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Permissive recognition of a mycobacterial T-cell epitope: localization of overlapping epitope core sequences recognized in association with multiple major histocompatibility complex class II I-A molecules

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SUMMARY

Most T-cell epitopes are recognized in the context of a single or limited number of major histocompatibility complex (MHC) class II molecules. We have shown previously, however, that the immunodominant p61–80 epitope from the *Mycobacterium tuberculosis* 19 000 MW protein is recognized in a genetically permissive manner. In this study, permissive recognition of p61–80 was analysed in three murine MHC haplotypes (H-$2^{b,d}$ and ^{k}) with respect to: (i) T-cell-epitope core structure; (ii) I-A/I-E class II MHC restriction; and (iii) the identification of critical amino acid residues within the core region. Overlapping epitope core sequences composed of 6 to 8 amino acids were identified for each of the three H-2 haplotypes by T-cell epitope scanning (PEPSCAN) using peptide-specific T-cell lines. The epitope core sequences recognized by peptide and 19 000 MW protein-specific T cells were similar. In all three haplotypes, responses to p61–80 were restricted by class II MHC I-A molecules. To identify residues within the epitope core critically required for recognition, single substitution (alanine or leucine) analogue peptides were tested for their capacity to stimulate p61–80-specific T-cell hybridomas. A heterogeneous pattern of reactivity was observed, even among individual hybridomas derived from the same H-2 haplotype. Although every core residue could be defined as critical for at least one hybridoma, only one critical substitution (^{74}Val → Ala) was common to all hybridomas. The identification and structural analysis of genetically permissive epitopes of mycobacteria may be a useful strategy for the rational design of peptide-based vaccines for tuberculosis.

INTRODUCTION

Antigen recognition is mediated by $CD4^+$ T cells through the clonotypic $\alpha\beta$ T cell receptor (TCR) which interacts with peptide-major histocompatibility complexes (MHC) displayed on the surface of antigen-presenting cells (APC).[1] The crystallographic structure of the MHC class II molecule HLA-DR1[2] indicates that peptides are bound in an extended conformation within a peptide binding groove which, unlike the class I molecule, is open-ended and can thus accommodate longer peptides. Acid elution and sequencing of naturally processed peptides derived from both murine[3,4] and human[5,6] class II MHC molecules indicates that bound peptides are heterogeneous in length, ranging from 10 to 34 amino acids, and possess nested amino and carboxy-terminal ends. Although allele-specific anchor residues have been more difficult to identify in class II binding peptides due to heterogeneity in peptide length, conserved motif patterns have emerged.[7,8] However, these motifs do not appear to be as stringent as the allele-specific binding motifs described for class I molecules.[9] Most of the allelic variability that distinguishes different MHC molecules is located within the polymorphic peptide-binding groove.[10] A functional consequence of allelic variability that has been demonstrated in peptide-binding studies,[11] and from the sequencing of naturally processed peptides,[5,9] is that distinct sets of peptides tend to associate with a particular MHC allele, thereby accounting for the MHC-restricted nature of T-cell recognition.[12] Nevertheless, a small subset of promiscuous peptides have been described that bind to MHC molecules in a degenerate fashion or elicit T-cell responses in the context of multiple MHC class II antigens.[13–15] Although the mechanisms that account for such degenerate binding have not been fully elucidated, it has been postulated that promiscuous-binding self-peptides could play an immunoregulatory role as competitors, helping to prevent overpresentation of foreign peptide epitopes *in vivo*.[5]

Recently, we analysed the promiscuous recognition of an immunodominant peptide epitope (residues 350–369) from

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Abbreviations: APC, antigen presenting cell; ECS, Epitope core sequence(s); LNC, lymph node cell; MHC, major histocompatibility complex; TCR, T-cell receptor.

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the 38 000 MW antigen of *Mycobacterium tuberculosis* (*M. tuberculosis*) which is recognized in the context of multiple human and mouse MHC class II antigens. We showed that certain peptide-specific T-cell hybridomas were able to recognize this epitope when presented by either syngeneic or allogeneic APC and speculated that the peptide interacts with polymorphic residues on H-2 I-A molecules.[16] The identification of promiscuously recognized mycobacterial epitopes that have the capacity to elicit T-cell immune responses in a genetically diverse human population has obvious implications for the rational design of anti-tuberculosis diagnostic reagents and subunit vaccines.

In this present study, we have analysed a permissively recognized mycobacterial epitope, residues 61–80, from the immunodominant 19 000 MW lipoprotein antigen of *M. tuberculosis*.[17,18] Overlapping epitope core sequences (ECS) recognized in the context of I-A^b, ^{d} and ^{k} have been defined by epitope scanning (PEPSCAN) and we examine the role of individual amino acids within this epitope core with a series of substituted analogue peptides.

MATERIALS AND METHODS

Mice

Recombinant inbred female C57BL/10 (H-2^b), BALB/c (H-2^d), and BALB.K (H-2^k) strains of mice were obtained from Olac Harlem Ltd, (Shaws Farm, Bicester, Oxon, UK) and used when 6–8 weeks old.

Synthetic peptides

Peptides werre made in a fully automatic MilliGen/Bioresearch 9050 pepsynthesiser (Millipore UK, Watford, UK) using standard methods for Fmoc amino acid pentafluorophenyl esters on a PepSyn KB resin and according to conditions recommended by the manufacturers for synthesis and cleavage of the peptides which were amidated at the carboxy end. Efficiency of the coupling cycles was monitored by ionic dye equilibrium distribution and was maintained at >98% for each residue.[19] Peptide homogeneity was confirmed by analytical reverse phase high-performance liquid chromatography (HPLC) and selected peptides were subjected to amino acid composition and sequencing by Edman degradation. Peptide purity levels were estimated to be ⩾90%. For analysis of ECS within residues 61–80 of the 19 000 MW protein, a series of 15mer peptides, overlapping by 1 amino acid residue (47–61 → 80–94; PEPSCAN), were synthesized by simultaneous multiple pin synthesis technology[20] and obtained from Cambridge Research Biochemicals Ltd (Northwich, UK). Peptides were cleaved from the pins by exposure to 0·05 M hepes buffer (pH 7·8), freeze-dried and resuspended in sterile phosphate-buffered saline (PBS) prior to use. The average peptide yield was 55 nmol (~130 μg).

Mycobacterial proteins

Two preparations of recombinant *M. tuberculosis* 19 000 MW protein were used: (i) partially purified recombinant glutathione-S-transferase (rGST)—19 fusion protein prepared from cell lysates of the overexpressing *Escherichia coli* (*E. coli*) strain MC1061;[17] and, (ii) purified 19 000 MW protein (19K-PC-2) (Hewinson *et al.*, manuscript in preparation).

Antibodies

The following anti-MHC class II antibodies were obtained from culture supernatants and used at a 1/10 dilution; M5/114 (anti-H-2Aβ^b), MK-D6 (anti-H-2Aβ^d), and 14.4.4S (anti-H-2Eα) (kindly provided by Professor R. Lechler, Department of Immunology, Royal Postgraduate Medical School, Hammersmith Hospital, London UK). H40.483.1 (anti-H-2A^k) was obtained as a purified antibody and used at a final concentration of 10 μg/ml (kindly provided by Dr D. Oliveira, University of Cambridge, Cambridge, UK).

Immunization procedure and T-cell proliferation assay

Mice (groups of three) were immunized subcutaneously in the hind footpads with 30 nmol synthetic peptide, 5 nmol recombinant 19 000 MW protein or PBS emulsified in incomplete Freund's adjuvant. Eight to 10 days later the draining popliteal lymph nodes (LN) were aseptically removed, pooled and lymph node cells (LNC) (4×10^5 cells/well) cultured with antigen in 96-well microtitre plates (Nunc, Roskilde, Denmark) in complete tissue culture medium (RPMI-1640 supplemented with 10% heat-inactivated fetal calf serum (FCS) (Gibco, Paisley, UK), 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin sulphate and 5×10^{-5} M 2-mercaptoethanol (2-ME), or HL-1 serum-free medium (Ventrex Laboratories, Portland, OR) supplemented with L-glutamine, 2-ME and penicillin–streptomycin as indicated above. Cultures were incubated for 3 days at 37°, radiolabelled with 37 kBq/well [^{3}H]thymidine (Amersham International, Amersham, UK) and 6–8 hr later harvested onto glass-fibre filter paper. [^{3}H]thymidine incorporation was quantitated by liquid scintillation counting.

T-cell lines

Long-term peptide and protein-specific murine T-cell lines were established from primed popliteal LNC by repeated rounds of stimulation with antigen followed by a resting period as described in detail elsewhere.[21] For analysis of specificity, T-cell lines (2×10^4 cells/well) were cultured *in vitro* for 3 days in the presence of antigen (0–50 μg/ml) and irradiated syngeneic spleen cells (3×10^5 cells/well) and [^{3}H]thymidine incorporation determined by liquid scintillation counting.

T-cell hybridomas

Hybridomas specific for peptide p61–80 were established by a standard fusion protocol[22] using polyethylene glycol 1500 (PEG 1500, Boehringer, Mannheim, Germany) in conjunction with the TCR-negative fusion partner BW5147$_{\alpha\beta-}$. Briefly, popliteal LNC from peptide p61–80-primed mice were cultured (4×10^6 cells/ml) in 25 cm^2 flasks (Costar, Cambridge, MA) in the presence of 12 μM peptide p61–80. After 4 days, blast cells were isolated by centrifugation over Ficoll–Hypaque (Pharmacia, Uppsala, Sweden), fused with BW5147, and distributed into 96-well flat-bottom microtitre plates (Nunc, Roskilde, Denmark). Hybridomas were selected by culturing in complete RPMI-1640 medium supplemented with hypoxanthine aminopterin thymidine (HAT) (Boehringer, Mannheim, Germany). Growing hybridomas were transferred to 24-well plates (Nunc) and subsequently screened for specificity by incubation with peptide (10 μM) and irradiated syngeneic spleen cells (3×10^5 cells/well) in 96-well plates (Nunc). After 24 hr, culture supernatants (50–100 μl/well) were removed and tested for interleukin-2 (IL-2) content using the HT-2 indicator cell line.

Peptide-specific hybridomas were further expanded and routinely maintained by growing in 25 cm^2 flasks in complete medium supplemented with hypoxanthine thymidine (HT) (Boehringer, Mannheim, Germany). Prior to assay, hybridoma cells were washed extensively with culture medium to remove HT and then incubated ($2-5 \times 10^4$ cells/well) in the presence of irradiated spleen cells (3×10^5 cells/well) and antigen at appropriate concentrations. After 24 hr culture supernatants (50–100 μl/well) were removed, frozen for at least 2 hr and the IL-2 content subsequently assayed with the HT-2 indicator cell line.

Relative stimulatory capacity of peptide analogues

Analogues of the truncated 15mer peptide, p65–79, containing single amino acid substitutions at residues 70–77, were synthesized by standard Fmoc technology. Analogue peptides were titrated over a range of concentrations (0·03 to 26 μM) and assayed for their capacity to stimulate T-cell hybridomas specific for peptide p61–80. The relative stimulatory capacity of each analogue peptide was calculated as the ratio of the concentration of the native unsubstituted peptide (p65–79) required for 30% maximal response to the same value determined for the analogue peptide.[13] Data are presented from a single experiment which was repeated at least two times and gave similar results.

RESULTS

Immunodominant T-cell recognition of the p61–80 epitope

A T-cell line specific for the 19 000 MW protein of *M. tuberculosis* was established from LNC of C57BL/10 mice primed with rGST-19 protein. When stimulated with a series of overlapping 20mer synthetic peptides spanning the entire sequence of the molecule, the T-cell line responded almost exclusively to an immunodominant epitope contained within residues 61–80 (Fig. 1). A considerably weaker, but consistent response was also elicited by peptide 111–130. The response to this epitope was unexpected given that previous studies have shown that this peptide, unlike p61–80, is non-immunogenic in C57BL/10 mice.[17] The remaining peptides elicited either weak or negligible T-cell proliferative responses.

Localization of p61–80 epitope cores in three H-2 haplotypes

We have previously shown that the immunodominant p61–80 epitope is immunogenic in a diverse range of inbred mouse strains including H-$2^{b, k, d, s}$ and ^{f} haplotypes.[18] Consequently, it was of interest to determine the localization of ECS recognized within p61–80, in the context of different H-2 molecules. To address these questions, p61–80-specific T-cell lines were established from three different inbred strains of mice (C57BL/10, BALB/c and BALB.K) and tested for recognition of a panel of synthetic peptides (34 in total; prepared by multiple pin synthesis technology), each overlapping by a single amino acid and spanning residues 61 to 80. The ECS recognized by peptide-specific T cells of H-2^b (residues 71–76), H-2^d (70–76) and H-2^k (69–76) haplotypes were highly overlapping (Tables 1 and 2). To determine whether antigen processing influenced the specificity of the ECS recognized, 19 000 MW protein-immune LNC from the H-2^b compatible strains C57BL/10 or BALB.B were cultured *in vitro* with the same series of overlapping peptides. No major differences were observed in the ECS recognized by peptide and protein-immune T cells (Tables 1 and 2).

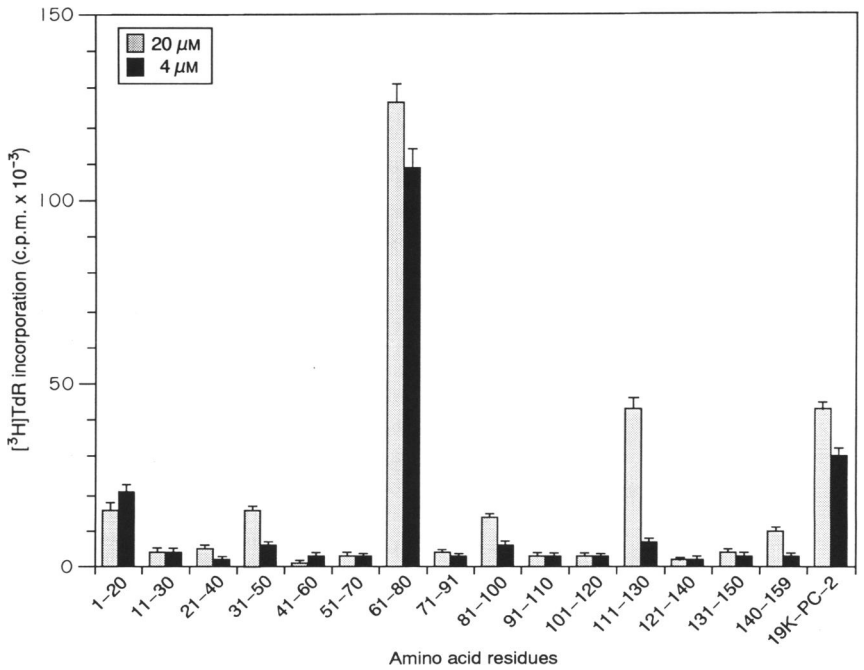

Figure 1. T-cell recognition of the immunodominant p61–80 epitope from the 19 000 MW prtoein. A T-cell line derived from rGST-19-immune LNC was stimulated (2×10^4 cells/well) with overlapping 20mer peptides (20 and 4 μM) or purified 19 000 MW protein in the presence of irradiated syngeneic (C57BL/10) APC (3×10^5 cells/well). Data are represented as mean incorporation of [^{3}H]thymidine of triplicate microcultures ± SEM following 3 days *in vitro* culture.

Table 1. Pepscan analysis of epitope cores within residues 61–80

Pepscan peptide	Sequence	Peptide immune*			Protein immune†	
		C57BL/10	BALB/C	BALB.K	C57BL/10	BALB.B
61–75	VTGSVVCTTAAGNVN	3·4	2·0	4·1	0·1	1·5
62–76	TGSVVCTTAAGNVNI	**66·4**	**9·9**	**12·1**	**18·7**	**9·9**
63–77	GSVVCTTAAGNVNIA	**50·2**	**14·4**	**13·1**	**22·0**	**26·1**
64–78	SVVCTTAAGNVNIAI	**82·1**	**15·1**	**30·3**	**24·4**	**20·6**
65–79	VVCTTAAGNVNIAIG	**72·1**	**29·2**	**24·4**	**17·4**	**16·6**
66–80	VCTTAAGNVNIAIGG	**73·5**	**15·5**	**30·6**	**22·3**	**27·9**
67–81	CTTAAGNVNIAIGGA	**66·6**	**12·3**	**20·9**	**20·8**	**31·7**
68–82	TTAAGNVNIAIGGAA	**121·8**	**4·3**	**19·9**	**13·4**	**16·7**
69–83	TAAGNVNIAIGGAAT	**56·8**	**4·5**	**6·7**	**6·2**	**3·1**
70–84	AAGNVNIAIGGAATG	**21·0**	**4·0**	2·9	2·4	0·7
71–85	AGNVNIAIGGAATGI	**9·5**	2·5	0·5	0·4	0·2
72–86	GNVNIAIGGAATGIA	1·6	2·0	3·7	0·8	0·5
61–80	VTGSVCTTAAGNVNIAIGG	**85·0**	**58·1**	**49·2**	**28·3**	**37·2**
19 000 MW protein		nd	nd	nd	**26·8**	**47·2**

* Peptide p61–80-specific T-cell lines or † LNC from 19 000 MW protein-immune (19K-PC-2) mice were cultured *in vitro* with pepscan peptides (13 μM). p61–80 (20 μM), or 19 000 MW protein (2·5 μM) and proliferative responses (Δ c.p.m. $\times 10^{-3}$) determined. Background c.p.m. (cells without antigen) were: (C57BL/10, 2325; BALB/c, 2104; and BALB.K, 6793 for T-cell lines) and (C57BL/10, 7852; BALB.B, 6640) for protein-immune LNC. Positive responses (c.p.m. with peptide > background c.p.m. + 3 × SD) indicated in bold type. Responses to pepscan peptides that mapped outside the epitope core were negative and are not shown. nd = not determined.

T-cell response to truncated peptides

Based on the results of the epitope-core mapping experiments, a series of truncated p61–80 peptides were synthesized and assayed for their capacity to stimulate p61–80-specific T-cell hybridomas derived from H-$2^{b, d}$ and ^{k} mice (Table 3). The H-2^{b}-derived hybridoma B10.7.6 responded with equal sensitivity when stimulated with either the p61–80 20mer peptide or the truncated 15mer peptide (residues 65–79). Although peptides shorter than 15 amino acids in length retained their capacity to stimulate, a significant decrease (10–100 times) in stimulatory capacity was observed with the 12, 10 and 9mer truncated peptides. Hybridoma B10.7.6 failed to recognize the 8mer truncated peptide (residues 70–77). Hybridomas derived from H-2^{d} (BC.7.31) and H-2^{k} (BK.7.46) mice respectively, responded in an identical fashion to the 20 and 15mer peptides but failed to recognize any other truncated peptides.

Table 2. Localization of T-cell epitope core sequences

Priming antigen*	In vitro antigen†	Haplotype	Epitope core sequence‡
p61–80	p61–80 PS	d	VTGSVVCTT**AAGNVNI**AIGG
p61–80	p61–80 PS	k	VTGSVVCTT**AAGNVNI**AIGG
p61–80	p61–80 PS	b	VTGSVVCTT**AAGNVNI**AIGG
19 000 MW protein	p61–80 PS	b	VTGSVVCTT**AAGNVNI**AIGG

*Antigen-specific T cells were cultured *in vitro* with p61–80 † PEPSCAN (PS) peptides (13 μM). ‡ The localization of epitope core sequences within the 20mer peptide (p61–80) are shown in bold type.

Class II MHC restriction

To investigate whether responses to p61–80 were restricted by I-A or I-E class II MHC molecules when recognized in the context of different H-2 alleles, p61–80-specific T-cell hybridomas derived from H-$2^{b, d}$ and ^{k} haplotypes were stimulated in the presence of anti-MHC class II antibodies. All three hybridomas failed to respond in the presence of anti-I-A-specific antibodies, in contrast anti-I-E antibodies had only a marginal effect on recognition (Table 4). These results demonstrate that p61–80 is recognized in the context of multiple I-A class II MHC molecules.

Stimulatory capacity of p61–80 analogues

A series of 15mer analogue peptides containing single alanine or leucine amino acid substitutions within the p61–80 epitope core (residues 70 to 77) were tested for their capacity to stimulate a panel of p61–80-specific T-cell hybridomas derived from H-$2^{b, d}$ and ^{k} mice (Table 5). Analysis was restricted to these eight core residues as amino acid substitution (alanine or leucine) of residues flanking the epitope core did not significantly alter the stimulatory capacity of the peptide (data not shown). Analogue peptides were titrated over a range of concentrations (26 to 0·03 μM) and the stimulatory capacity of each analogue, relative to the unsubstituted peptide (p65–79) determined. Although each of the eight core residues was critically required for recognition by at least one of the nine hybridomas tested, the only critical substitution common to all hybridomas was ^{74}V → A. BALB/c-derived hybridomas responded in a relatively uniform manner and were characterized by their ability to tolerate an alanine substitution at residue ^{73}N, unlike the B10 and BALB.K-derived hybridomas which were generally more

Table 3. T-cell recognition of truncated peptides

Peptide	Amino acid sequence	T-cell recognition B10.7.6	BC.7.31	BK.7.46
61–80	VTGSVVCTTAAGNVNIAIGG	0·35*	0·5	5·5
65–79	----VVCTTAAGNVNIAIG-	0·25	0·5	6·0
67–78	------CTTAAGNVNIAI--	2·5	>100	>100
69–78	--------TAAGNVNIAI--	3·0	>100	>100
69–77	--------TAAGNVNIA---	22	>100	>100
70–77	---------AAGNVNIA---	>100	>100	>100

*Data are presented as the concentration of peptide (μM) required for 50% of the maximum stimulation of each p61–80-specific T-cell hybridoma.

sensitive to substitution at this residue. Although we have restricted our analysis to only three hybridomas from each strain, the pattern of reactivity of B10 and BALB.K-derived hybridomas was, unlike the BALB/c hybridomas, more heterogeneous with regard to the effect of substitutions.

DISCUSSION

Because of allelic variability within the peptide-binding site of MHC class II molecules, most epitope-specific T-cell responses are restricted by a single H-2 allele.[11] In contrast, previous analysis of the immunodominant p61–80 T-cell determinant from the *M. tuberculosis* 19 000 MW protein has revealed recognition in a genetically permissive manner.[17,18] One of the main aims of the present study was to characterize the structural basis of permissive recognition of p61–80 by detailed analysis of epitope core structure in three murine haplotypes, H-$2^{b, d}$ and ^{k}, using the pepscan method of epitope scanning. The ECS recognized by peptide-specific T cells from each haplotype were highly overlapping and composed of 6–8 amino acid residues (Table 2). Similar ECS were also recognized following priming of H-2^{b} mice with intact 19 000 MW protein, indicating that antigen processing did not alter T-cell specificity. Differences in the fine processing of an immunodominant ovalbumin epitope by MHC-matched APC lymphoblastoid B-cell lines have been reported.[23] In our study however, identical ECS were recognized by 19 000 MW protein-immune T cells from C57BL/10 and BALB.B mice (both H-2^{b}), indicating that the different non-MHC background genes expressed in these congeneic strains did not influence T-cell fine specificity.

Table 4. MHC class II restriction of responses to p61–80

Hybridoma	Haplotype	Proliferation (c.p.m. × 10^{-3}) No Ab	anti-I-A*	anti-I-E	MHC restriction
B10.7.6	b	116·14†	0·10	118·32	I-A^{b}
BC.7.13	d	150·98	2·94	99·12	I-A^{d}
BK.7.46	k	238·26	1·32	218·07	I-A^{k}

*Specificities of anti-class II antibodies used are described in the Materials and Methods. † Proliferative response of T-cell hybridomas cultured with p61–80 (0·4–4 μM) in the presence or absence of anti-class II antibodies and irradiated syngeneic APC. Background proliferation (c.p.m. × 10^{-3}) of T-cell hybridomas in the absence of antigen were <0·2.

Although p61–80-specific T cells from different haplotypes recognized overlapping ECS, significant differences were observed in the minimal peptide length required for the stimulation of T-cell hybridomas. For example, while the truncated 9mer peptide (residues 69–77) retained the capacity to stimulate the C57BL/10-derived hybridoma B10.7.6, albeit only at higher peptide concentrations, hybridomas derived from BALB/c and BALB.K mice were more stringent in their requirement for additional flanking residues. These results indicate that the structural requirements for recognition of truncated p61–80 peptides vary among different haplotypes. It is apparent from this and other studies[24] that the minimal ECS is not necessarily sufficient for the activation of all T-cell clones and additional flanking residues may be important in that they increase the relative binding affinity of the peptide for MHC class II molecules[25] or increase the stability of TCR interactions with peptide-MHC molecules.

Peptide p61–80 is recognized in the context of multiple H-2 I-A molecules as antibodies specific for I-$A^{b, d}$ or ^{k} completely inhibited the proliferative response of T-cell hybridomas. Allele-specific I-A binding motifs[7,12,26] were not observed in either the p61–80 ECS or in the promiscuously recognized p350–369 epitope from the *M. tuberculosis* 38 000 MW protein.[16] In contrast, the majority of epitopes previously described as permissive or promiscuous are recognized in the context of either human leucocyte antigen (HLA)-DR or the murine H-2 I-E molecule, both of which express a conserved α chain. A number of possibilities have been suggested to explain permissive binding of peptides to HLA-DR. Analysis of DR-binding peptides produced from M13 phage libraries[8] has revealed highly conserved anchor residues at relative positions 1 and 4 (designated promiscuous anchors) and variable anchors at position 6 (designated allele-specific anchors). It has been postulated that anchor residue side chains at positions 1 and 4 interact with a MHC binding pocket composed of conserved α- and β-chain residues, while the allele-specific anchor residues interact with polymorphic β-chain residues. According to this scenario, promiscuous binding would be favoured by peptides containing dominant anchor residues at positions 1 and 4 and non-allele-specific residues at position 6.[8] Alternatively, studies with polyalanine substituted peptides[27] have shown that

Table 5. Relative stimulatory capacity of p61–80 analogues*

T cell hybridoma	Residue p65–79	Amino acid substitution 70 A→L	71 A→L	72 G→A	73 N→A	74 V→A	75 N→A	76 I→A	77 A→L
B10.7.6	+ + (0·26)†	+ +	+ +	+	↓	↓	+	↓	↓
B10.7.37	+ + (0·12)	↓	+	+	↓	↓	↓	↓	+
B10.7.29	+ + (0·09)	↓	+	↓	↓	↓	+ +	↓	↓
BC.7.13	+ + (1·0)	↓	↓	↓	+ +	↓	↓	↓	↓
BC.7.24	+ + (2·2)	+	+ +	↓	+ +	↓	↓	↓	↓
BC.7.31	+ + (0·24)	+	↓	↓	+ +	↓	↓	↓	↓
BK.7.14	+ + (2·8)	+ +	↓	↓	↓	↓	+ +	+ +	↓
BK.7.46	+ + (3·0)	↓	↓	+ +	+ +	↓	↓	+	+
BK.7.19	+ + (2·0)	+ +	↓	↓	↓	↓	↓	↓	↓

*Peptides containing single alanine or leucine substitutions at residues 70 to 77 were incubated with T-cell hybridomas and the *relative stimulatory capacity* of each analogue determined (= ratio of concentration of the native unsubstituted peptide, p65–79 required for 30% maximal response to the same value determined for the substituted peptide. †Values shown in parentheses = μM concentration of the unsubstituted peptide p65–79 required for 30% maximal response. Relative stimulatory capacity $\geq 0{\cdot}1$ = + +; $< 0{\cdot}1 \geq 0{\cdot}01$ = +; $< 0{\cdot}01$ = ↓.

the majority of amino acid side chains do not contribute significantly to peptide–MHC binding and that a significant amount of the binding energy may be derived from hydrogen bonding between the MHC molecule and the peptide backbone as reported with class I binding peptides.[28] This idea is supported by reports indicating that promiscuous-binding self-peptides derived from the invariant chain are characterized by a lack of aromatic residues. The absence of large side chains would be expected to promote efficient hydrogen bonding and may contribute to the high binding affinities reported for these promiscuous peptides.[5]

The mechanisms underlying I-A mediated permissive recognition of p61–80 are likely to be more complex given the polymorphic nature of both α and β chains. Analysis of individual hybridomas derived from the same haplotype revealed unexpected differences in the response to alanine substituted peptides (Table 5). Given that each individual hybridoma is presented with the same peptide–MHC complex, differential recognition of a particular substituted peptide implies that hybridomas may utilize different TCR. Differential V_β gene usage has previously been observed among hybridomas responsive to a defined peptide epitope from myoglobin.[29] However, we cannot discount that a given peptide may interact with the same MHC molecule in different configurations as reported in a number of other studies.[30,31] From the differential response of hybridomas in respect of certain amino acid substitutions (e.g. B10. ^{70}A-L) we infer that lack of recognition results from a failure to engage the TCR appropriately, as opposed to a failure in binding the MHC molecule. Additional experiments in which substitutions other than alanine were introduced at the same position provide support for this hypothesis (unpublished observations). However, in cases where a substitution resulted in a complete loss of recognition for all three hybridomas (e.g. B10 ^{74}V-A) it is not possible to determine whether this effect is MHC or TCR related and alternative strategies will be required for more precise definition of the epitopic, agretopic or dual role of residues within the epitope core.

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