-protected L amino acids. Peptide mixture resins were prepared using a predetermined ratio of 19 of the 20 L natural amino acids (except cysteine) (19). Mixture resins used as equimolar standards for amino acid analysis were prepared by the divide, couple, and recombine method (20). The cleavage and extraction of the mixtures from the resin were conducted as described previously (17). The solutions were lyophilized and resuspended in water at 10 mg/ml. Individual peptides were synthesized either by simultaneous multiple peptide synthesis (18), F-moc-, or Merrifield’s solid phase technology as described (21). The purity and identity of each peptide were characterized by reversed-phase HPLC and matrix-assisted laser desorption/ionization-time-of-flight mass spectrometry.

Each of the 10 positional peptide libraries making up this decapeptide PS-SCL is composed of 20 peptide mixtures, in which a single position is defined with 1 of the 20 natural L amino acids (represented as O), whereas the remaining nine positions of the 10-residue sequence are composed of mixtures (represented as X) of 19 amino acids (cysteine omitted). The 10 positional peptide libraries have N-terminal acetyl and C-terminal amide groups. Each positional library contains the same diversity of peptide sequences; they differ only in the location of their defined position. Theoretically, each mixture is made up of $3.22 \times 10^{11}$ (19) individual peptides. Assuming an average m.w. of 1200 for a peptide mixture and a concentration of 10 mg/ml, the concentration of each individual decapeptide is 25.8 femtomolar. The PS-SCL can be represented as follows: acetylated (Ac-XXXO4XXXXXX-NH2), Ac-XXXXO5XXXXX-NH2, Ac-XXXXO6XXXXX-NH2, Ac-XXXXO7XXXXX-NH2, Ac-XXXXO8XXXXX-NH2, Ac-XXXXO9XXXXX-NH2, Ac-XXXXO10XXXXX-NH2, Ac-XXXXO11XXXXX-NH2, Ac-XXXXO12XXXXX-NH2, Ac-XXXXO13XXXXX-NH2, Ac-XXXXO14XXXXX-NH2, Ac-XXXXO15XXXXX-NH2, Ac-XXXXO16XXXXX-NH2, Ac-XXXXO17XXXXX-NH2, Ac-XXXXO18XXXXX-NH2, Ac-XXXXO19XXXXX-NH2, Ac-XXXXO20XXXXX-NH2.
The single letter code for amino acids is used throughout this manuscript.

TCC and APCs

TCC GDBP, TL3A6, and TL5F6 were established from peripheral blood lymphocytes by a limiting dilution split-well technique or from long-term T cell lines stimulated with MBP and characterized as described before (22). TCC TL3A6 is restricted by DR2a (DRα + DRB5*0101), GDBP by DR6 (DRα + DRB1*1302), and TCC TL5F6 by DR2b (DRα + DRB1*1501). The TCC are specific for MBP (87–99). Cloneability was demonstrated for all TCC by RT-PCR and/or monoclonal TCRV family-specific Abs. TCR usage is TCRBV5S1 for TL3A6, TCRBV21S3 for GDBP, and TCRBV6S2 for TL5F6.

PBMC were used as APC for proliferation assays. The PBMC were derived from leukocytapheresis of human donors after Ficoll separation. HLA typing was done by sequence-specific oligonucleotides and kindly performed by the Department of Transfusion Medicine, National Institutes of Health (Bethesda, MD). Autologous or allogeneic PBMC from different donors and matched for single HLA-DR alleles were used. To define the importance of the MHC molecule for recognition, additional donors with other than those in question, each experiment was performed with PBMC from at least two different donors who expressed the desired MHC molecule but did not overlap in the others.

T cell proliferation

TCC were rested for 8–12 days, washed, and resuspended at 2.5 $\times 10^5$ cells/ml in complete medium (IMDM containing 5% human serum, 1% penicillin/streptomycin, and 0.2% gentamicin). A total of 100 $\mu$g of this cell suspension was added to each well of 96-well U-bottom plates containing $1 \times 10^5$ irradiated (3000 rad) PBMC and varying concentrations of mixtures of peptide PS-SCL or individual peptides. Cells were incubated for 48 h at 37°C. During the last 8 h of culture, 1 $\mu$Ci of $[^3]H$thymidine was added to each well. Cells were then harvested and incorporated radioactivity was measured by scintillation counting. The proliferative response to deduced peptides was measured using conditions described above with various dilutions of peptides. The peptide concentrations inducing half-maximal (EC50) or 20% maximal proliferative response (EC100) were determined by curve fitting using the program GraphPad Prism (GraphPad, San Diego, CA).

Results

CD4+ TCC respond differentially to peptide mixtures

To address whether T cells differ in the degeneracy of their TCRs, we tested several human Ag-specific TCC for their response to various concentrations of a 10-amino acid long N-terminal Ac peptide mixture (X10). The peptide mixture contained $6.12 \times 10^{12}$ different decapeptides. Although the TCC responded similarly to their nominal Ag MBP(87–99) (Fig. 1A, left panel), the response to the X10 peptide mixture differed (Fig. 1A, right panel). Approximately 80–90% of MBP-specific TCC did not respond at all to any concentration of the X10 mixture tested or showed only weak responses at the highest concentrations. However, some TCC responded at concentrations as low as 1 $\mu$g/ml of the peptide mixture (Fig. 1A, TCC TL5F6). At this concentration, each individual peptide in the mixture is present at a concentration of 0.00258 femtomolar. The differential response of various TCC to the complex peptide mixture demonstrates variation in the extent of peptide recognition. This suggests that 1) the response of TCC to peptide mixtures is not predictable by the response to their nominal Ag, and 2) the extent of degeneracy in T cell Ag recognition may differ among these TCC.

To study length requirements for recognition, TCC TL5F6 was then tested for its response to two sets [differing in their N terminus: one being Ac and the other nonacylated (non-Ac)] of sizing mixtures ranging from tripeptides to decapeptides (X3–X10). As shown in Fig. 1B, TCC TL5F6 showed no response to Ac X3–X7, a weak response to Ac X8, and a strong response to Ac X9 and X10. More than 10-fold higher concentrations of the mixture were required for similar responses when the non-Ac mixtures were used. The N-terminal acetylation of the peptide mixtures may enhance recognition in at least some TCC, most likely owing to better binding of the peptides to the MHC molecule (23). Therefore, an Ac decapeptide PS-SCL was used for further studies.

Positional scanning peptide combinatorial libraries and peptide analogues identify optimal amino acids for the different positions of the TCR epitope

Two of the TCC, one responding to a low concentration of the X10 mixture (TL5F6) and one giving no response (TL3A6), were selected for further studies. The TCC were tested for their proliferative response to an Ac decapeptide, PS-SCL.

The TCC were tested in parallel for their responses to a set of single amino acid-modified peptide analogues of MBP (87–99). The results obtained with these two methods were compared for different positions of the epitope. The positions P1–P10 of the PS-SCL correspond to the sequence MBP(89–98) for TCC TL3A6. As shown in Fig. 2A for the DR2a-restricted TCC TL3A6 using single amino acid-modified peptides, only K was tolerated in position 91 (upper panel). In the corresponding position P3 of the PS-SCL, the only (but highly significant) response was observed for the mixture having K defined at this position. In contrast, several amino acids were tolerated in position 92 as shown by single amino acid-substituted peptides. Although the natural MBP peptide carries the amino acid N in position 92, the substituting L, S, and A in this position resulted in more potent peptides (Fig. 2A, middle panel). Similarly, in P4 the response to PS-SCL mixtures having aliphatic, aromatic, or small amino acids L, I, Y, A, and S defined elicited stronger responses. In contrast, the mixture having...
N in P4 did not elicit any positive response. In position 97, the positively charged amino acids K and R were tolerated, although K improved recognition compared with R in the native sequence (Fig. 2A, lower panel). The mixtures of the PS-SCL with K and R in P9 were recognized, with the mixture with K giving a stronger response than the mixture having R. Interestingly, the number of tolerated amino acids in a given position inversely correlated with the magnitude of the response to the mixtures with defined amino acids in this position of the PS-SCL (i.e., the only tolerated amino acid K in P3 induced a much stronger response than L, S, and I in P4; Fig. 2A).

Similar responses were observed with TCC TL5F6. However, in contrast to TCC TL3A6, the TCC responded very strongly to the PS-SCL. In most instances, one tolerated amino acid was found to elicit a response in up to three adjacent positions (strong responses in the first two positions and weak responses in the third position) similar to previous observations (11). This phenomenon is likely due to binding of the peptide to the MHC in different registers (“sliding effects”). The sliding effects had to be taken into account when analyzing these data (Fig. 2B). In this case, whenever a specific amino acid elicited a response in two adjacent positions, we considered it optimal only for the first position. Accordingly, when a strong response to a specific amino acid was observed in three adjacent positions, the amino acid was considered to be optimal in the first two positions. Similar to TCC TL3A6, a strong correlation was observed when comparing the results for TCC TL5F6 obtained with the PS-SCL and peptide analogues of MBP(87–99). Unlike TCC TL3A6, the TCC responded very strongly to the PS-SCL. In most instances, one tolerated amino acid was found to elicit a response in up to three adjacent positions (strong responses in the first two positions and weak responses in the third position) similar to previous observations (11). This phenomenon is likely due to binding of the peptide to the MHC in different registers (“sliding effects”). The sliding effects had to be taken into account when analyzing these data (Fig. 2B). In this case, whenever a specific amino acid elicited a response in two adjacent positions, we considered it optimal only for the first position. Accordingly, when a strong response to a specific amino acid was observed in three adjacent positions, the amino acid was considered to be optimal in the first two positions. Similar to TCC TL3A6, a strong correlation was observed when comparing the results for TCC TL5F6 obtained with the PS-SCL and peptide analogues of MBP(87–99). Unlike TCC TL3A6, the TCC responded very strongly to the PS-SCL. In most instances, one tolerated amino acid was found to elicit a response in up to three adjacent positions (strong responses in the first two positions and weak responses in the third position) similar to previous observations (11). This phenomenon is likely due to binding of the peptide to the MHC in different registers (“sliding effects”). The sliding effects had to be taken into account when analyzing these data (Fig. 2B). In this case, whenever a specific amino acid elicited a response in two adjacent positions, we considered it optimal only for the first position. Accordingly, when a strong response to a specific amino acid was observed in three adjacent positions, the amino acid was considered to be optimal in the first two positions. Similarly, mixtures defined with T and K elicited a response in P8 but not the T and K modifications in position 96. These findings might be a result of different peptides in these peptide mixtures.
bind in a different frame to the MHC-binding groove, since strongest responses were found when K was present in P3 (adjacent to P2 and P4), T in P7 (adjacent to P6 and P8), and K in P9 (adjacent to P8). Since the response of the TCC was much weaker compared with TCC TL5F6, it is possible that these effects with weak responses in positions 2 and 1 are only seen with highly discriminative amino acids, such as K in P3, T in P7, and K in P9.

Although the response of TL5F6 to the PS-SCL was much stronger, it was also more difficult to analyze, since tolerated amino acids elicited responses in two to three adjacent positions. In addition, TCC TL5F6 showed a slightly weaker response to MBP(87–99) peptide (Fig. 2), possibly due to its low binding capacity to HLA-DRB1*1501 (25), thus allowing a slightly less precise discrimination of the response to the peptide analogues. Nevertheless, the results obtained by both approaches matched in positions P3/89–P9/95 (Figs. 2B and 4A). Although few substituted peptides were available for position 87, a correlation was found also in P1/V87. The data did not match for single amino acids in position P2/H88 (responses were best with E and Q in position 88 of the peptide analogues but mixtures with those amino acids defined did not give the strongest responses with the PS-SCL approach). Similarly, for P10/P96, responses were best with Q, G,
and T in position 96 of the peptide analogues but mixtures defined with those amino acids did not give the strongest responses. This may be due in part to the fact that P10 is the C-terminal amino acid in the decapeptide PS-SCL, whereas in the MBP(87–99) peptide it is followed by three amino acids.

Overall, the PS-SCL data not only matched very well with the data obtained with single amino acid-modified peptides, but also with the known binding motifs for HLA-DR2a and HLA-DR2b (22, 25, 26). The responses of TCC TL3A6 to the PS-SCL demonstrate the importance of aromatic amino acids in pocket 1 (pocket 2), aliphatic residues in pocket 4 (pocket 5), and positively charged amino acids in position 8 (pocket 9) in the DR2a-binding motif. Similarly, the response of TCC TL5F6 to the PS-SCL matched the known peptide-binding motif of DR2b; aliphatic amino acids in position 1 (pocket 1), aromatic amino acids in position 4 (pocket 4), and aliphatic amino acids in position 7 (pocket 7). Based on the results obtained with the decapeptide PS-SCL, recognition motifs were deduced for both TCC (Table I). Only amino acids corresponding to the mixtures that induced a response significantly stronger than the average are displayed and ranked according to their stimulatory potencies for the given position. Responses that were most likely the result of sliding are displayed in brackets.

Influence of MHC structure on Ag recognition

After determining the TCR motifs for peptide recognition of both TCC, we wanted to define the contribution of the MHC molecule to Ag recognition. For this reason, we tested the response of the TCC to the decapeptide PS-SCL and peptides presented by variant MHC molecules. Both TCC did not respond to MBP(87–99) presented by any mismatched HLA-DR molecule, not even by closely related MHC molecules. In addition, TCC TL3A6 and TL5F6 showed no response to any of the mixtures of the PS-SCL when APC were used that expressed HLA-DR molecules with a larger number of differing amino acids in exon 2 of the DRβ-chain, such as HLA-DRB1*0101, even though the MBP peptide binds well to this allele (27) (Table II and data not shown). TCC TL5F6 responded strongly to the PS-SCL in the presence of the autologous class II restriction element DRB1*1501 and was tested for its response to the decapeptide PS-SCL in the presence of closely related HLA-DR molecules (Table II). The difference of one amino acid in position 30 (H for Y in pocket 4, see Table II) of the HLA-DR1*1503 molecule resulted in significant changes in the response of the TCC to the PS-SCL (Fig. 4). None of the mixtures of the PS-SCL in positions 2–7 elicited any response, whereas only mixtures having Q, R, S, T, and V defined in position 1, mixtures defined with V in positions 8 and 9, and T in position 9 induced significant and reproducible responses. This is particularly interesting since the modification affecting pocket 4 had a strong effect on the recognition of positions 2–7 of the decapeptide PS-SCL, whereas residues that directly interacted with pockets 1 and 9 showed the least effect. In the presence of APC expressing HLA DRB1*1601, only the mixtures of the PS-SCL defined with V in positions 8 and 9 were stimulatory for the TCC. The HLA...
FIGURE 4. Comparison of TCR motifs in the context of different HLA molecules. TCC TL5F6 was tested for its proliferative response to a complete decapeptide PS-SCL in the context of HLA-DRB1*1501 (A) and HLA-DRB1*1503 (B). One representative experiment of five is shown. The mean cpm and SD of duplicates are shown.
DRB1*1601 was different from the HLA-DRB1*1501 molecule by four amino acids, three of them influence the peptide-binding pockets [G for V86 (pocket 1), R for A71 (pockets 4 and 7), and Y for F47 (pocket 7); see Table II]. Position 9 of the PS-SCL was the least affected since the response to V was still present. More distant HLA-DR molecules (HLA-DR1, HLA-DR4, and HLA-DR3) failed to stimulate the TCC in combination with any of the mixtures of the decapeptide PS-SCL (Table II and data not shown).

In contrast to TL5F6, TCC TL3A6 did not show any response to the PS-SCL even in the context of HLA-DRB5*0202, a molecule that is related to HLA-DRB5*0101 and differs by 13 amino acids. The results document the importance of the restriction element for the two TCC and indicate the outstanding contribution of the MHC molecule to the overall affinity of the TCR for its MHC-peptide ligand. Although it is theoretically possible that the TCC recognize peptides in the context of other restriction molecules that we did not examine, the observed lack of responses to most mixtures of the PS-SCL in the context of closely related HLA-DR suggests that the natural restriction element is most suitable for TCR activation, whereas MHC mutations in the context of the native peptide or closely related peptides may create weak/partial agonist ligands (28). If TCR recognition is sufficiently degenerate, highly diverse peptide mixtures are sufficient to trigger activation. In the case of MHC molecules differing from the natural restriction element, a much higher contribution of the peptide would be required to allow productive engagement by the TCR. In most instances,

Table I. TCR motifs for TCC TL3A6 and TCC TL5F6

|        | PS-SCL | P1 | P2 | P3 | P4 | P5 | P6 | P7 | P8 | P9 | P10 |
|--------|--------|----|----|----|----|----|----|----|----|----|-----|
| TL3A6  |        | W  | K  | K  | L  | L  | P  | I  | K  | K  | L   |
|        |        | H  | F  | [K]| L  | L  | [I]| [I]| [I]| [I]| ALL |
|        |        | R  | Y  | I  | M  | L  | E  | C  | R  | C   |
|        |        | F  | F  | K  | N  | I  | V  | T  | R  | T   |
|        | MBP(89-96) | 89 | 90 | 91 | 92 | 93 | 94 | 95 | 96 | 97 | 98  |
|        |        | F  | F  | K  | N  | I  | V  | T  | R  | T   |

* The amino acids corresponding to the most active mixtures at each position of the epitope were defined by the proliferative response of the TCC to the decapeptide PS-SCL. The corresponding positions of the MBP epitope are displayed in gray shaded boxes. Responses to amino acids most likely due to sliding effects are displayed in brackets. MHC-binding residues are indicated by empty boxes. Amino acids that were used to deduce peptides are underlined.

Table II. Amino acid differences of HLA-DR molecules and their influence on T cell activation

| DRB Residue | Pocket | DRB1*1501 | DRB1*1503 | DRB1*1601 | DRB1*0101 |
|-------------|--------|-----------|-----------|-----------|-----------|
| 11          | P6     | P         |           |           |           |
| 13          | P6, P4 | R         |           |           |           |
| 26          | F      |           | L         |           |           |
| 28          | D      |           |           | E         |           |
| 30          | P4     | Y         |           | H         |           |
| 33          | N      |           |           |           |           |
| 37          | S      |           |           |           |           |
| 47          | P7     | F         |           |           |           |
| 57          | P7, P4 | A         |           | R         |           |
| 67          | I      |           | F         |           | L         |
| 71          | P1     | V         |           | G         |           |
| 96          | Q      |           |           | E         |           |
| 133         | L      |           |           | R         |           |
| 142         | M      |           |           |           | V         |

Affected pockets | None | P4 | P1, P4, P7 | P1, P4, P7 | None |
T cell response of TCC to PS-SCL | All positions | P1, P8, P9 | P9 | None |

* Shown are differences in the amino acid composition of four HLA-DR molecules that were used in the T cell assays. The pockets that were affected by amino acid exchanges are marked for each individual HLA-DR molecule (reference molecule, HLA-DRB1*1501). TCC TL5F6 was tested for its response to PS-SCL presented by different HLA-DR molecules. Observed responses of the TCC are displayed for the different positions.
**Table III. Proliferative response of TCC TL3A6 to deduced peptides**

| No. | Sequence | EC<sub>50</sub>   | No. | Sequence | EC<sub>50</sub>   |
|-----|----------|-----------------|-----|----------|-----------------|
| 37  | Ac-FFKN1VTPT-NH2 | 0.034 | 19  | Ac-WFKLIPTKKL-NH2 | 0.0017 |
| 1   | Ac-WKKLIPPTKL-NH2 | >100  | 20  | Ac-WFKLIPTKKG-NH2 | 0.0037 |
| 2   | Ac-WKKLIPPTKL-NH2 | 7     | 21  | Ac-WFKLIPTTTK-NH2 | 0.0028 |
| 3   | Ac-WKKLIPPTKL-NH2 | >100  | 22  | Ac-WFKLIPTTKG-NH2 | 0.085  |
| 4   | Ac-WKKLIPPTKL-NH2 | 10    | 23  | Ac-WFKLIPTTKL-NH2 | 0.0018 |
| 5   | Ac-WKKLIPPTKL-NH2 | >100  | 24  | Ac-WFKLIPTTKG-NH2 | 0.31   |
| 6   | Ac-WKKLIPPTKL-NH2 | 88    | 25  | Ac-WFKLIPTTTK-NH2 | 0.0014 |
| 7   | Ac-WKKLITPTKL-NH2 | 26    | 26  | Ac-WFKLIPTTTK-NH2 | 0.0026 |
| 8   | Ac-WKKLITPTKL-NH2 | 2     | 27  | Ac-WFKLIPTTTK-NH2 | 0.000034 |
| 9   | Ac-WKKLITPTKL-NH2 | 66    | 28  | Ac-WFKLIPTTTG-NH2 | 0.0052 |
| 10  | Ac-WKKLITTTK-NH2 | 3     | 29  | Ac-WFKLIPTTPK-NH2 | 0.0012 |
| 11  | Ac-WKKLITTTK-NH2 | >100  | 30  | Ac-WFKLIPTTPK-NH2 | 0.0012 |
| 12  | Ac-WKKLITTTK-NH2 | 8     | 31  | Ac-WFKLIPTTTLK-NH2 | 0.6486 |
| 13  | Ac-WKKLITTTK-NH2 | 12    | 32  | Ac-WFKLIPTTTK-NH2 | 0.00013 |
| 14  | Ac-WKKLITTTK-NH2 | 5     | 33  | Ac-WFKLITTTKL-NH2 | 0.0013 |
| 15  | Ac-WKKLITTTK-NH2 | 7     | 34  | Ac-WFKLITTTG-NH2 | 0.0033 |
| 16  | Ac-WKKLITTTK-NH2 | 3     | 35  | Ac-WFKLITTPK-NH2 | 0.0006 |
| 17  | Ac-WKKLITTTK-NH2 | 108   | 36  | Ac-WFKLITTPK-NH2 | 0.0037 |
| 18  | Ac-WKKLITTTK-NH2 | 174   | 157 | Ac-YVKQNTLKL-NH2 | >100   |

*Proliferative responses of TCC TL3A6 to deduced peptides are shown. The responses are displayed as EC<sub>50</sub> (μ/ml determined by regression analysis (antigen concentration required to reach 50% of maximum cpm obtained after stimulation with MBP(87–99)). Representative results of one of five experiments are shown.

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this threshold would not be surpassed by the peptide concentrations present in the mixtures of the PS-SCL.

**Deduction of peptides**

Recent studies have demonstrated that the PS-SCL approach allows the identification of optimal ligands for a given TCC. However, few systematic studies have been conducted to determine whether the stimulatory potency of the deduced peptide is predictable and strictly dependent on one TCR motif, or whether it is similar to Ab recognition, where several nonidentical motifs may be recognized. To address these questions, we used the response of TCC TL3A6 to the PS-SCL to systematically deduce 36 Ac decapeptides (selected amino acids are underlined in Table I). TCC TL3A6 was tested in proliferation assays for its response to the deduced peptides. As shown in Table III, most peptides that carried an F at position 2 exerted stronger responses than MBP(87–99) (peptides 19–36). In contrast, the same peptides with only one change (F to K in position 2) were only weakly or not stimulatory for the TCC (peptides 1–18). Most peptides that carried L in position 10 were more active than peptides carrying a G in position 10 (Table IV). However, the peptide that carried L in P6, K in P8 and P9, and G in P10 (peptide 32) was more active than the same peptide that carried L in P10 (Table IV, peptide 31). The situation was more complex in position P8. Three peptides that carried K in P8 were more active than the peptides that carried T or P in this position. Two of them had the -----P<sub>8</sub>---(L<sub>10</sub>/G<sub>10</sub>) motif (peptides 19 and 20) and one the -----T<sub>8</sub>---G<sub>10</sub> motif (peptide 32, numbers in subscript represent the position of the amino acid within the sequence). In contrast, when a -----T<sub>8</sub>---(L<sub>10</sub>/G<sub>10</sub>) motif was present, the responses were more complex for the TCC (peptides 1–18).

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**Table IV. Synergistic effects of different optimal amino acids within the peptide sequence**

| No. | Sequence | EC<sub>50</sub>   | No. | Sequence | EC<sub>50</sub>   |
|-----|----------|-----------------|-----|----------|-----------------|
| 19  | Ac-WFKLIPTTKL-NH2 | 0.00017 | 19  | Ac-WFKLIPTTKL-NH2 | 0.00017 |
| 21  | Ac-WFKLIPTTKL-NH2 | 0.0028 | 25  | Ac-WFKLIPTTKL-NH2 | 0.0014 |
| 23  | Ac-WFKLIPTTKL-NH2 | 0.0018 | 31  | Ac-WFKLIPTTKL-NH2 | 0.6488 |
| 25  | Ac-WFKLIPTTKL-NH2 | 0.0014 | 21  | Ac-WFKLIPTTKL-NH2 | 0.0026 |
| 27  | Ac-WFKLIPTTKL-NH2 | 0.000034 | 27  | Ac-WFKLIPTTKL-NH2 | 0.000034 |
| 29  | Ac-WFKLIPTTKL-NH2 | 0.00012 | 33  | Ac-WFKLIPTTKL-NH2 | 0.0013 |
| 31  | Ac-WFKLIPTTKL-NH2 | 0.6488 | 23  | Ac-WFKLIPTTKL-NH2 | 0.0018 |
| 33  | Ac-WFKLIPTTKL-NH2 | 0.0013 | 29  | Ac-WFKLIPTTKL-NH2 | 0.00012 |
| 35  | Ac-WFKLIPTTKL-NH2 | 0.0006 | 35  | Ac-WFKLIPTTKL-NH2 | 0.0006 |
| 20  | Ac-WFKLIPTTKG-NH2 | 0.0037 | 20  | Ac-WFKLIPTTKG-NH2 | 0.0037 |
| 22  | Ac-WFKLIPTTKG-NH2 | 0.085  | 26  | Ac-WFKLIPTTKG-NH2 | 0.0026 |
| 24  | Ac-WFKLIPTTKG-NH2 | 0.31   | 28  | Ac-WFKLIPTTKG-NH2 | 0.00013 |
| 26  | Ac-WFKLIPTTKG-NH2 | 0.0026 | 22  | Ac-WFKLIPTTKG-NH2 | 0.085  |
| 28  | Ac-WFKLIPTTKG-NH2 | 0.00052 | 28  | Ac-WFKLIPTTKG-NH2 | 0.00052 |
| 30  | Ac-WFKLIPTTKG-NH2 | 0.0012 | 34  | Ac-WFKLIPTTKG-NH2 | 0.0033 |
| 32  | Ac-WFKLIPTTKG-NH2 | 0.0013 | 24  | Ac-WFKLIPTTKG-NH2 | 0.31   |
| 34  | Ac-WFKLIPTTKG-NH2 | 0.0033 | 30  | Ac-WFKLIPTTKG-NH2 | 0.0012 |
| 36  | Ac-WFKLIPTTKG-NH2 | 0.037  | 36  | Ac-WFKLIPTTKG-NH2 | 0.037  |

*Proliferative responses of TCC TL3A6 to various deduced peptides are shown (EC<sub>50</sub>). The peptides are arranged to demonstrate the dependency of peptide potency on certain amino acid combinations within the sequence. The amino acids that are used to group the sequences are shown in bold, and the amino acids that vary among the groups are underlined.
T (peptides 27 and 28) was much more active in P8 than K and P (peptides 25, 26, 29, and 30). When the \(-L_6--L_{10}\) motif was present, P in P8 (peptide 35) was the most active amino acid. Similar observations were obtained in P6. In the case of the \(-T_{9}--\)

\(-----P_{9}--\) motif, T was the most active amino acid in P6 (peptides 27, 28, 29, and 30). When the \(-K_9--L_{10}\) motif was present, P was most active in P6 (peptide 19), and in the \(-K_9--G_{10}\) motif, L was most active in P6 (peptide 32).

The identification of highly active peptides demonstrates the importance of the deconvolution strategy used in this and previous studies on T cell specificity. With the positional scanning approach, data derived from library scans are used to synthesize peptide sequences representing the combinations of the most active amino acids (10, 11, 29) rather than the approach of using library scan data to alter the native ligand in the search for more active ligands (8).

Peptides deduced from the results with the decapetide PS-SCL are recognized by the TCC, and many of them have much higher potencies than MBP(87–99), an observation in agreement with our previous studies (11, 30). However, among the deduced peptides, major differences in ligand potency were detected. Interestingly, for a given position, not all of the amino acids had the same effects on peptide potency. In a variety of instances, certain combinations of amino acids within the peptide sequence had positive or negative influences on ligand potency that were not entirely predicted based on their independent contributions.

Discussion

The concepts of how T cells recognize peptide Ags have evolved rapidly during recent years (31–34). Although TCR recognition was initially believed to be highly specific for a single or few MHC-peptide complexes, it is now, considered highly flexible or degenerate, at least in some instances (1, 2, 35). However, major potency differences are found among different MHC-peptide ligands recognized by the same TCR. The potency of the antigenic peptides is related to many factors, including its binding affinity to the MHC, the affinity of the TCR for the resulting MHC-peptide complex, and the off-rate of the complex from the TCR (34, 36). The stimulatory potency of peptides for a given TCR ranges from those that efficiently activate T cells in the picomolar range to those that fail to stimulate T cells even at the highest concentration, but still antagonize T cell activation (1, 37, 38). Although it is still believed that only a few high-affinity ligands will be able to activate and expand T cells under physiological conditions, it has been demonstrated that low-affinity ligands play an important role during thymic selection (39) and survival of T cells in the periphery (4, 40, 41). Significant progress has been made not only in determining the flexibility of the TCR, but also in understanding the physicochemical interactions within the trimolecular complex (42–44). However, it is still not clear how the different residues of peptide and MHC contribute to recognition, i.e., what the relative contributions of both components of the ligand are, although the crystal structures of several class I-restricted TCR MHC-peptide complexes have been elucidated (42–45).

New techniques have been developed to address the above questions on T cell recognition. The use of systematically arranged peptide PS-SCL is particularly promising, since such libraries allow one to dissect the interaction of a TCR with the entire spectrum of potential peptides in an unbiased fashion. In this study, we demonstrate a differential response of various TCC to complex peptide mixtures, despite similar responses to their nominal Ag. The finding that some T cells can recognize mixtures of \(6 \times 10^{12}\) peptides at concentrations as low as 1 \(\mu\)g/ml, while others do not recognize these mixtures even at a 250-fold higher concentration, provides an estimate of the degree of degeneracy of such TCRs. Given the immense diversity and the minute molar concentrations of individual peptide species present in the PS-SCL mixtures, a T cell can only be activated if a high number of different peptides productively engage the TCR (2, 11, 46). In contrast to the apparent tolerance toward mutations of the peptide, a change of one amino acid in the MHC molecule resulted in an almost entire loss of responses to peptide mixtures. This underscores the importance of the MHC in providing structural constraints for Ag recognition. The use of “altered MHC ligands” demonstrates that the exchange of even one amino acid in the MHC dramatically alters the recognition efficacy of the TCR (28). A significant decrease in flexibility, but not necessarily in the recognition pattern, results from MHC-variant stimulation (Table II and Fig. 4). The MHC molecule seems to set an affinity threshold that determines how much the peptide ligand will have to contribute to the interaction to reach the threshold for T cell activation. The MHC contribution may be very low for individual TCRs, and in this case even binding of optimal peptides may not allow recognition of the MHC-peptide complex. At the opposite end of the spectrum, the MHC contribution may be very high, and in this case very little contribution of an exogenous peptide is needed for recognition of the complex (i.e., certain allogeneic T cell responses) (47). The latter situation was recently shown to exist when T cells are selected in the context of low MHC expression (48). T cells that had been positively selected in such an environment exhibited a markedly more flexible specificity repertoire than those selected under conditions of normal MHC expression (48).

It is important to note that the intrinsic MHC reactivity of the TCR germline repertoire is entirely consistent with this finding (49). Using mixtures of peptides (X10) we may directly obtain information about how much the MHC contributes to the overall interaction between TCR and MHC-peptide ligand. The higher the contribution of the MHC, the higher the number of peptides that will be recognized by the TCR. The resulting response to mixtures within the peptide combinatorial libraries will consequently be strong. In contrast, if a T cell fails to respond to peptide mixtures, we hypothesize that its TCR is less flexible due to a lower affinity contribution of the MHC. In the latter situation, only a few peptides may be able to more specifically increase the overall avidity beyond the threshold of activation.

Independent of the relative contributions by the MHC molecule, a peptide will be needed in most instances to surpass the activation threshold; this may be due largely to the requirement of a peptide for stabilization of the HLA-DR heterodimer (50). We used two different approaches to dissect the contribution of the peptide, a decapetide PS-SCL and peptide analogues. Both approaches provided similar information on TCR motifs, although a few additional optimal amino acids were discovered from the PS-SCL. High potency peptides were identified for the TCC on the basis of the response to mixtures from the SCL with only one defined amino acid in specific positions. Although all peptides that were identified by this approach were recognized by the TCC, potency differences among the different peptides were not entirely predictable from the response to the PS-SCL. Certain combinations of amino acids synergistically enhanced the potency of the peptide, whereas others did not. This observation indicates that, besides the individual contribution of a single amino acid, certain combinations increase the peptide potency beyond the expected additive effects (51, 52). This finding is similar to recent findings on the interaction of antipeptide (53) or anti-protein Abs (54) and pigeon cytochrome T cell mimics (15). Similar to our data, different recognition motifs were discovered for the Ab, indicating that optimal
peptides can be identified that are not necessarily homologous with respect to the primary amino acid sequence. Based on the methodology employed here, we can only speculate about the underlying physicochemical mechanisms. Most likely, the interface between TCR and MHC-peptide ligand displays a degree of flexibility in vivo that permits interactions in slightly different positions, e.g., an induced fit (42).

In summary, we have demonstrated that Ag recognition by CD4+ T cells is defined by at least three different parameters: 1) the baseline affinity of the TCR for its MHC ligand, 2) the individual contribution of each amino acid residue of the peptide to the overall affinity of the trimolecular complex, and 3) synergistic effects of certain amino acid combinations within the antigenic peptide. These considerations do not take into account TCR-independent mechanisms such as Ag processing or co-receptor-dependent mechanisms.

This study not only sheds new light on the interactions within the TCR-MHC-peptide complex, but also raises intriguing questions about the influence of TCR degeneracy for development and maintenance of the immune system. Furthermore, it will be of interest whether degeneracy in TCR Ag recognition relates to physiological and/or pathological immune responses in T cell-dependent diseases (55). These questions will be the focus of further work.

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