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Role of Peptide Backbone in T Cell Recognition

Sébastien Calbo,* Gilles Guichard,† Philippe Bousso,* Sylviane Muller,† Philippe Kourilsky,* Jean-Paul Briand,† and Jean-Pierre Abastado**

T cells recognize self and nonself peptides presented by molecules of the MHC. Amino acid substitutions in the antigenic peptide showed that T cell specificity is highly degenerate. Recently, determination of the crystal structure of several TCR/MHC-peptide complexes suggested that the peptide backbone may significantly contribute to the interaction with the TCR. To directly investigate the role of the peptide backbone in T cell recognition, we performed a methylene-amino scan on the backbone of an antigenic peptide and measured the capacity of such pseudopeptides to bind their cognate MHC molecule, to sensitize target cells for T cell lysis, and to stimulate IL-2 secretion by two T cell hybridomas. For one of these pseudopeptides, we prepared fluorescent tetramers of MHC molecules and compared the staining of two T cell hybridomas. Our results demonstrate that the peptide backbone has an important contribution to TCR binding and suggest that some interactions between the peptide backbone and the TCR may be partially conserved. We discuss this finding in the perspective of TCR plasticity and T cell function. The Journal of Immunology, 1999, 162: 4657–4662.

Maj or histocompatibility complex I molecules bind short peptides mainly derived from the cytosol (1). Mass spectroscopy analyses revealed that a single MHC molecule expressed on the cell surface can present up to $10^4$ different peptides (2). In addition, protein composition is not random, and proteasomes and TAP transporters are highly selective (3, 4). Therefore, the degeneracy of peptide binding by MHC molecules may be even greater. Crystallography and mutagenesis studies showed that this woolly specificity is facilitated through conserved interactions between nonpolymorphic residues of the MHC and conserved structures of the peptide, such as peptide ends and backbone (5–8). In a previous study, we directly demonstrated the importance of the peptide backbone in MHC binding by introducing defined alterations in the different peptide bonds of a H-2Kd binding peptide PbCS derived from Plasmodium berghei (9).

T cell recognition is also degenerate. Most T cells develop in the thymus, where they must recognize self peptides to be positively selected (10–12). Once in the periphery, T cells must interact with self MHC/peptide complexes to survive and with self MHC loaded with foreign pathogenic peptides to expand and differentiate into effector or memory cells (13). MHC/peptide interaction with TCR is therefore inherently promiscuous. The recent determination of the crystal structures of several TCR/MHC-peptide complexes shed some light on the structural basis of this plasticity (14–18). In all complexes analyzed so far, a similar angle between the TCR and the MHC-peptide was found, suggesting that conserved interactions may contribute to TCR degeneracy. The precise role of the peptide backbone in the interaction with the TCR has been more controversial. Only three structures displayed a sufficient resolution to identify the atomic contacts with certainty. Interestingly, two of them (14, 15) revealed a significant number of conserved contacts between the TCR and the peptide backbone, especially with the carbonyl groups. However, these two studies bore on the same MHC-peptide complex. In a third unrelated complex, the TCR only contacted the distal tips of the up-facing side chains of the peptide (17).

To directly assess the contribution of the peptide backbone to the interaction with the TCR and its potential importance in TCR plasticity, we performed a methylene-amino scan. Starting from the H-2Kd-restricted peptide, CW3, derived from HLA-Cw3, we systematically replaced one peptide bond at a time by a reduced peptide bond $\psi$(CH2-NH). We measured the capacity of the resulting analogues to bind H-2Kd and tested their recognition by one T cell clone and two T cell hybridomas raised against the parent peptide. Our results provide direct evidence for a large contribution of the peptide backbone to TCR recognition.

Materials and Methods

Peptide and peptide analogues

The CW3 and S9I peptides have been previously described (19) and were purchased from NeoSystem (Strasbourg, France). Reduced peptide bond analogues were synthesized by the stepwise solid-phase methodology on a multichannel peptide synthesizer as previously described (9, 20).

Peptide binding assay

Soluble single chain Kd (SC-Kd)3 was purified by affinity chromatography from transfected chinese hamster ovary cells, as previously described (21). Peptide binding to SC-Kd was determined as previously described by competition with the radiolabeled S9I peptide (9, 22). The concentration leading to 50% inhibition binding of S9I (IC50) was used to calculate the relative affinity following the Cheng and Prussoff relationship (23).

Cells

The CAS 1 CTL clone and the 9.4 T hybridoma, which are specific for CW3 and restricted by Kd, were previously described (24, 25). P815 cells (H-2d; TIB64, American Type Culture Collection (ATCC), Manassas, VA)

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3 Abbreviations used in this paper: SC-Kd, single chain Kd; MFI, mean fluorescence intensity.
were maintained in RPMI 1640 medium supplemented with 10% FCS. HIP was produced by fusion between CAS 1 and the 58 α β thymoma (26).

**Cytolytic assay**

Lysis of P815 cells loaded with different peptides or pseudopeptides was measured in a 4-h 51Cr chromate release assay as described (27). The percentage of specific lysis was calculated by dividing the concentration of CW3 required for half-maximal lysis by that required for the pseudopeptide. The relative antigenic activities of the compounds were normalized by dividing the relative antigenic activity by the corresponding relative affinity for SC-Kd, as previously described (28). The normalized relative antigenic activity of CW3 was defined as 1.

**T cell hybridoma stimulation**

T cells (10^5) and peptide loaded P815 (10^5) were cultured in 200 μl of RPMI 1640 medium supplemented with 10% FCS. Culture supernatant was collected at 24 h, and IL-2 secretion was monitored by its capacity to sustain the proliferation of the IL-2-dependent cell line CTLL-2 (TIB214, ATCC).

**Staining with Kd tetramers**

Kd heavy chain and human β2-microglobulin were prepared from bacterial inclusion bodies, as previously described, and refolded by dialysis in the presence of CW3 or C7 (29–31). Complexes were purified by gel filtration on Superdex 200 HR 10/30 run by an Akta purifier 10 using the Unicorn 3.0 software (Pharmacia-Biotech, Uppsala, Sweden). Complexes were enzymatically biotinylated and tetramerized by incubation with phycoerythrin-labeled Ultravidin (Leinco Technologies, Ballwin, MI). T cells (10^6) were incubated for 2 h at 4°C with labeled tetramers, washed, and analyzed by flow cytometry on a FACScan using CellQuest software (Becton Dickinson, San Jose, CA). TCR expression was monitored using phycoerythrin-labeled Ultravidin (Leinco Technologies, Ballwin, MI). T cells (10^6) were incubated for 2 h at 4°C with labeled tetramers, washed, and analyzed by flow cytometry on a FACScan using CellQuest software (Becton Dickinson, San Jose, CA). TCR expression was monitored using phycoerythrin-labeled Ultravidin (Leinco Technologies, Ballwin, MI).

**Results**

**Synthetic pseudopeptides**

Table I shows the sequence of the parent CW3 peptide and the structure of nine analogues (C1–C9) with reduced peptide bond. These analogues were synthesized using 9-fluorenylmethyloxycarbonyl (F-moc) chemistry on Wang resin. The reduced peptide bond was incorporated directly on the resin according to Sasaki and Coy (32). The N-F-moc-protected a-amino-aldehydes used in this reductive amination step were found to be less reactive than their corresponding N-Boc derivatives, and longer reaction times (up to 3 h with double coupling) were often required for the reaction to be complete. Stepwise elongation of the peptide chain followed by trifluoroacetic acid cleavage and HPLC purification yielded C1-C9. All final compounds were identified by matrix-assisted laser desorption/ionization mass-spectroscopy (MALDI-MS), and their homogeneity was assessed by analytical reverse phase HPLC.

**Relative affinity of pseudopeptides for Kd**

The parent CW3 peptide and the nine pseudopeptides were compared to determine the effect of the reduced peptide bond on the affinity for Kd. Affinities were measured in a competitive binding assay using purified soluble SC-Kd and the 125I-labeled peptide S9I of known affinity for SC-Kd. The concentration of competing pseudopeptide inhibiting 50% binding of 125I-S9I (IC50) was determined and used to calculate the relative affinity following the Cheng and Prusoff relationship (23).

Two analogues, C6 and C7, had an affinity for Kd equivalent to that of the parent peptide (Fig. 1). The C2, C4, and C5 analogues had an affinity ~25 times lower, while the C1, C3, C8, and C9 analogues had an affinity ~100 times lower than the parent peptide. Thus, the introduction of a reduced amide bond in the N-terminal or in the C-terminal part of the parent peptide CW3 decreased the binding of resulting analogues to SC-Kd, while

### Table 1. Structures of the various pseudopeptides used in this study

<table>
<thead>
<tr>
<th>Pseudopeptides</th>
<th>Structure</th>
</tr>
</thead>
<tbody>
<tr>
<td>CW3</td>
<td>H-Arg^{170} Tyr-Leu-Asn-Gly-Lys-Glu-Thr-Leu^{170}OH</td>
</tr>
<tr>
<td>C1</td>
<td>H-Arg-{(CH2)n}Tyr-Leu-Asn-Gly-Lys-Glu-Thr-Leu-OH</td>
</tr>
<tr>
<td>C2</td>
<td>H-Arg-Tyr-{(CH2)n}Leu-Asn-Gly-Lys-Glu-Thr-Leu-OH</td>
</tr>
<tr>
<td>C3</td>
<td>H-Arg-Tyr-Leu-{(CH2)n}Asn-Gly-Lys-Glu-Thr-Leu-OH</td>
</tr>
<tr>
<td>C4</td>
<td>H-Arg-Tyr-Leu-{(CH2)n}Asn-Gly-Lys-Glu-Thr-Leu-OH</td>
</tr>
<tr>
<td>C5</td>
<td>H-Arg-Tyr-Leu-Asn-{(CH2,n)}Gly-Lys-Glu-Thr-Leu-OH</td>
</tr>
<tr>
<td>C6</td>
<td>H-Arg-Tyr-Leu-Asn-{(CH2,n)}Gly-Lys-Glu-Thr-Leu-OH</td>
</tr>
<tr>
<td>C7</td>
<td>H-Arg-Tyr-Leu-Asn-{(CH2,n)}Gly-Lys-Thr-Leu-OH</td>
</tr>
<tr>
<td>C8</td>
<td>H-Arg-Tyr-Leu-Asn-{(CH2,n)}Gly-Lys-Thr-Leu-OH</td>
</tr>
<tr>
<td>C9</td>
<td>H-Arg-Tyr-Leu-Asn-{(CH2,n)}Gly-Lys-Thr-Leu-OH</td>
</tr>
</tbody>
</table>

**FIGURE 1.** Relative affinity of pseudopeptides for H-2Kd. Increasing concentrations of the different peptides and analogues were compared in a competitive binding assay for SC-Kd with radiolabeled S9I. The concentration yielding 50% inhibition of S9I binding was used to compute relative affinities. The figure shows the ratio of these relative affinities to that of the parent CW3 peptide. Data are the mean of two to five independent experiments for each peptide. The sequence shown on the right is that of the CW3 peptide. Variation of free energy (ΔΔG) between the parent peptide and each pseudopeptide is indicated.
target cells in a 51 Cr chromate release assay with CAS 1, a T cell clone were loaded with increasing concentration of pseudopeptides and used as

increased by 100-fold. For C2, C3, C4, C6, and C9, the efficiency of recognition by CAS 1 was decreased between 4-fold and 100-fold.

Pseudopeptide recognition by two independent T cell hybridomas

The CAS 1 T cell clone expresses the CD8 coreceptor. Consequently, minor alterations in the stability of the TCR/MHC-peptide complex may be blurred by the stabilizing effect of CD8 (33). Therefore, we derived the CD8− T cell hybridoma HIP. Fig. 3 shows the effect of reducing CW3 peptide bonds on HIP recognition and on 9.4, another previously derived T cell hybridoma specific for CW3 but expressing a different TCR. As expected, both hybridomas were more sensitive to peptide bond reduction: C5, C6, C8, and C9 were unable to trigger HIP and 9.4 to secrete IL-2.

In addition, C3 and C7 did not stimulate 9.4. Interestingly, C1, C3, C4, and C7 stimulated HIP 2–3 times more efficiently than the parent CW3.

Binding of tetramers of Kd-C7 and Kd-CW3 to T hybridoma cells.

To directly confirm the differential effect of C7 on HIP and 9.4, we produced fluorescent tetramers of Kd loaded with either CW3 or C7 and stained 9.4 and HIP cells (Fig. 4A). Kd-CW3 tetramers yielded a similar fluorescence signal on both hybridomas (MFI = 16.3 and 13.3). In contrast, while no significant staining was observed with Kd-C7 tetramers on 9.4 (MFI = 4.8), staining of HIP cells with Kd-C7 tetramers resulted in a signal stronger than with Kd-CW3 tetramers (MFI = 36.9). Moreover, when the stability of the staining was assessed at 17°C, binding of Kd-C7 tetramers on HIP (Koff = 0.027·10−3 s−1, and t1/2 = 415 min) was more stable than binding of Kd-CW3 on HIP (Koff = 0.091·10−3 s−1, and t1/2 = 126 min) and than binding of Kd-CW3 tetramers on 9.4 (Koff = 0.075·10−3 s−1, and t1/2 = 153 min). This suggests that a reduced Koff is at least in part responsible for the stronger interaction between the Kd-C7 complex and the HIP TCR (Fig. 4B). These results are in complete agreement with cytotoxicity and IL-2 secretion experiments and further confirm that, depending on the T cell, reducing the seventh peptide bond of CW3 can either enhance or decrease TCR binding.

Discussion

The present study was undertaken to address the role of the peptide backbone in the binding of MHC-peptide complexes to TCR. Most alterations introduced in the peptide backbone of CW3 (7 out of 9 tested) reduced the affinity for Kd by at least 10-fold. This observation confirms the importance of the peptide backbone in MHC binding, as found in our previous study on the PbCS peptide (9) and suggested in several crystallographic studies (6, 34–38). PbCS and CW3 bind to the same MHC molecule, but differ in length, CW3 being one residue longer. Longer peptides has been shown to bulge out in the middle (5). Consistent with this observation, we found that the middle portion of CW3 (altered in C6 and C7) is

FIGURE 2. Pseudopeptide recognition by CAS 1. A and B, P815 cells were loaded with increasing concentration of pseudopeptides and used as target cells in a 51Cr chromate release assay with CAS 1, a T cell clone specific for the parent CW3 peptide. E:T cell ratio was 10. Experiments were done in triplicate. C, From the efficiency of lysis (A and B) and the affinity of the pseudopeptide for Kd (Fig. 1), we calculated the normalized relative antigenic activity as indicated in Materials and Methods.

normalized relative antigenic activity

Heteroclitic activity as indicated in Materials and Methods

Pseudopeptide recognition by a CTL clone

Recognition of the different pseudopeptides by CAS 1, a CTL clone specific for the parent CW3 peptide, was assessed in a 51Cr release cytolytic assay using P815 as target cells. As shown in Fig. 2, A and B, half-maximal lysis of P815 cells by CAS 1 CTL was observed at a concentration of CW3 of about 5·10−10 M. Reducing the fifth and the eighth peptide bonds (C5 and C8) obliterated recognition by CAS 1. For C2, C4, and C6, recognition was decreased by ~100-fold. For C3 and C9, recognition by CAS 1 was decreased by ~2000-fold, whereas the reduced peptide bond in C1

affected the recognition by only 10-fold, and recognition of C7 was even better than that of the parent peptide.

To take into account the differences in the binding to Kd (Fig. 1), we calculated the normalized antigenic activities (Fig. 2C) obtained by dividing the relative affinity for Kd by the concentration of pseudopeptide required to get half-maximal lysis, as previously described (28). This parameter represents more faithfully the effect of the modifications on the interaction with the TCR. Both C1 and C7 displayed a heteroclitic activity (relative antigenic activity 10 and 5 times higher, respectively), while C5 and C8 were not recognized by CAS 1 CTL. For C2, C3, C4, C6, and C9, the efficiency of recognition by CAS 1 was decreased between 4-fold and 100-fold.
more tolerant to peptide bond reduction than both ends. In addition, for both peptides (CW3 and PbCS), one of the most critical carbonyls is at the C-terminal end (9). When Guo et al. (5) determined the crystal structure of HLA-A68 loaded with a mixture of peptides, they found that this last carbonyl was at hydrogen bonding distance from the indole nitrogen atom of the Trp147. This Trp residue is highly conserved in mouse and human MHC class I molecules (39, 40). Therefore, some interactions between the MHC molecule and the peptide backbone may be conserved in different human and mouse complexes and, as previously suggested (6, 41), could contribute to the promiscuous selectivity of MHC molecules in peptide binding.

C6, C7, and CW3 bind Kd with identical affinities (Fig. 1). Therefore, comparison of their recognition by T cells should only reflect differences in TCR binding. Remarkably, target cell lysis by CAS 1 required 100 times higher concentrations of C6 but 5 times lower concentrations of C7 than CW3. It is interesting to note that reducing backbone carbonyls can either increase or decrease TCR recognition. These observations directly demonstrate a role of the peptide backbone carbonyls in TCR interaction.

The fact that only C6 and C7 bind to Kd with an affinity comparable to CW3 could have seriously limited our analysis of the role of the peptide backbone. However, using CAS 1, a T cell clone recognizing the CW3 peptide at nanomolar concentrations, we could circumvent this difficulty in two ways. First, the equilibrium constant (KD) of CW3 for Kd is in the order of 300 nM (S.C., unpublished observation). We compared the recognition by CAS 1 of the different pseudopeptides at concentrations ranging from 1 pM to 10 µM. At the latter concentration, loading of the different pseudopeptides on Kd should not be limiting, and differences in T cell recognition should mainly reflect differences in the interaction of the TCR with the MHC-peptide complex. Second, we determined the concentration of peptide yielding half maximal lysis by CAS 1, measured affinity for Kd, and used these two values to calculate a normalized relative antigenic activity, as previously described by Luescher et al. (28). Remarkably, both approaches gave a similar hierarchy and showed that several carbonyls of the peptide backbone significantly contribute to the interaction with the TCR, the most important carbonyls being those reduced in C5 and C8.

CD8 stabilizes the interaction between the TCR and the MHC-peptide complex (33, 42) and contributes to the adhesion of T cells to APC (43). Since CAS 1 expresses CD8, minor alteration in TCR binding could have been obliterated. Therefore, we compared the
recognition of the different pseudopeptides by two CD8⁺ T cell hybridomas named 9.4 and HIP and specific for CW3. Four carbonyls (reduced in C5, C6, C8, and C9) are essential for HIP recognition. Interestingly, these four carbonyls are also essential for 9.4 recognition, although 9.4 is also dependent on the third and seventh carbonyls. The latter has, in fact, an opposite effect on HIP recognition, although 9.4 is also dependent on the third and seventh carbonyls. These four carbonyls are also essential for HIP recognition of the different pseudopeptides by two CD8⁺ T cell hybridomas named 9.4 and HIP and specific for CW3. Four carbonyls (reduced in C5, C6, C8, and C9) are essential for HIP recognition. Interestingly, these four carbonyls are also essential for 9.4 recognition, although 9.4 is also dependent on the third and seventh carbonyls. The latter has, in fact, an opposite effect on HIP recognition, although 9.4 is also dependent on the third and seventh carbonyls. These four carbonyls are also essential for HIP recognition.

The present study demonstrates that the peptide backbone has a critical contribution to TCR recognition. Comparison with available crystallographic studies suggests that some TCR residues may directly contact the peptide backbone. Moreover, some of these contacts may be conserved between different TCR/MHC-peptide complexes, thereby contributing to TCR plasticity. With regard to TCR specificity, previous studies have stressed the importance of dominant negative effects of amino acid side chain substitution in MHC-peptide molecule interactions and T cell recognition (47). Similarly, MHC molecules exhibit a broad specificity for peptides by using conserved contacts to stabilize binding, while selected polymorphic bulky residues restrain the repertoire of bound peptides (6, 41). In both cases, conserved interactions ensure that each ligand binds the receptor with an optimal affinity, while dominant negative interactions restrict the repertoire of ligands and delineate the specificity. Therefore, a similar strategy may be used by both MHC molecules and TCR to achieve broad specificity and plasticity of recognition.

Acknowledgments

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