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Binding of alanine-substituted peptides to the MHC class I protein, K^d

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Peptides eluted from the native MHC class I molecule, K^d, are generally nonamers that display a strong preference for Tyr in position 2. We investigated the molecular basis for this 'consensus motif' by synthesizing a virally derived peptide, NP 147-155, that is known to be presented by K^d on living cells, and peptide variants of NP 147-155 in which the amino acids in the different positions were sequentially replaced by Ala. All of the peptides bound to purified K^d molecules in vitro with high affinity, except for the peptide in which Tyr^2 was replaced by Ala, for which the affinity for K^d decreased at least 100-fold. These results confirm the interpretation of the in vivo studies; namely, that Tyr^2 is a critical anchor residue for binding to K^d.

Major histocompatibility complex; Cytotoxic T lymphocyte; Antigen presentation

1. INTRODUCTION

Cytotoxic T lymphocytes (CTL) lyse virally infected and cancerous cells through a contact-dependent mechanism [1]. CTL receptors recognize aberrant antigens from the target cells in the form of short proteolytic fragments displayed on the target cell surface in association with major histocompatibility complex (MHC) class I molecules. The latter are heterodimers composed of a polymorphic, transmembrane heavy chain to which the light chain, β_2-microglobulin, is attached non-covalently [2,3].

Crystallographic analysis of the MHC class I structure has revealed that the heavy-chain N-terminal domains fold into a groove that can accommodate antigenic peptides [4–8]. Peptides bound to native class I molecules have recently been eluted and sequenced. The peptides are 8–10 residues long and contain a 'consensus motif' that depends on the particular MHC molecule purified [9]. In the case of the MHC molecule K^d, the motif consists of a 9-mer sequence with Tyr in position 2 (Tyr^2) and a hydrophobic amino acid in position 9 (the C-terminal residue).

Competition studies using functional CTL assays have also demonstrated the importance of Tyr^2 for effective inhibition of target cell lysis by antigen-specific CTL clones [10–12]. These results have been interpreted in terms of the ability of the different peptides to compete for binding to target cell surface-associated K^d molecules, but direct evidence for binding to K^d was not available. We have re-addressed this issue by synthesizing seven variants of a virally derived antigenic peptide known from in vivo studies to be presented by K^d, in which each residue of the 9-mer peptide, except Ala^7 and the C-terminal Val, was sequentially replaced by Ala. We then measured binding of each peptide to purified K^d molecules in vitro in order to assess the role of each residue in binding. We found that the binding affinities are not significantly affected by any of the Ala substitutions, except when Tyr^2 is replaced.

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Abbreviations: CTL, cytotoxic T lymphocyte; DAN, dansyl; DAN-peptide, dansylated peptide; MHC, major histocompatibility complex; SC-K^d, single-chain K^d.

2. MATERIALS AND METHODS

A single-chain K^d molecule (SC-K^d), produced by the baculovirus system, was purified by affinity chromatography as previously described [13]. The SC-K^d molecule consisted of a truncated heavy chain, lacking the transmembrane domain, to which β_2-microglobulin had been covalently linked through a 15-residue molecular bridge. This protein was soluble in the absence of detergents, and was secreted into the supernatant by the infected insect cells.

The peptides were synthesized by the standard *t*-butoxycarbonyl method [14] on an Applied Biosystems model 430A peptide synthesizer. Deprotected peptides were purified by HPLC, lyophilized, dissolved in saline buffer, and stored frozen. All peptide concentrations were determined by the BCA assay.

N-Terminal dansylation was performed as previously described [15]. Briefly, 100 μl of 2 mg/ml dansyl isothiocyanate (*p*-(5-dimethylaminonaphthalene-1-sulfonyl)aminophenylisothiocyanate, from Molecular Probes) in dimethylformamide was added dropwise to 4 mg peptide in 1 ml 50 mM phosphate buffer, pH 7.4, at 4°C. The reaction was carried out at room temperature for 2 h, after which dansylated peptide (DAN-peptide) was separated from unmodified peptide and free dansyl by HPLC. Labelled peptide was lyophilized and stored frozen in saline buffer.

The fluorescence of DAN-peptide was measured as a function of time at an excitation wavelength of 340 nm and an emission wavelength of 520 nm in a Perkin-Elmer spectrometer using a magnetically stirred, thermostatted cuvette.

3. RESULTS AND DISCUSSION

A soluble, single-chain molecule of K^d, SC-K^d, consisting of the heavy chain of K^d covalently linked to β_2-microglobulin, was chosen for the peptide binding studies. This molecule has the advantage of efficiently binding low concentrations of peptides with the expected specificities in the absence of exogenously added β_2-microglobulin [13], thus simplifying the analysis of measurements in which the class I molecules must be diluted.

Peptide binding was determined using a competition assay with dansylated peptides (DAN-peptide). It was recently shown [15], that binding of DAN-peptide to SC-K^d results in fluorescence enhancement, due to transfer of the dansyl moiety from water to the peptide binding groove of the class I molecule. The binding is MHC allele specific since fluorescence enhancement could be inhibited by SC-K^d pre-incubation with unlabelled peptides known to bind to K^d, but not by peptides that bind to other MHC molecules. Furthermore, the spontaneous dissociation of the SC-K^d/DAN-peptide complex could be conveniently measured by pre-incubating 2 μM DAN-peptide with a high concentration (2 μM) of SC-K^d, and then diluting this mixture 10-fold directly into the spectrofluorometer cuvette. The spontaneous dissociation is extremely slow, but can be accelerated by addition of unlabelled peptides that bind to K^d. This observation became the basis for a 'dilution assay' in which the binding affinities of a series of unlabelled peptides could be evaluated by comparing their ability to accelerate the time–course of the fluorescence decrease due to dissociation of the SC-K^d/DAN-peptide complex [15].

We first compared three peptides with known binding affinities in the dilution assay. Two of the peptides, PbCS 253-260 and PbCS 249-260, are derived from the circumsporozoite protein of *Plasmodium berghei*, and have previously been shown to sensitize target cells for lysis by K^d-restricted CTL. The former peptide binds to K^d-expressing cells with high affinity, whereas the latter, a 12-mer, was estimated to be about 2% as effective as PbCS 253-260 in functional competition assays in vivo [12]. The third peptide, NP 366-374, chosen as a negative control, is derived from the influenza nucleoprotein, and is known to be presented by the class I molecule D^b [16]. Dansylated PbCS 253-260 was used for the pre-incubations in all of the dilution assays described here.

In order to distinguish qualitatively between peptides of different affinities, the dilution assays was performed at varying concentrations of competitor peptide. Immediately after MHC/DAN-peptide dilution, 0.1 μM of the unmodified peptide was added. Peptides that accelerate the fluorescence decrease at this concentration were defined as 'high-affinity' peptides. In the absence of a response, 1 μM competitor peptide was added 15 min later. Peptides that had an effect at this point were defined as 'intermediate-affinity' peptides. Finally, peptides that had an effect only at 10 μM were defined as 'low-affinity' peptides. All other peptides were defined as 'zero-affinity' peptides, meaning that their affinity is at least 100-fold lower than for the high-affinity peptides.

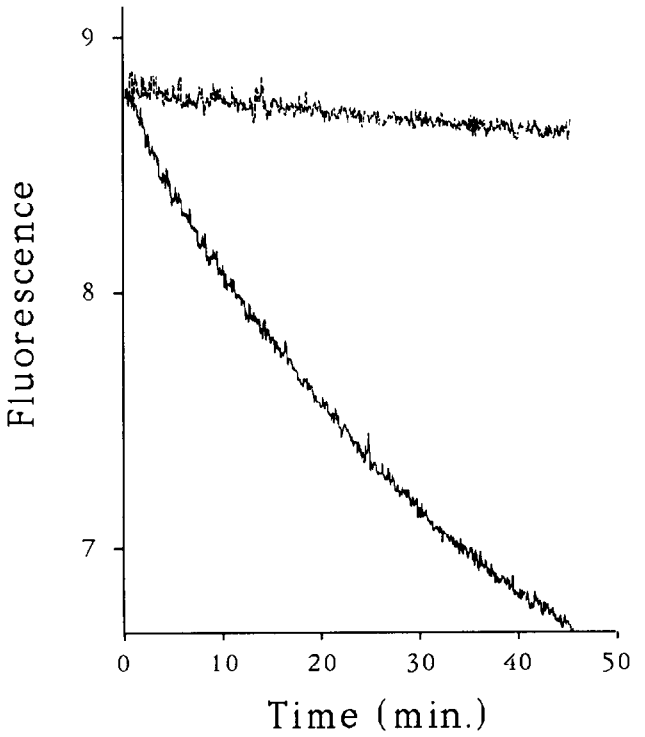

Fig. 1. Fluorescence decrease due to the dissociation of the K^d/DAN-peptide complex. The soluble K^d protein (2 μM) was pre-incubated with 2 μM DAN-peptide, and the mixture was then diluted 10-fold into a cuvette containing either 0.1 μM of an unlabelled peptide known to bind to K^d, PbCS 253-260 (lower trace), or a large excess (10 μM) of a peptide, NP 366-374 (upper trace), that binds to another MHC molecule. The trace obtained in the presence of NP 366-374 was the same as that obtained in the complete absence of competitor peptide.

This protocol was tested with the three peptides of known affinity. As expected, 0.1 μM PbCS 253-260 was highly effective in decreasing the fluorescence (Fig. 1), and was thus classified as a high-affinity binder. On the other hand, the non-optimal peptide, PbCS 249-260, had no effect at either 0.1 or 1 μM, but set off an effect at 10 μM (not shown), consistent with it being a low-affinity binder. The negative control, NP 366-374, had no perceptible effect even at 10 μM (Fig. 1); the kinetics of the fluorescence decrease in the presence of NP 366-374 was identical to that observed in the complete absence of competitor peptide. These results are summarized in Table I. Extension of these studies to a larger series of peptides with known in vivo activity confirmed that this protocol can reliably distinguish between peptides of high-, intermediate-, low-, and zero affinity (manuscript in preparation).

Table I

Affinities of peptides used in this study, determined by the dilution and equilibrium binding assays

Peptide	Sequence	MHC restriction	Binding affinity to K^d	
			Dilution assay	Equilibrium K_D
NP 366-374	ASNENMETM	D^b	zero	
PbCS 249-260	NDDSYIPSAEKI	K^d	low	
PbCS 253-260	YIPSAEKI	K^d	high	
NP 147-155	TYQRTRALV	K^d	high	1.2×10^{-7} M
NP ($T^1 \rightarrow$ A)	AYQRTRALV		high	1.1×10^{-7} M
NP ($Y^2 \rightarrow$ A)	TAQRTRALV		zero	$> 10^{-5}$ M
NP ($Q^3 \rightarrow$ A)	TYARTRALV		high	1.1×10^{-7} M
NP ($R^4 \rightarrow$ A)	TYQATRALV		high	4.0×10^{-7} M
NP ($T^5 \rightarrow$ A)	TYQRARALV		high	2.5×10^{-7} M
NP ($R^6 \rightarrow$ A)	TYQRTAALV		high	2.8×10^{-7} M
NP ($L^8 \rightarrow$ A)	TYQRTRAAV		high	6.8×10^{-8} M

A series of Ala-substituted peptides was next evaluated. The original peptide, NP 147-155, is a K^d-restricted peptide from the influenza virus nucleoprotein [17] and has a sequence (TYQRTRALV) consistent with the consensus motif identified for K^d. The other seven peptides synthesized had each of the residues replaced separately by an Ala. Positions 7 and 9 were not tested because the original amino acids are Ala and Val, respectively. Alanine was used for the substitutions since it has the smallest lateral chain with chirality, which at the same time does not excessively perturb the local secondary structure of the peptide [18].

Table I gives the results for the eight peptides. The original NP 147-155 peptide and six of the Ala-substituted peptides were high-affinity binders. The only peptide for which the binding affinity was diminished is NPY($Y^2 \rightarrow$ A), which became a zero-affinity binder. In this sense it became indistinguishable from the negative control, NP 366-374.

We subsequently attempted to quantify the binding affinities by determining the association and dissociation rates for DAN-peptide binding to SC-K^d, since we have previously shown [15] that the fluorescence time-course can be fit by single exponentials. However, the apparent association rate constants were independent of the peptide concentration (not shown), suggesting that the association on-rate may be limited by the rate at which the complex of K^d and previously bound peptide can dissociate.

Thus, although the dilution assay has the advantage of consuming less material, quantitative binding affinities were obtained under equilibrium conditions using 0.2 μM SC-K^d, 0.2 μM dansylated PbCS 253-260, and varying concentrations of unlabelled competitor pep-

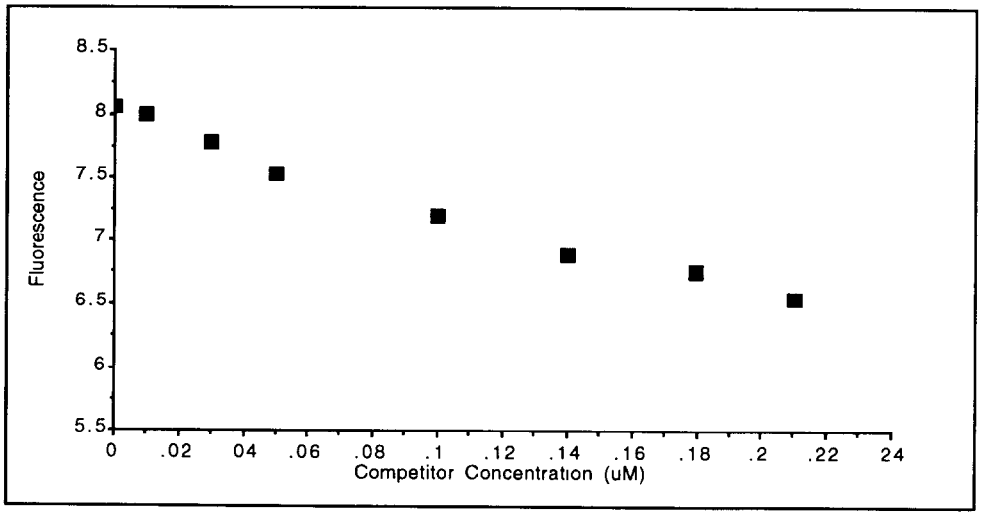

Fig. 2. Inhibition activity of NP 147-155 on the fluorescence enhancement due to 0.2 μM dansylated PbCS 253-260 incubated in the presence of 0.2 μM SC-K^d. In the absence of competitor peptide, the relative fluorescence level was 8.1, corresponding to 0% inhibition. In the presence of 10 μM unlabelled PbCS 253-260, it was 5.6, defined as 100% inhibition. Thus, 50% inhibition was obtained at 0.16 μM NP 147-155.

tide. Following Cheng and Prusoff [19], the competitor peptide affinity can then be calculated from the relationship:

$$K_D = \frac{F_{50}}{1 + [\text{DAN-peptide}]/K_{DAN}},$$

where [DAN-peptide] = 0.2 μM; K_{DAN} is the affinity of dansylated PbCS 253-260 (6.25×10^{-7} M) (Ojcius et al, submitted manuscript); and F_{50} is the competitor peptide concentration that inhibits the dansyl fluorescence enhancement by 50%. A large excess (10 μM) of unlabelled PbCS 253-260 was used to determine the level of 100% fluorescence enhancement inhibition.

A representative fluorescence titration is shown in Fig. 2 for wild-type NP 147-155. The peptide concentration at which there was 50% fluorescence inhibition, 0.16 μM, gave a K_D of 1.2×10^{-7} M. Similar K_D values were measured for the other peptides (summarized in Table I), except for NP($Y^2 \rightarrow A$). Up to 100 μM NP ($Y^2 \rightarrow A$) had no effect on the fluorescence, allowing us to safely assign a lower limit of 10^{-5} M for the affinity. The dramatic drop in the NP affinity due to replacement of Tyr^2 thus confirms, in a direct binding assay, the critical role of this residue for proper binding to K^d.

Based on functional competition assays using CTL clones, it was previously shown that substitution of Tyr^2 in different K^d-restricted peptides results in a substantial diminution in the competitor activity of the peptides [10–12]. It was subsequently shown [20–23] that synthetic peptides used as antigens in vivo are susceptible to proteolytic cleavage due to proteases present in serum. The extent of cleavage varies among different antigenic peptides [20], and the proteolysis can lead to either an increase or decrease in the antigenicity of the peptides [23]. Interestingly, it has been reported [21] that a serum protease, angiotensin-converting enzyme (ACE), is responsible for the K^d-binding activity in vivo of the influenza antigen, NP 147-158. This antigen is inactive until cleaved by ACE, which converts it into NP 147-155, i.e. the nonamer conforming to the consensus motif. Taken together, these results underscore the importance of confirming the results from in vivo studies with direct binding studies with purified proteins in vitro.

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