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Efficient binding to the MHC class I K^d molecule of synthetic peptides in which the anchoring position 2 does not fit the consensus motif

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Abstract Peptides eluted from the MHC class I K^d molecule are generally nonamers that display a strong preference for Tyr in position 2 and Ile or Leu in position 9. We investigated the binding ability of several synthetic peptides which did not fit this consensus motif. In our peptides, Tyr^2 was substituted by other amino acids, i.e. Leu, Ile or Met. These peptides were variants of the 252–260 K^d-restricted peptide SYIPSAEKI derived from the *Plasmodium berghei* circumsporozoite protein. They bound to purified K^d molecules in vitro with intermediate affinity. One of them was tested for in vivo stimulation of T cells and induced a cytotoxic response. These results demonstrate the importance of binding motif refinement to discover new binding characteristics and new ligands such as low-affinity peptides.

Key words: Major histocompatibility complex I; Antigenic peptide; Binding motif; MHC affinity; Cytotoxic T lymphocyte; H-2K^d

1. Introduction

Major histocompatibility complex (MHC) class I molecules bind short peptides derived mainly from endogenously synthesized proteins [1]. Peptide binding to the class I molecule occurs in the endoplasmic reticulum (ER) and is followed by the egress of MHC/peptide complex to the cell surface. Allele specific cytotoxic T lymphocytes (CTLs) can recognize complexes expressing virus- or tumour-derived peptides.

Crystallographic studies of the MHC molecules in complex with peptides [2,3], chemical analyses of mixtures of naturally occurring peptides bound to MHC alleles [4] and binding tests performed either with individual synthetic peptides [5,6] or with peptide libraries [7] revealed biochemical details of MHC-peptide interactions. These studies identified allele-specific motifs containing critical or 'anchor' residues at defined positions within the sequences of peptides that bind to each particular allele. Peptides bound to class I molecules are 8–10 residues long. In the case of the murine MHC class I molecule K^d, the motif consists of a 9-mer sequence with Tyr or Phe in position 2 counting from the N-terminus (Tyr^2 or Phe^2) and an aliphatic amino acid in the C-terminus [8]. All studies carried out so far have demonstrated the importance of Tyr^2 for efficient binding to H-2K^d [5,9-11]. However, allele-specific motifs are necessary for binding but not sufficient by themselves [12]. Several studies were performed to determine the influence at non-anchor positions of positive and negative interactions. These studies consisting of binding motif refinement used individual peptides in competitive assays. Results indicated that some residues have a harmful effect on binding. In this way especially Pro or acidic residues should be avoided at position 3 as well as Phe or charged residues at position 5 in the sequences of particular K^d ligands [13,14].

Therefore, to confirm these observations and extend the refinement at other positions, we recently used synthetic peptide libraries in binding assays with the K^d molecule [15]. In a parent K^d binding sequence, SYIPSAEYI named 2Y in this study, an analog of the 252–260 antigenic peptide derived from the *Plasmodium berghei* circumsporozoite protein, each position was replaced by all natural amino acids (except Cys). Each library obtained for the substitution of one position in the parent sequence was then tested for selection by the K^d molecule. Our work showed that when the library binding tests were carried out in noncompetitive conditions a much broader set of peptides binds to H-2K^d than expected from previous studies. The pattern of accepted residues varied at each position. Positions 2, 3 and 9 were quite restrictive while others were more permissive. In addition to the selection of Tyr and Phe at position 2, the selection of three other residues, i.e. Leu, Ile and Met, was the most surprising result of this study.

Here we report that the new H-2K^d ligands identified in our former study, which do not fit the consensus motif because Tyr^2 was substituted by Leu, Ile or Met, are intermediate-affinity peptides. The affinity of the new ligands was compared with the affinity of the parent peptide. This peptide was previously shown to be K^d restricted [12]. The binding assays were performed with a single-chain, soluble H-2K^d molecule (SC-K^d) [16]. The C-terminal end of the α_3 domain of this molecule is connected to the N-terminal end of mouse β_2-microglobulin. This fusion protein had been previously shown to be functionally empty, to bind the same repertoire as the native cell-surface expressed H-2K^d molecule [12] and to specifically stimulate K^d-restricted T cells [17]. The relative affinities of the new ligands were evaluated by competitive binding of radiolabeled control K^d-restricted peptide to purified SC-K^d. The binding affinities were slightly affected by the substitution of Tyr^2 by Leu, Ile or Met and the new ligands

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Abbreviations: CHO, Chinese hamster ovary; CTL, cytotoxic T lymphocyte; ER, endoplasmic reticulum; FCS, fetal calf serum; HBSS, Hank's balanced salt solution; HPLC, high-performance liquid chromatography; IFA, incomplete Freund's adjuvant; mAb, monoclonal antibody; MALDI, matrix-assisted laser desorption and ionization; MHC, major histocompatibility complex; PBS, phosphate-buffered saline; TAP, transporter associated with antigen processing

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correspond to intermediate binders according to the classification of Grey et al. [18]. The best competitor was tested for its ability to induce CTLs in vivo as well as positive and negative controls and a peptide with a very weak binding affinity. The identification of such intermediate- or even low-affinity peptides capable of eliciting CTL response may be useful for developing new therapeutic approaches.

2. Materials and methods

2.1. Peptides and peptide synthesis

Peptides (Table 1) were prepared by automated solid-phase synthesis using Fmoc chemistry and Wang resins on an Applied Biosystems Peptide Synthesizer (Model 431A, Foster City, CA, USA). Side-chain protecting group was OtBu for Glu, Ser and Tyr. Fmoc amino acids were coupled after activation with dicyclohexylcarbodiimide/1-hydroxybenzotriazole. Fmoc deprotection of the peptidyl-resin before each coupling was performed with 20% piperidine in *N*-methylpyrrolidone. Side-chain deprotection and cleavage from the solid support were achieved in trifluoroacetic acid with phenol as a scavenger [19]. Peptides were precipitated in diethyl ether. They were purified by reversed-phase HPLC (RP-HPLC). Their purity was then assessed by analytical RP-HPLC and MALDI mass spectrometric analysis.

2.2. Peptide binding assays

The soluble single chain K^d, SC-K^d, molecule was expressed in transfected CHO cells as previously described [20]. SC-K^d was purified from the culture medium by affinity chromatography using the K^d-specific SF1-1.1.1 monoclonal antibody (mAb).

The relative affinities of peptides were determined in a competition binding assay. The K^d-restricted control peptide, 2Y, was labeled using chloramine T-catalyzed iodination to a specific activity of about 60 Ci/mmol [21]. Free iodine was removed by ion exchange chromatography on an AG11A8 column (BioRad, France). 10 ng (9.6 pmol) of labeled peptide 2Y* was incubated in PBS with 100 ng (2 pmol) of SC-K^d in the presence of increased concentration of the test peptide for 1.5 h at room temperature. Peptide-MHC complexes were immunoprecipitated using SF1-1.1.1 mAb. Radioactivity in the precipitated pellet was measured in a γ-counter (Gammamatic, Kontron). Reduction of the radioactivity in the pellet indicates competitive binding of the test peptides. The binding capacity of each competitor is determined according to the IC_{50} and the relative affinity. IC_{50} is the concentration of the competitor peptide that inhibits the radiolabeled control peptide binding by 50%. The relative affinity of each competitor was calculated as previously described [22]. Briefly, $K_{rel} = IC_{50}/(1 + [2Y^*]/K_D^*)$ where $[2Y^*] = 0.4$ μM and K_D^* is the affinity of radiolabeled control peptide (30 nM) [12].

2.3. Mice

6–8-week old BALB/c (H-2^d) female mice were obtained from the National Institute of Medical Research (Mill Hill, London, UK).

2.4. Cells

Target cells used in cytotoxicity assays were P815 (H-2^d, MHC class I^+ and MHC class II^-) mastocytoma cells and were grown in RPMI 1640 medium supplemented with 10% fetal calf serum. Peptide-pulsed P815 cells were prepared for use as targets by prior incubation with a 100 μl volume of the appropriate concentration of peptide.

2.5. Immunizations

BALB/c mice were injected subcutaneously (s.c.) with 100 nmol of 2Y and 2L dissolved in PBS and emulsified in incomplete Freund's adjuvant (IFA) (1:1). After 14 days, mice were killed and spleen cells were removed for in vitro lymphocyte restimulation with designated peptides for 7 days, in 10% concanavalin A supernatant containing medium, which acts as a source of interleukin-2.

2.6. CTL assay

P815 (H-2^d) target cells were labeled with ^{51}Cr by incubation with 200 μCi $Na^{51}CrO_4$ (NEN Research Products, Boston, MA) for 1 h. After washing, 10^6 P815 cells were pulsed with each peptide (50 nmol in 100 μl HBSS) at 37°C for 1 h. The unbound peptide was removed by three washes with HBSS (Gibco Laboratories, Uxbridge, UK). Graded numbers of effector cells were mixed with 10^4 peptide pulsed labeled target cells in RPMI 1640 medium containing 10% FCS in the wells of round-bottom microtiter plates, followed by incubation at 37°C for 6 h. Supernatant (100 μl) was taken from each well and the radioactivity detected in a γ-counter. The percentage of specific lysis was determined using the formula: [(cpm released by CTL−cpm released with medium alone)/(cpm released by detergent−cpm released with medium alone)] × 100. The detergent used to determine maximum release was 5% Triton X-100. Spontaneous release was determined from target cells incubated without the addition of effector cells. The assay was performed in triplicate.

3. Results

3.1. Peptide binding to SC-K^d

Different analogs of the K^d-restricted 2Y peptide were synthesized (Table 1) and their binding capacity to K^d was assessed by competition binding assay with SC-K^d. Competitive binding activities of 2L and 9A are represented in Fig. 1. With increasing concentration of 2L, the binding of ^{125}I-labeled control peptide SYIPSAEYI was significantly reduced. In the case of 9A, the bound radioactivity was displaced only when a large concentration of competitor was used. When a 7.5 molar excess of competitor was used (i.e. 3 μM of competitor), the binding capacity of 2L, 2M and 2I was slightly lower than that of the unlabeled control peptide (Fig. 2). However, they were high enough to displace more than 50% of the bound radioactivity while the other peptides 2A, 9A and 2A9A, under the same conditions, showed no significant binding. When a large excess of competitor was used (i.e. 150 molar excess that is equivalent to a competitor concentration of 60 μM), 2A and 9A were able to bind while 2A9A had still no competitive binding activity. IC_{50} values ranged from 1 to 2 μM for 2L, 2M and 2I. 2L bound to SC-K^d most efficiently with an IC_{50} of 1 μM (relative affinity ~70 nM). 2A and 9A bound less efficiently with IC_{50} values over 20 μM and were weak binders for SC-K^d.

3.2. Induction of CTL

The immunogenic activities of a strong, an intermediate, a weak and a non-binder to SC-K^d (i.e. respectively 2Y, 2L, 9A and 2A9A) were assessed using CTL from BALB/c mice immunized with 100 μg of the designated peptide emulsified in IFA. CTL activity was tested in a ^{51}Cr release assay against peptide loaded P815 cells.

Lymphocytes from mice immunized with 2Y expressed high level CTL activity while lymphocytes from mice immunized with 2L and 9A showed moderate to weak cytolytic activity (Fig. 3). Target cells without peptide and cells loaded with the

Table 1
Peptide sequences used in this work and their binding affinity for SC-K^d

Code	Residue position									K_D(nM)
	1	2	3	4	5	6	7	8	9	
2Y	S	Y	I	P	S	A	E	Y	I	30[a]
2L	–	L	–	–	–	–	–	–	–	70[b]
2I	–	I	–	–	–	–	–	–	–	140[b]
2M	–	M	–	–	–	–	–	–	–	77[b]
2A	–	A	–	–	–	–	–	–	–	2094[b]
9A	–	L	–	–	–	–	–	–	A	1396[b]
2A9A	–	A	–	–	–	–	–	–	A	n.d.

[a]K_D was determined previously by Scatchard analysis [12].
[b]Relative binding affinity was evaluated as described in section 2.

non-binder 2A9A peptide were lysed to a very weak extent (Fig. 3).

4. Discussion

Many studies have already shown that peptides, in spite of bearing the allele-restricted motif, did not bind to the considered allele. For example, several peptides found in the sequence of antigenic proteins, which displayed the reported K^d-restricted motif, had no affinity for K^d [13,23]. Therefore, the screening for MHC binding peptides in the sequence of antigenic proteins merely based on the detection of anchor residues cannot ensure that peptides thus selected, then resynthesized by chemical methods, and tested for their capacity to induce a CTL response could prove efficient binders. Several additional refinements must be carried out and particularly positive and negative interactions at non-anchor positions must be elucidated in order to allow good prediction of binding peptides.

In the work reported here, we confirm that some peptides devoid of the Tyr^2 anchor residue are nevertheless able to bind to K^d. The peptides 2L, 2M and 2I were first identified in a previous study in which interaction tests with the K^d molecule were carried out with peptide libraries under non-competitive conditions [15]. These peptides have a reasonable binding capacity to K^d and can be considered as intermediate binders according to the classification of Grey et al. [18]. In order to confirm the role of Leu^2, Met^2 and Ile^2 in binding, the affinities of the three peptides containing these residues were compared with the affinities of peptides in which one of the two anchor positions or both were substituted by Ala. Disubstituted 2A9A peptide was unable to bind to K^d, indicating that, in this sequence, there is no other effective anchoring position than 2 and 9. Monosubstituted peptides 2A and 9A both bind significantly to K^d but could compete with the labeled control peptide only when added in large amounts (Fig. 2). Thus, the presence of a single anchor residue confers to the ligand a pattern of weak binder. Now comparing the affinities of 2Y, 2L, 2M and 2I with the weak ligand 2A, it appears that the presence of Tyr, Leu, Met or Ile in position 2 enhances greatly the binding to nearly the same order of magnitude. These results confirm that position 2 is an integral part of the K^d motif. Leu, Ile or Met at this position are efficient substitutes of the Tyr^2 anchor residue. The affinity of the best competitor (i.e. 2L) identified in this study is only 2.5 times weaker than this of the control peptide. It seems surprising therefore that the three amino acids identified in position 2 in our previous work had never been described by the method in which naturally occurring peptides are eluted from K^d and sequenced [4]. Several reasons could be put forward: (i) no or a few peptides with Leu, Met or Ile in position 2 are processed in the cytosol by the proteasome; (ii) processed peptides with L^2, M^2 or I^2 cannot bind to TAP transporters and therefore cannot reach the ER where the association with the K^d molecule takes place; (iii) as our binding test was performed with synthetic libraries, the composition of the libraries did not necessarily resemble the natural peptide mixture existing in the ER so that our synthetic libraries could contain some peptides not naturally processed and presented.

We show in the present study that several new K^d ligands could induce a cytotoxic response. The efficacy of CTL induction seems to parallel closely the binding capacity to K^d as the

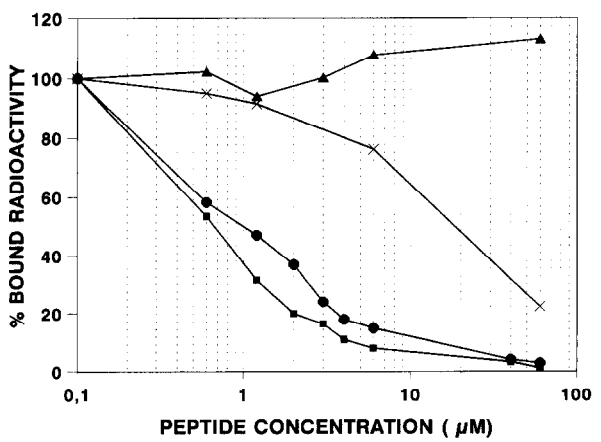

Fig. 1. Labeled control peptide binding to K^d. Different concentrations of test peptides (Table 1) were used to displace the labeled peptide. 2L (●) and 9A (×) test peptides were compared to unlabeled positive control K^d-restricted peptide 2Y (■) and to negative control 2A9A peptide (▲), which does not contain K^d-anchor residues. Each data point is the mean of duplicate experiments.

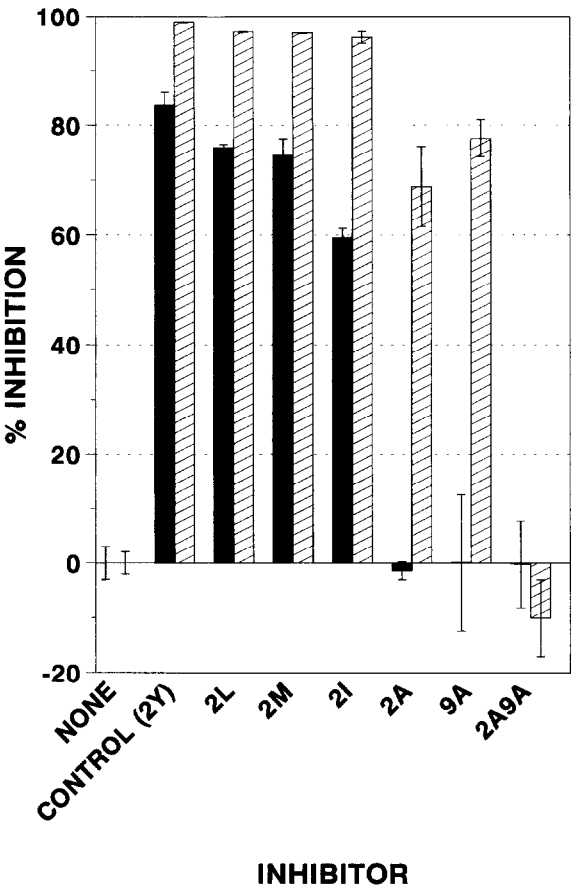

Fig. 2. Control peptide binding inhibition. Peptide binding to K^d was assayed in the competition test described in Section 2. SC-K^d (100 ng) was incubated with 0.4 μM ^{125}I-labeled K^d-restricted peptide in the presence of 3 μM (closed bar) or 60 μM (hatched bar) of unlabeled test peptide. Results are expressed as percent inhibition of labeled peptide binding.

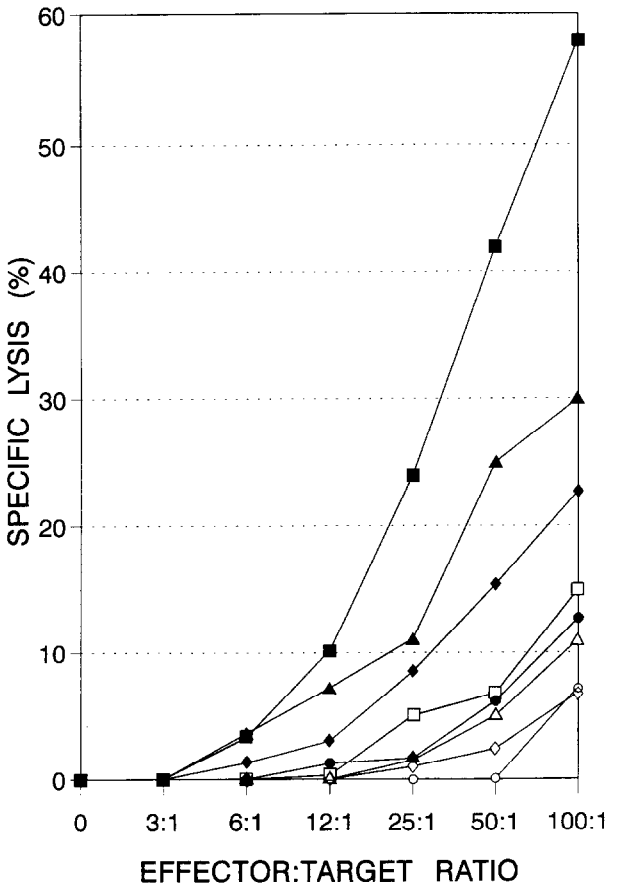

Fig. 3. Induction of CTL with SC-K^d binding peptides. BALB/c mice were injected s.c. in the tail base with 100 μg of 2Y (square), 2L (triangle), 9A (lozenge) or 2A9A (circle) emulsified in IFA. Spleen cells were removed 14 days after injection and were restimulated in vitro with designated peptides for 7 days. CTL activity was measured by the lysis of target cells with (closed symbols) or without (open symbols) specific peptide.

cytolytic activity of the strongest binder 2Y was higher than all others. Lymphocytes from mice immunized with 2L, 9A and 2A9A showed decreasing cytolytic activity in proportion with their decreasing binding affinities. Peptide affinity for the MHC was reported to be a factor contributing to the number of specific complexes expressed on the antigen presenting cell surface [20]. Peptides with high affinity induce a strong expression of peptide-MHC complexes. On the other hand, low-affinity interactions do not generate enough complexes or complexes sufficiently stable to trigger a response. However, in our work, the cytolytic activity against the weak-binder 9A was not non-existent. Therefore, by enhancing its immunogenicity either by encapsulation in liposomes or by covalent association with lipids or by co-immunization with a T-helper epitope as recently reported [24], it would certainly be possible to obtain a high cytolytic activity against it.

In the work reported here, we show that many more peptides than these described by Rammensee et al. bind to K^d [8]. In addition to the results of Rammensee et al., ours will be useful for the identification of many more T epitopes in viral or tumoral protein sequences. However, to induce a cytolytic activity in vivo, it remains to be determined whether the intermediate- and low-affinity peptides identified in this work are naturally processed and presented. If this is the case, they seem to be presented by K^d at concentrations too low to succeed in being identified by the extraction and sequencing method. However, as only a few complexes are necessary for cytolysis, these peptides may become the in vivo target of highly reactive and specific CTLs pulsed with these peptides ex vivo [25].

The recognition of peptides by class I-restricted CTLs offers the unique opportunity to develop immunotherapeutics for treating or preventing viral infections and tumour growth. Therefore, methods leading to the identification of class I binding peptides and putative T cell epitopes are of particular interest. Our method using synthetic peptide libraries with known compositions and non-competitive binding tests with the class I molecule seems the technique of choice for this purpose. It should now be exploited for the identification of new ligands of other class I molecules.

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