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Identification of residues necessary for clonally specific recognition of a cytotoxic T cell determinant

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The residues in an influenza nucleoprotein (NP) cytotoxic T cell determinant necessary for cytotoxic T cell (CTL) recognition, were identified by assaying the ability of hybrid peptides to sensitize a target cell to lysis. The hybrid peptides were formed by substituting amino acids from one determinant (influenza NP 147–158) for the corresponding residues of a second peptide (HLA CW3 171–182) capable of binding to a common class I protein (H-2K^d). Six amino acids resulted in partial recognition; however, the presence of a seventh improved the potency of the peptide. Five of the six amino acids were shown to be required for recognition. The spacing of the six amino acids was consistent with the peptide adopting a helical conformation when bound. The importance of each amino acid in CTL recognition and binding to the restriction element was investigated further by assaying the ability of peptides containing point substitutions either to sensitize target cells or to compete with the natural NP sequence for recognition by CTL. The T cell response was much more sensitive to substitution than the ability of the peptide to bind the restriction element. Collectively the separate strategies identified an approximate conformation and orientation of the peptide when part of the complex and permitted a potential location in the MHC binding site to be identified. The model provides a rationalization for analogues which have previously been shown to exhibit greater affinity for the class I molecule and suggests that the binding site in major histocompatibility complex (MHC) class I molecules might have greater steric constraints that the corresponding area of class II proteins.

Key words: amino acid substitutions/cytotoxic T cell/major histocompatibility complex/T cell receptor/complex

Introduction

The binding of fragments of protein immunogens by MHC class I and class II molecules appears to be a necessary requirement for antigen specific immune responsiveness (Guillet *et al.*, 1987; reviewed in Moller (ed.), 1987). Binding of peptides to purified, detergent solubilized, class II proteins initially was shown using equilibrium dialysis and gel filtration (Babbitt *et al.*, 1985; Buus *et al.*, 1986, 1987). Subsequently, peptide–class II complexes have been detected when the MHC proteins are present in lipid monolayer (Watts and McConnell, 1986) and on the surface of cells (Busch *et al.*, submitted; Ceppelini *et al.*, submitted).

In contrast, the direct interaction between peptide and MHC class I molecules has been much more difficult to demonstrate. However, addition of exogenous peptides can be used to sensitize target cells for lysis by CTL in much the same way as peptides are used to generate proliferative responses in class II restricted T cells (Townsend *et al.*, 1986; McMichael *et al.*, 1986; Maryanski *et al.*, 1986; Taylor *et al.*, 1987; Bastin *et al.*, 1987; Oldstone *et al.*, 1987). In addition, specific competition between peptides seen by class I restricted T cells has recently been demonstrated (Maryanski *et al.*, 1988; Bodmer *et al.*, 1988; Pala *et al.*, 1988) as has previously been shown for class II restricted determinants (Guillet *et al.*, 1986).

The solution of the three dimensional structure of HLA A2 (Bjorkman *et al.*, 1987a,b) has produced important structural information on the proposed antigen binding sites for MHC class I molecules. It has also led to a working model of the T cell receptor–peptide–MHC complex (Davis and Bjorkman, 1988). Inherent in this hypothesis is that the peptide contacts both the MHC protein and the T cell receptor. Previously, we have postulated that residues which exclusively contact the T cell should be clonally specific, while those that interact with a common MHC protein might display a degree of physical and chemical similarity (Rothbard and Taylor, 1988). The degree of similarity will depend upon the diversity of peptide–MHC interactions. Empirically we have identified putative sequence motifs in many T cell determinants which, if valid, argue that many peptides bind class I and class II proteins in a similar fashion.

We have assayed a series of analogues of a previously defined murine H-2K^d restricted determinant in the nucleoprotein of influenza to be recognized by the T cell receptor and/or compete with the natural determinant for binding to the restriction element. In earlier experiments the determinant necessary for lysis by both NP specific CTL lines and clones was shown to be residues 147–158 (Bodmer *et al.*, 1988). Deletion of arginine-156 resulted in a peptide that was recognized by T cells at dramatically low concentrations. Assaying analogue peptides with individual substitutions revealed that the critical modifications which resulted in improved recognition were both the substitution of threonine-157 by glycine and the removal of an amino acid at position 158. The changes were shown to result in an improved interaction with the class I protein and not the T cell receptor because the peptide containing the deletion bound both the target cells far more rapidly than the natural sequence (Bodmer *et al.*, 1988), and was a superior competitor for another K^d restricted determinant (HLA CW3 171–182) in assays using a different T cell clone (Pala *et al.*, 1988).

In the current set of experiments the residues necessary

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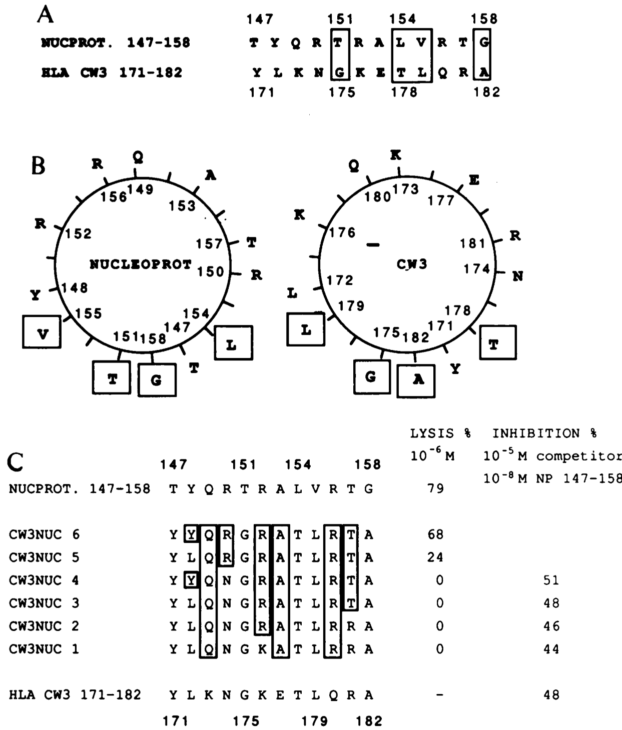

Fig. 1. Alignment of two defined H-2K^d restricted cytotoxic T cell determinants which led to the construction of the hybrid peptides used in these experiments. (**A**) The alignment used as a basis of the exchange of residues between the two determinants. Structurally similar residues at relative positions 1, 4, 5 and 8 (enclosed in boxes). (**B**) The sequences of the two determinants, aligned as in (A), displayed in helical wheels, demonstrating the boxed residues would be juxtaposed if the peptide adopts a helical conformation. (**C**) Sequences of the hybrid peptides synthesized by substituting residues from the nucleoprotein sequence (enclosed in boxes) into the corresponding positions of the CW3 peptide and a summary of their ability either to be recognized by a nucleoprotein specific CTL or to compete with the natural nucleoprotein peptide sequence for recognition.

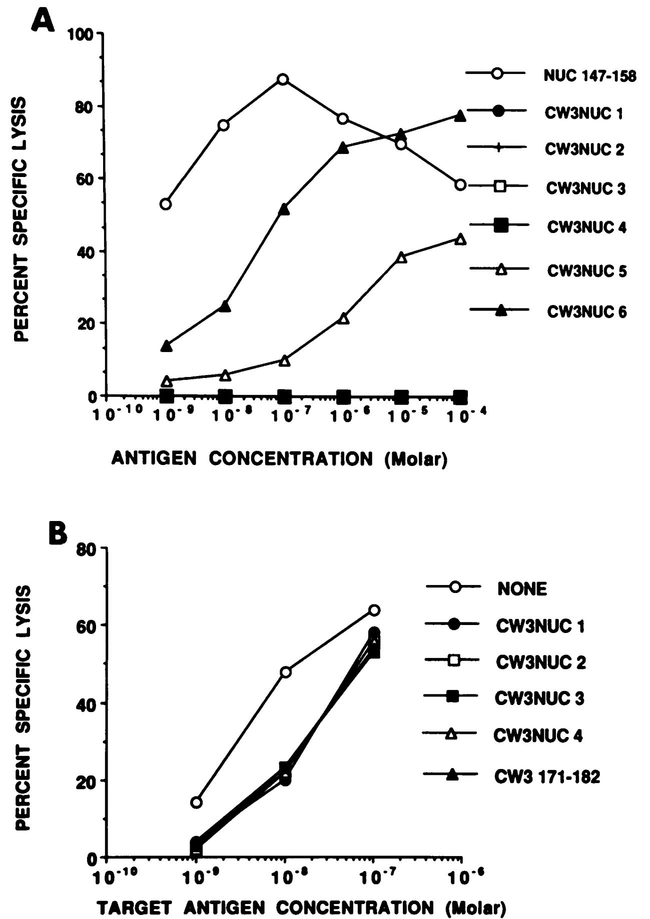

Fig. 2. The ability of hybrid peptides composed of residues from both the nucleoprotein and CW3 determinants to sensitize target cells to lysis and/or compete with the native nucleoprotein determinant for recognition by a nucleoprotein specific CTL clone. (**A**) Percentage specific lysis of P815 (H-2K^d) cells in the presence of differential amounts of the target peptides shown. (**B**) Percentage specific lysis of P815 (H-2K^d) cells in the presence of a constant concentration of each of the competitor peptides shown (10^{-5} M) with varying concentrations of the natural nucleoprotein peptide (147–158).

for clonal specific T cell recognition were identified by assaying the ability of a family of hybrid peptides either to be targets for a CTL clone or to compete with the natural sequence for binding to the restriction element. Variants of the hybrid peptide, which was best recognized by the NP specific clone, were tested to confirm that the pattern of substitutions was optimal. The results were consistent with the peptide adopting a helical conformation when complexed with the class I protein. The importance of each amino acid in MHC binding was examined further by assaying the effects of point mutations in the peptide on cytotoxic and competition assays. Finally, based upon the proposed conformation and orientation of the bound peptide, a complementary surface in the proposed binding site was identified allowing a tentative model of the complex to the constructed.

The strategy used to examine the recognition of peptide antigens by both H-2K^d and the T cell receptor of a NP specific cytotoxic clone was to test whether the residues that interact with the T cell receptor could be exchanged between two determinants recognized by K^d restricted T cells in a manner previously demonstrated for two peptides recognized by DR1 restricted T cells (Rothbard *et al.*, 1988). The success of this strategy is conditional upon the following assumptions: (i) the two peptides adopt a similar conformation when bound; (ii) both peptides bind in the same location in the binding site, and contact many common MHC residues; (iii) substitution of amino acids from one sequence into the second will not prevent the hybrid peptide from adopting the appropriate conformation for binding, and (iv) substitution of a sufficient number of amino acids results in the retention of an intact surface necessary for binding to either the MHC protein or the T cell receptor.

Results

Identification of clonally specific amino acids in a K^d restricted determinant

To investigate exhaustively the exchange of all groups of amino acids between two determinants requires the synthesis of an extremely large number of hybrid peptides. For example, if two 12 amino acid peptides are aligned colinearly there are 2^{12} possible ways the residues can be combined. If alternative alignments are also considered, then the possibilities are further increased. To make the approach practical, the two defined K^d restricted cytotoxic determinants (Bodmer *et al.*, 1988; Maryanski *et al.*, 1986) (Figure 1A) were analysed for similar structural

Table I. Ability of hybrid peptides CW3NUC 5.0–5. and 6.0 to sensitize target cells to lysis and compete with the natural sequence for recognition relative to the two determinants from which they were formed

		Percentage specific lysis				Percentage competition
		10^{-5}	10^{-6}	10^{-7}	10^{-8} M	10^{-8} M NP:10^{-5} M competitor
	147 151 154 158					
NP 147–157	T Y Q R T R A L V R T G	71	76	81	79	
CW3NUC 5.0	Y L Q R G R A T L R T A	38	18	10	6	Recognized
CW3NUC 5.1	Y Y K R G R A T L R T A	0	0	0	0	30
CW3NUC 5.2 (4)	Y Y Q N G R A T L R T A	0	0	0	0	51
CW3NUC 5.3	Y Y Q R G K A T L R T A	0	0	0	0	52
CW3NUC 5.4	Y Y Q R G R E T L R T A	0	0	0	0	16
CW3NUC 5.5	Y Y Q R G R A T L Q T A	77	71	19	0	Recognized
CW3NUC 5.6	Y Y Q R G R A T L R R A	20	5	8	0	Recognized
CW3NUC 6.0	Y Y Q R G R A T L R T A	77	73	52	23	Recognized
CW3 171–182	Y L K N G K E T L Q R A					
	171 175 179 182					

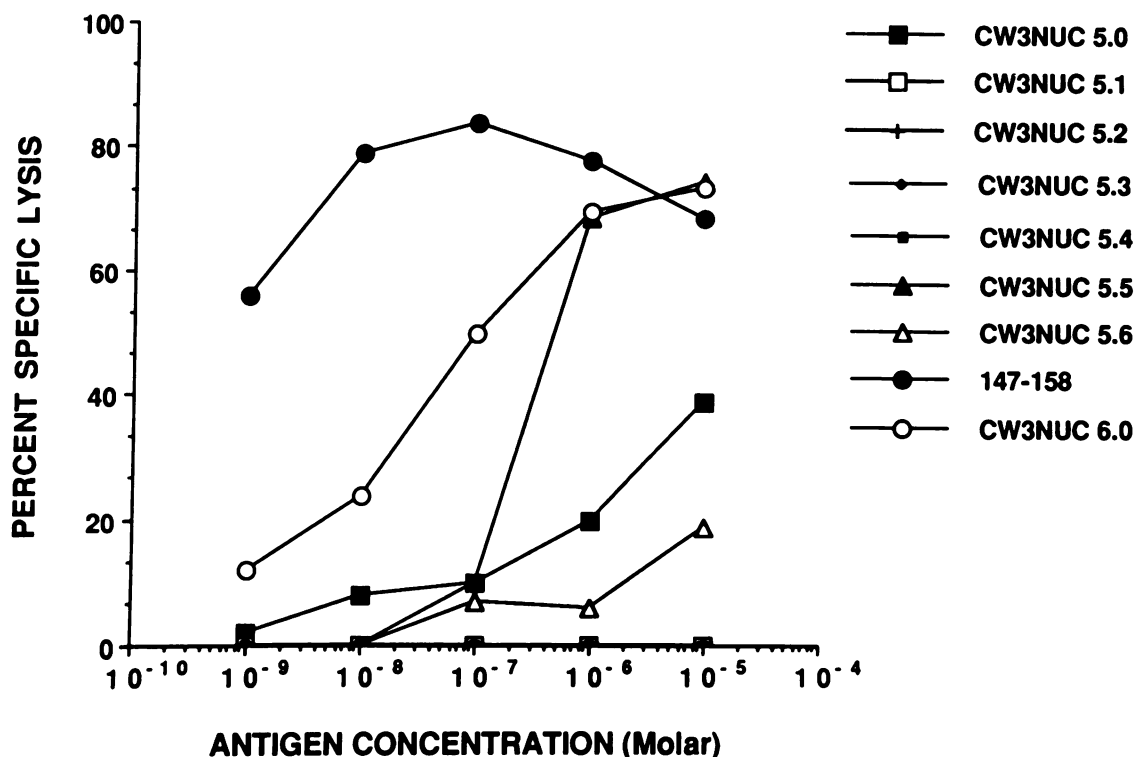

Fig. 3. Ability of the set of peptides CW3NUC 5.0–5.6 to be recognized by a NP specific cytotoxic T cell clone. Each peptide containing six of the seven NP amino acids in CW3NUC 6.0 was assayed for its ability to sensitize target cells over the range of concentrations shown.

features which could arise from their interaction with a common histocompatibility protein. When the sequences were placed colinearly, a reasonable alignment of the polar and nonpolar residues was apparent. In particular, both sequences contained adjacent hydrophobic residues with flanking small amino acids at relative positions 1, 4, 5 and 8 (enclosed in boxes in Figure 1A). As previously postulated (Rothbard and Taylor, 1988), these residues would form a structurally similar surface if the two peptides adopted a helical conformation (Figure 1B). A positively charged residue at each of 152 and 176 and polar residues at 150 and 174 could also be important. If valid, then the residues forming the upper face of the helix, as shown in Figure 1B, would interact with the T cell receptor.

Our initial construct to test this hypothesis, CW3NUC 1 (Figure 1C), involved substituting three residues from the NP sequence for the corresponding amino acids in the CW3 peptide. This peptide was not recognized by the CTL clone (Figure 2A); however, it did compete with the NP peptide equally well as the intact CW3 sequence in a competition assay (Figure 2B). As in previous studies (Maryanski *et al.*, 1988; Bodmer *et al.*, 1988; Pala *et al.*, 1988), the inhibition was very inefficient; 1000-fold excess of the competitor only reduced the specific cytotoxicity by approximately half. Nevertheless, CW3NUC 1 was as effective as the parent CW3 peptide (Table I and Figure 2B). Equivalent competition was still observed when an additional three residues were substituted in a stepwise manner, to form CW3NUC 2, CW3NUC 3 and CW3NUC 4, indicating that the substitutions in these peptides interfered with recognition

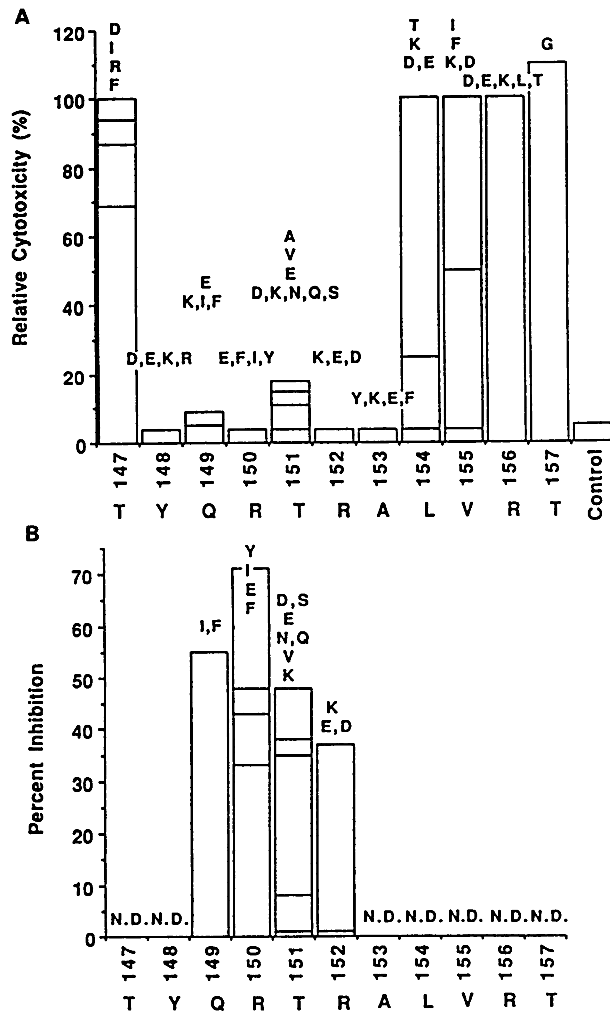

Fig. 4. Ability of analogues of the nucleoprotein determinant containing point substitutions either to sensitize target cells (P815) or to compete with the natural nucleoprotein sequence for recognition. (**A**) Relative ability of analogues to sensitize target cells compared with the natural sequence at 10^{-7} M. The analogues are listed in decreasing order of effectiveness for each position. If two analogues were recognized equally they are shown on the same line. (**B**) Percentage inhibition of specific cytotoxicity of P815 target cells incubated with 10^{-8} M of the natural NP sequence in the presence of 10^{-5} M of each of the analogues containing point substitutions. Only those analogues that were not recognized at 10^{-5} M could be used. If all substitutions at a particular position resulted in peptides that were recognized at these concentrations, competition could not be determined (ND).

by the T cell receptor of the clone and not their ability to interact with the restriction element.

A second peptide, CW3NUC 5, containing six NP amino acids, with arginine-150 and not tyrosine-148, was recognized by the T cell. However, when both amino acids were present, the resulting peptide, containing seven NP residues, CW3NUC 6, sensitized target cells to lysis by the clone equally well as the natural sequence at 10^{-5} M, although was not as effective at lower concentrations.

To demonstrate that the success of identifying this hybrid peptide was not fortuitous, and that all seven NP residues present in CW3NUC 6 were necessary for recognition, variants of these peptides were systematically investigated. If each of the seven NP residues in CW3NUC 6 are required, then no analogue containing only six of these seven should be recognized equally well. Therefore all hybrid peptides were generated by the reversion of each NP residue in CW3NUC 6 to its CW3 equivalent. This produced a set of seven peptides, that are referred to as CW3NUC 5.0 to 5.6, including CW3NUC 4 (5.2) and 5 (5.0) (Table I). Each of these peptides was tested for its ability both to sensitize target cells and to compete with the natural NP peptide for recognition (Table I and Figure 3A). Four, CW3NUC 5.1, 5.2, 5.3 and 5.4 were not recognized by the T cell clone, even at high concentrations. Of the remaining two, CW3NUC 5.6 produced a very weak response, while 5.5 was the most effective of the set, but approximately an order of magnitude less potent than CW3NUC 6. These experiments demonstrate that no combination of six of these substitutions was as potent as all seven. The only residue that could be exchanged with minimal loss of recognition was the conversion of arginine-156 to a glutamine.

Additional information about the contribution of each amino acid in the response was obtained by assaying the peptides for their ability to compete with the NP peptide for recognition. Only CW3NUC 5.2 and 5.3 were as effective inhibitors as the CW3 peptide, demonstrating that substitution of arginine -150 or -152 by asparagine or lysine respectively removes all recognition by the T cell receptor, but does not interfere with binding to the class I molecule. CW3NUC 5.1 competed ~60% as effectively as the natural CW3 peptide, whereas CW3NUC 5.4 was a less effective inhibitor. CW3NUC 5.0, 5.5 and 5.6 could not be assayed for their ability to compete because they were recognized.

Collectively these data demonstrate that only five of the seven NP residues present in CW3NUC 6.0 are necessary for clonal specific recognition. Partial recognition by CW3NUC 5.0 and 5.5 indicated that only amino acids 149, 150, 152, 153 and 157 were essential. Reversion of tyrosine-148 to leucine resulted in greater loss of recognition than replacement of arginine at 156. Tyrosine at 148 could improve the potency of the peptide by stabilizing either the peptide–MHC complex or peptide interaction with the T cell receptor, but these two possibilities cannot be distinguished using these experimental techniques.

Effect of point substitutions on CTL recognition and MHC binding

To examine both the physical and chemical requirements at each residue for T cell recognition and binding to the class I protein, a series of peptides containing point mutations were assayed for their ability to sensitize target cells and/or compete with the natural NP sequence for recognition. Even though only a limited number of substitutions were examined, several general patterns were apparent (Figure 4A and B). As previously seen for other peptides recognized by either class I or class II restricted T cells (Sette *et al.*, 1987; Gotch *et al.*, 1988; J.B.Rothbard *et al.*, submitted), the majority of substitutions affected T cell recognition significantly more than the ability of the peptide to bind the MHC protein. Of the 45 analogues examined, only three were unable to be recognized either at high concentrations (10 μM) or compete with the natural sequence for recognition (Figure 4A and data not shown). In contrast 23 substitutions prevented the peptide from being seen by the T cell receptor at 1 μM and only one change (glycine for threonine at 157) resulted in a more potent peptide. Residues forming the core of sequence (residues 148–153) appear

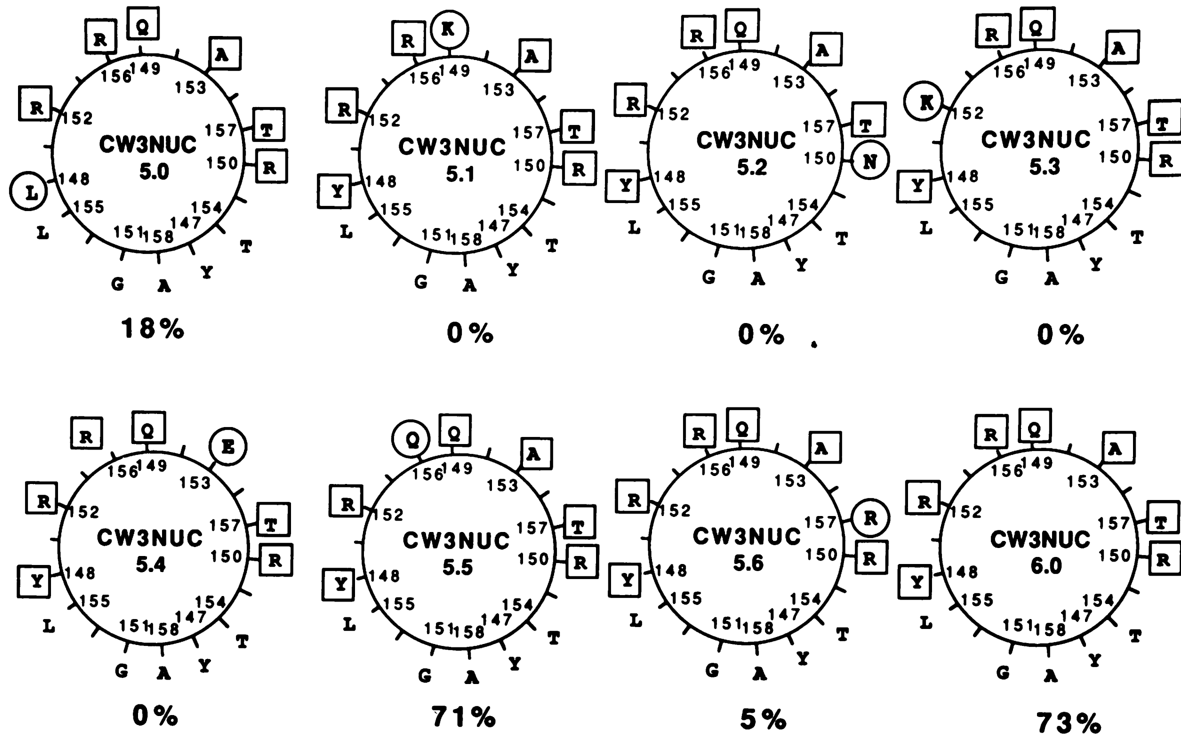

Fig. 5. Interpretation of the results of recognition of CW3NUC 5.0–5.6 and 6.0 in the context of a helical model for the bound peptide. Summary of the ability of CW3NUC 5.0–5.6 and 6.0 to sensitize target cells to lysis at 10^{-7} M and comparison with the proposed orienation of each residue if they adopt a helical conformation. The sequences are displayed in helical wheels to represent the relative positions of the α carbons in a perfect α-helix. The residues enclosed in boxes are from the NP sequence, while those in circles are the individual amino acids reverted to the corresponding CW3 residue.

to be more sensitive to substitution than those composing the amino and carboxyl termini of the peptide which exhibit greater latitude for substitution.

Inhibition of the T cell response with the peptides containing point substitutions provided much less information. Detectable reduction of T cell recognition by coincubation with competing peptides required a much larger excess of competitor (between 100- and 1000-fold molar excess) than with other receptors, most likely due to the very slow rate of association and dissociation of the peptide–MHC complex. Consequently only those peptides that were not recognized at 10 μM could be used in these experiments. Of the 16 analogues that satisfied this criterion, only four substitutions could not compete: lysine or valine at 151 and both aspartic and glutamic acid at 152 (Figure 4B).

When the effects of the individual substitutions were examined in detail and compared with the results of the hybrid peptides a number of interesting aspects were apparent (Table I, Figure 4A and B). Threonine-147: this position was quite tolerant of substitutions, however the presence of this amino acid was important for T cell recognition because its deletion resulted in a peptide that exhibited only one-tenth the potency of the natural sequence (Bodmer *et al.*, 1988). Tyrosine-148: addition of this residue in CW3NUC 6 resulted in superior recognition. The corresponding amino acid in the CW3 sequence is leucine. The hydrophobic character of this residue might be important because substitution with any of the four charged amino acids eliminated recognition. Glutamine-149: replacement with lysine in CW3NUC 5.1 resulted in loss of cytotoxicity. This result was confirmed by demonstrating that a single substitution by glutamic acid or lysine at 149 dramatically reduced the potency of the peptide, sensitizing target cells only at high concentrations (10^{-5} M). Substitution with isoleucine or phenylalanine generated peptides that failed to sensitize target cells at all concentrations. These substitutions apparently did not obviate the ability of the peptide to bind the MHC protein because they competed with the natural sequence. Arginine-150: this residue cannot be substituted by the corresponding amino acid, asparagine, in the CW3 peptide and still be recognized (CW3NUC 5.2), and replacement by glutamic acid, tyrosine, isoleucine or phenylalanine also resulted in loss of recognition. As with position 149, the failure to stimulate was not due to their inability to bind because they were all capable of inhibiting recognition of the natural sequence. Threonine-151: the corresponding residue in CW3 was a glycine that was successfully exchanged in the hybrid peptides. However all eight individual substitutions at this position, including alanine and serine, resulted in dramatic reductions of the percentage of specific lysis. Only lysine and valine substituted at 151 appeared to affect significantly the ability of the peptide to bind. Arginine-152: substitutions at this position had interesting effects. Replacement of arginine with either an acidic amino acid or lysine removed all recognition. The lysine substitution generated a peptide that could compete with the natural sequence and consequently bound well to the restriction element. In contrast, analogues with an acidic amino acid at this position were not recognized, nor could they compete. Evidently, a positive residue at this position was sufficient for binding, but a guanidinium group and not a primary amine enabled the bound peptide to be recognized by the T cell receptor. Interestingly, the corresponding position in the CW3 peptide (176) is a lysine,

indicating that charge conservation is important at this position. Alanine-153: CW3NUC 5.4 which reverted alanine to glutamic acid was not recognized. The point substitutions confirmed this sensitivity, but because they reduced, but did not eliminate, recognition at 10^{-5} M they could not be assayed for competition. Leucine-154 and valine-155 appear to tolerate substitutions with hydrophobic amino acids, but replacement with charged residues interfered with recognition. Arginine-156: like residue 147, this position can tolerate both positively and negatively charged amino acids as well as those with hydrophobic character. Nevertheless, shorter peptide analogues lacking a residue at this position, i.e. 147–155, were not stimulatory. Threonine-156: a glycine at this position improved the potency of the peptide by approximately an order of magnitude, indicating the size of the side chain at this position might be an important factor. The corresponding position in the CW3 sequence is a relatively large amino acid, arginine, which when substituted in the context of CW3NUC 5.6, reduced the ability of the peptide to compete, consistent with a preference for a small amino acid. Glycine-158: no substitutions were done at this position because deletion of this residue dramatically improved the ability of the peptide to sensitize target cells providing that it is not essential for recognition. This position also is a small amino acid, alanine, in the CW3 sequence.

Discussion

A central issue in understanding the detailed interaction between peptides and MHC proteins is how a single combining site can accommodate a wide variety of ligands. One possible explanation is that a large percentage of the peptides that can bind share a number of structural details that allow them to bind in a preferred site with similar conformation. This premise was tested by identifying the critical residues necessary for T cell recognition using these structural similarities as a guide to align two peptides recognized by K^d restricted T cell clones. Substitution of amino acids from an influenza NP determinant for the corresponding amino acids in a peptide from HLA CW3 resulted in a set of hybrid peptides, some of which were recognized by a NP specific cytotoxic T cell clone. In addition, by assaying the ability of the hybrid peptides, which were not recognized, to inhibit the ability of the native NP sequence to sensitize cells, the replacements were shown not to interfere with the ability of the hybrid peptides to bind the restriction element. Consequently, these studies provided information both about the structural requirements for MHC binding and recognition and the orientation of the bound peptide relative to the restriction element and the T cell receptor. They also generated constraints on the possible conformation the bound peptide could adopt and its possible interactions in the binding site.

The difficulty in inhibiting cytotoxicity probably was due to two separate factors: (i) the extremely small number of peptides necessary for recognition required abnormally high concentrations of an antagonist to modulate the response, and (ii) the unusual kinetics of the formation of MHC–peptide complexes. Even though binding of peptides to MHC class I molecules has not been published at this time, a number of laboratories are investigating the interaction. As with MHC class II proteins, the rates of binding and dissociation appear to be extremely slow (Parham and Levy, personal communication). If generally true, the time course of the described experiments might not be sufficient to allow the system to reach equilibrium, which could also contribute to the poor competition. These issues are examined in greater detail in separate publications (Bodmer *et al.*, 1988; Maryanski *et al.*, 1988; Pala *et al.*, 1988).

The details of recognition of the hybrid peptides by the T cell clones were similar to those published in our previous investigation of DR1 restricted recognition (Rothbard *et al.*, 1988). In both cases, the initial substitutions removed all ability of the peptide to be recognized by the T cell; however additional substitutions eventually led to a species that was capable of acting as a target for the CTL. By itself, the recognition of CW3NUC 6.0 and 5.0 does not constitute proof of the proposed conformation of the bound peptide because of the similarity between the unsubstituted amino acids and the corresponding residues of the NP sequence. This also was a limitation in the earlier study. In contrast to that study, the ability to assay peptide binding to the class I protein indirectly, by using a competition assay, eliminated many ambiguities. In this paper, the hybrid peptides that were not recognized by the T cell clone, CW3NUC 1, 2, 3 and 4, were shown to bind the restriction element due to their ability to compete with the natural NP sequence as well as the intact CW3 peptide. This could only arise if the unaltered residues form the principal interactions with the H-2 molecule and/or the substituted residues maintain important contacts. In contrast, the cytotoxic T cell clone failed to recognize these analogues because they did not contain sufficient residues of the influenza peptide to form a competent interaction with the T cell receptor. These results are consistent with the proposed orientation of the bound peptide shown in Figure 1B, with the residues composing the lower facade, as shown, interacting with H-$2K^d$ while those forming the upper surface contact the T cell receptor.

To discuss the importance of each amino acid in binding the restriction element and/or the T cell receptor in the context of the proposed model, a schematic summary of the results of the hybrid series CW3NUC 5.0–5.6 in cytotoxicity assays has been compiled (Figure 5). The seven NP amino acids present in CW3NUC 6.0 and the six in CW3NUC 5.0 both constitute a continuous arc of the helical wheel. CW3NUC 5.5, which was also recognized, contains a substitution in the middle of the arc; however, this position is near the carboxyl terminus of the peptide and was shown to be tolerant of substitution with a variety of residues. The remaining five positions, forming an arc from 152–149, 153, 157–150, were sensitive to substitution and constitute the minimum residues necessary for T cell recognition. Such specificity is characteristic of antibody recognition and contrasts with the demonstrated capacity of MHC molecules to bind a variety of divergent peptides and tolerate a large number of individual substitutions in defined T cell determinants (Sette *et al.*, 1987; Gotch *et al.*, 1988; Rothbard *et al.*, submitted). Even though residue 152 was necessary for T cell recognition it apparently also interacted with the restriction element based on the fact that substitution at this position with an acidic amino acid could not compete. Similar evidence implied that residue 151, and to a lesser extent 150, also interact with the restriction element. However, these conclusions assume that the substitutions do not markedly disrupt the conformation of the peptide backbone.

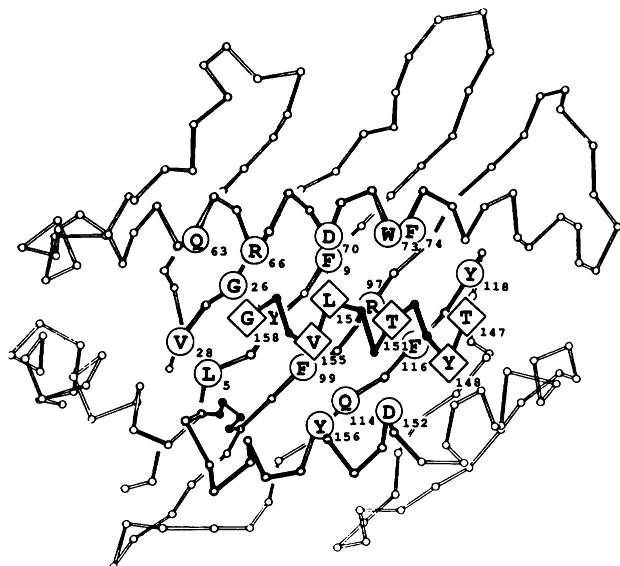

Fig. 6. Molecular model of the NP peptide (residues 147–158) in the proposed antigen combining site of H-2K^d. Schematic representation of the peptide antigen, folded as an α-helix, in the proposed combining site of H2K^d. Details of the site extrapolated from the published HLA A2 structure (Bjorkman *et al.*, 1987a,b) with some of the residues integral to the binding site enclosed in circles. For clarity, only those residues of the peptide (enclosed in diamonds) proposed to interact principally with the β-pleated sheet are highlighted. The peptide has been placed in a tentative location described in the text allowing 151, 150 and 152 of the peptide to be placed near 97, 70 and 152 of K^d. In this position residue 157 of the peptide is near arginine-66 of the restriction element.

The success of the hybrid peptides in both cytotoxicity and competition experiments supports the hypothesis that both peptides bind K^d in the orientation proposed by the alignment shown in Figure 1A and B, and also indicates that they both might bind in a common location in the combining site. The structural requirements of the peptides for binding might therefore be mirrored in the binding site.

The two positions for which there was strongest evidence for interaction with the class I molecule, threonine-151 and arginine 152, displayed different sensitivity to substitution. When a positively charged residue was present at position 151 or a negative amino acid was substituted at 152 the resulting analogues could not compete and therefore must not have been able to bind. However, reversal of the charge at each position did not interfere with binding. This suggested that a complementary pair of charged amino acids might be present in the proposed antigen combining site of H-2K^d.

Examination of a simple diagram of the proposed site in K^d (Figure 6), drawn based on the close sequence homology between H-2K^d and HLA A2 (Bjorkman *et al.*, 1987a,b), revealed two pairs of charged residues, arginine-97 with either glutamic acid-152 or aspartic acid-70, that fulfilled the geometric requirements of the peptide analogues. Depending on which contacts are made, two directions for the bound peptide in the proposed conformation are possible. In either direction the location of the two acidic amino acids were complementary with the arginine-150 and arginine-152 in the peptide. If arginine-152 of the peptide is opposite glutamic acid-152 of the MHC protein, then the helical axis of the peptide is parallel with the helical portion of the A2 domain and the peptide is central in the binding site. This places the two central hydrophobic residues in the peptide (154 and 155) over hydrophobic residues in the sheet, suggesting an energetically favourable interaction, which is consistent with their tolerance to substitution only by hydrophobic residues. In the reversed direction (not shown) the charge interactions are maintained and some hydrophobic contacts are still possible for residues 154 and 155. However, the peptide is now markedly displaced to one side of the binding site and, based on computer modelling studies, may have insufficient space for binding.

A further attractive feature of the proposed location of the peptide in the site was its ability to rationalize earlier work demonstrating that when threonine-157 was replaced by glycine, and/or residue 158 was deleted, the peptide was recognized at greatly reduced concentrations (Bodmer *et al.*, 1988). The most obvious rationalization for a glycine, an amino acid lacking a side chain, to improve the binding of a ligand to a receptor is that it relieves a steric constraint. Examination of the published electron density of HLA A2 revealed that the side chain of amino acid 66 extended well into the binding cleft and could constitute the source of steric hindrance to peptide binding. In the present model of the K^d–peptide complex, the equivlent residue, arginine-66, is near threonine-157 and the carboxy terminus of the peptide. Removal of bulk at this end of the peptide could, therefore, relieve the steric restraint and explain the improved binding. Furthermore, deletion of residue 158 and substitution of 157 by glycine also would expose the carboxyl terminal negative charge and place it closer to arginine-66, possibly allowing a salt bridge to be formed.

Support that steric constraint in this region of the site might be a general feature of class I molecules was found when other sequences were examined (Klein and Figueroa, 1986; Parham *et al.*, 1988). In all known class I molecules whose sequence has been determined, a bulky amino acid exists at the equivalent of position 66 in K^d, whereas in all class II sequences the corresponding position is either a glycine or an alanine, with a single exception where it is an aspartic acid (Klein and Figueroa, 1986; Bell *et al.*, 1987). This apparent steric restriction in the binding site, apparently not present in class II, might be a key factor in limiting the number of potential CTL epitopes. If, as we have shown, six residues of the antigen can determine specific T cell recognition, then the probability of a chance cross-reaction with a self antigen is quite great. Inappropriate stimulation or cross-reaction of cytotoxic T cells might be more harmful to the individual than with a cross-reactive helper T cell, and consequently restricting the number of potential determinants by sterically limiting the antigen combining site might be of great value to the organism. If true, then mutation of class I and class II antigens at this site might influence the repertoire of T cell determinants recognized by the individual.

Materials and methods

Cytotoxic T cell clone T5/5

The clone was obtained from BALB/c mice and maintained in culture as previously described (Taylor *et al.*, 1987).

Peptide synthesis

The peptides were synthesized by solid phase techniques (Barany and Merrifield, 1979) on an Applied Biosystems 430A peptide synthesizer, using commercially available t-boc protected amino acids and resins as previously described (Rothbard *et al.*, 1988). The peptides were assayed for purity

by amino acid analysis and analytical HPLC using an Aquapore RP-300 reverse phase column. Any peptides <90% pure were preparatively purified using the same reverse phase support and a 0.1% TFA-water-acetonitrile gradient.

^{51}Chromium release cytotoxicity assay

A standard procedure for cytotoxicity was used (Zweerink et al., 1977). Briefly, target cells (P815, H-2K^d) were labelled with chromium diluted to $2-4 \times 10^5$/ml, and added to the assay in a volume of 50 ml to give $1-2 \times 10^4$ cells/well. CTLs were diluted to give the appropriate killer to target ratio and added in 50 ml. All peptides were added directly to the assay. These were diluted to four times the concentration required and added in a volume of 50 ml. For competition assays the second peptide was added in 50 ml also giving a final volume of 200 ml. In recognition assays and controls the volume was made up to 200 ml/well with RPMI 1640 containing 10% fetal calf serum.

$$\text{Percentage target lysis} = \frac{\text{c.p.m. sample release} - \text{c.p.m. spontaneous release}}{\text{c.p.m. total release} - \text{c.p.m. spontaneous release}} \times 100$$

Spontaneous ^{51}chromium release of target cells in the absence of CTL was always <15% of the total release by 2.5% Triton X-100. For all graphs shown each point represents the average of triplicate wells. Incubation time for the assays was 4 h at killer; target ratios of 3 or 4:1. Percentage competition was calculated as the ratio between lysis of target cells in the presence of competitor compared with that found in the absence of the competing peptide.

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References

Babbitt,B., Allen,P., Matsueda,G., Haber,E. and Unanue,E. (1985) *Nature*, **317**, 359–361.
Barany,G. and Merrifield,R. (1979) In Gross,E. and Meienhofer,J. (eds.), *The Peptides*. Academic Press, NY, pp. 1–284.
Bastin,J., Rothbard,J., Davey,J., Jones,I. and Townsend,A. (1987) *J. Exp. Med.*, **165**, 1508–1523.
Bell,J., Denney,D., Foster,L., Belt,T., Todd,J. and McDevitt,H. (1987) *Proc. Natl. Acad. Sci. USA*, **84**, 6234–6238.
Bjorkman,P., Saper,M., Samraoui,B., Bennett,W., Strominger,J. and Wiley,D. (1987a) *Nature*, **329**, 506–512.
Bjorkman,P., Saper,M., Samraoui,B., Bennett,W., Strominger,J. and Wiley,D. (1987b) *Nature*, **329**, 512–518.
Bodmer,H., Pemberton,R., Rothbard,J. and Askonas,B. (1988) *Cell*, **52**, 253–258.
Bodmer,H., Bastin,J. and Townsend,A. (1989) *Immunology*, in press.
Buus,J., Sette,A., Colon,S., Jenis,D. and Grey,H. (1986) *Cell*, **47**, 1071–1077.
Buus,J., Sette,A., Colon,S., Miles,C. and Grey,H. (1987) *Science*, **235**, 1353–1358.
Davis,M. and Bjorkman,P. (1988) *Nature*, **334**, 395–402.
Figueroa,F. and Klein,J. (1986) *Immunol. Today*, **7**, 78–80.
Gotch,F., McMichael,A. and Rothbard,J. (1988) *J. Exp. Med.*, **168**, 2045–2058.
Guillet,J., Lai,M., Briner,T., Smith,J. and Gefter,M. (1986) *Nature*, **324**, 260–263.
Guillet,J., Lai,M., Briner,T., Buus,S., Sette,A., Grey,H., Smith,J. and Gefter,M. (1987) *Science*, **235**, 865–870.
Klein,J. and Figueroa,F. (1986) *Immunol. Today*, **7**, 41–44.
Maryanski,J., Pala,P., Corradin,J., Jordan,B. and Cerrotini,J. (1986) *Nature*, **324**, 578–579.
Maryanski,J., Pala,P., Cerottini,J. and Corradin,G. (1988) *J. Exp. Med.*, **167**, 1391–1405.
McMichael,A., Gotch,F. and Rothbard,J. (1986) *J. Exp. Med.*, **164**, 1397–1406.
Moller,G. (ed.) (1987) *Immunol. Rev.*, **98**, 1–187.
Oldstone,M., Whitton,J., Lewicki,H. and Tishon,A. (1987) *J. Exp. Med.*, **168**, 559–570.
Pala,P., Bodmer,H., Pemberton,R., Cerrotini,J., Maryanski,J. and Askonas,B.A. (1988) *J. Immunol.*, **141**, 2289–2294.
Parham,P., Lomen,C., Lawlor,D., Ways,J., Holmes,N., Coppin,H., Salter,R., Wan,A. and Ennis,P. (1988) *Proc. Natl. Acad. Sci. USA*, **85**, 4005–4009.
Rothbard,J. and Taylor,W. (1988) *EMBO J.*, **7**, 93–100.
Rothbard,J., Lechler,R., Howland,K., Bal,V., Eckels,D., Sekaly,R., Long,E., Taylor,W. and Lamb,J. (1988) *Cell*, **52**, 515–523.
Sette,A., Buus,S., Colon,S., Smith,J., Miles,C. and Grey,H. (1987) *Nature*, **328**, 395–399.
Taylor,P., Davey,J., Howland,K., Rothbard,J. and Askonas,B. (1987) *Immunogenetics*, **26**, 267–272.
Townsend,A., Rothbard,J., Gotch,F., Bahadur,B., Wraith,D. and McMichael,A. (1986) *Cell*, **44**, 959–968.
Watts,T. and McConnell,H. (1986) *Proc. Natl. Acad. Sci. USA*, **83**, 9660–9664.
Zweerink,H., Askonas,B., Millican,D., Courtneidge,S. and Skehel,J. (1977) *Eur. J. Immunol.*, **7**, 630–635.

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