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Non-canonical anchor motif peptides bound to MHC class I induce cellular responses

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ABSTRACT

The major histocompatibility complex (MHC) on the surface of antigen presenting cells functions to display peptides to the T cell receptor (TCR). Recognition of peptide–MHC by T cells initiates a cascade of signals, which results in the initiation of a T cell dependent immune response. An understanding of how peptides bind to MHC molecules is important for determining the structural basis for T cell dependent immune responses and facilitates the structure-based design of peptides as candidate vaccines to elicit a specific immune response. To date, crystal structures, immunogenicity and *in vivo* biological relevance have mainly been characterized for high affinity peptide–MHC interactions. From the crystal structures of numerous peptide–MHC complexes it became apparent what canonical sequence features were required for high affinity binding, which led to the ability to predict in most instances peptides with high affinity for MHC. We previously identified the crystal structures of non-canonical peptides in complex with MHC class I (one bound with low affinity and the other with high affinity, but utilizing novel peptide anchors and MHC pockets). It is becoming increasingly evident that other non-canonical peptides can also bind, such as long-, short- and glyco-peptides. However, the *in vivo* role of non-canonical peptides is not clear and we present here the immunogenicity of two non-canonical peptides and their affinity when bound to MHC class I, H2K^b. Comparison of the three-dimensional structures in complex with MHC suggests major differences in hydrogen bonding patterns with H2K^b, despite sharing similar binding modes, which may account for the differences in affinity and immunogenicity. These studies provide further evidence for the diverse range of peptide ligands that can bind to MHC and be recognized by the TCR, which will facilitate approaches to peptide-based vaccine design.

1. Introduction

Interaction of the major histocompatibility complex (MHC) with a peptide and its cognate T cell receptor (TCR) is a central event in T cell-mediated responses. Class I MHC molecules are found on nucleated cells (Zinkernagel and Doherty, 1979), where they associate as non-covalently linked heterodimers of a membrane-spanning variable heavy chain (44 kDa) and an invariant light chain β_2-microglobulin (12 kDa). Peptides with lengths of 8–10 amino acids are commonly presented by MHC molecules to CD8$^+$ T cells. Crystallographic studies of MHC class I complexed with high affinity peptides (8–10 amino acids) revealed that the N- and C-termini are held into the binding groove by conserved hydrogen bond networks. The side-chains of the bound peptides occupy various specificity pockets (A-F) that form in the binding groove between the α1 and α2 helices and the β-sheet platform of the MHC class I molecule (Falk et al., 1991; Fremont et al., 1992; Matsumura et al., 1992a; Zhang et al., 1992). Early biochemical studies with purified MHC class I molecules showed that peptides binding with high affinity shared conserved (consensus) amino acids at anchor positions. For example in H2K^b, the peptides usually have anchors at P2, P6, P9 for 9-mers or P2/3, P5, P8 for 8-mers, with the preferred anchor residues being the hydrophobic amino acids Phe/Tyr for the central P5/6 residues and Leu/Val/Met at the P8/9 positions (Rammensee et al., 1995, 1999; Rammensee, 1995). Typically, pockets B, C and F are occupied by residues at positions P2/3, P5/6 and P8/9, respectively. Although these anchor residues are required for stabilization and high affinity binding it has been shown that some peptides which do not contain the canonical anchor residues can bind and be presented by MHC class I and be recognized by CTL (Apostolopoulos et al., 1997b, 2000, 2001, 2002a, 2003; Apostolopoulos and Lazoura, 2004; Chelvanayagam et al., 1997; Daser et al., 1994; Gao et al., 1995; Lazoura and Apostolopoulos,

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2005a,b; Mandelboim et al., 1997; Ostrov et al., 2002; Tirosh et al., 1999).

Sequencing of the population of peptides isolated from particular MHC class I alleles suggested there are common features of peptides that interact with particular alleles (Falk et al., 1991; Rammensee, 1995; Rammensee et al., 1995). This information was pivotal in shaping the current view of the preferences of MHC in binding peptides. However, it is worth noting that the methods used to isolate peptides from MHC class I favored those with high affinity and the more abundant species. Algorithms designed to predict class I binding peptides based on the 'canonical' rules are at best ~80% accurate, suggesting that there are significant gaps in the available data regarding preferred sequences and binding modes of peptide ligands for MHC molecules. Previously, our group demonstrated low affinity and non-canonical peptides bound to MHC class I ($H2K^b$, $H2D^b$ and HLA-A2) (Apostolopoulos et al., 1997a,b). We also showed that peptides bulged at the central P5 position for peptide-$H2D^b$, looped at the C-terminus for peptide–$H2K^b$ and can be accessible to anti-peptide monoclonal antibodies (Apostolopoulos et al., 1998, 2000; Apostolopoulos and Lazoura, 2004). In addition, we determined the crystal structures of immunogenic non-canonical anchor motif containing peptides in complex with MHC class I, $H2K^b$ (Apostolopoulos et al., 2002b,c). The low affinity MUC1-8 peptide (SAPDTRPA) derived from MUC-1, a glycoprotein over expressed (up to 100-fold) on adenocarcinomas (Apostolopoulos and McKenzie, 1994; Apostolopoulos et al., 1996) bound $H2K^b$ via small non-polar Ala anchor residues at P2 and P8 and the small polar Thr residue at P5, i.e. the pockets B, C, F were not completely occupied (Apostolopoulos et al., 2002c). The affinity and immunogenicity of MUC1-8 could be increased considerably when Thr-P5 was glycosylated and GalNAc acted as an anchor or when Thr-P5 was replaced by Phe-P5 (and/or together with Ala-P8 replaced by Leu-P8) (Apostolopoulos et al., 2003; Lazoura et al., 2006). Furthermore, the high affinity YEA9 peptide (SRDHSRTPM), identified to be from the yeast α-D-glucosidase protein (maltase; residues 438–446), lacked the hydrophobic anchor at P6, but instead contained a large basic Arg residue. As a consequence, Arg-P6 made use of a new 'E pocket' and Arg-P2 bound to the B pocket. Binding of YEA9 via alternative anchor residues and the use of the B, E and F pockets provided an alternative mode of binding to the $H2K^b$ MHC class I molecule (Apostolopoulos et al., 2002b). Based on this mode of binding we identified the Strp9 (SRDNSRIPM) peptide, derived from the dextran glucosidase protein (residues 402–410) from *Streptococcus pneumoniae*.

Herein, we report the immunological responses and binding affinities of non-canonical peptides, YEA9 and Strp9, which demonstrates a functional role for these atypical MHC ligands. A molecular model was determined for the three-dimensional structure of Strp9 in complex with $H2K^b$. The Strp9 peptide is predicted to interact using a similar binding mode to YEA9 in the $H2K^b$ binding groove. However, the two peptides display different peptide–MHC hydrogen bonding patterns (Strp9 has fewer predicted hydrogen bonds compared to YEA9), which may explain the observed differences in binding affinity and induction of IFNγ secreting T cells.

2. Experimental

2.1. Materials and methods

2.1.1. Peptides

SRDNSRIPM (Strp9) and SRDHSRTPM (YEA9) were synthesized by Chiron Mimotopes (VIC, Australia). Peptide purity was >95% and molecular weights were confirmed by electrospray mass spectroscopy.

2.1.2. Generation of DC, immunization of mice and ELISpot assay

$H2K^b$ C57BL/6, female 6–8 week old mice, were used in the experiments. Bone marrow cells from C57BL/6 mice were cultured at 10^6 cells/ml in RPMI, supplemented with 10 ng/ml GM-CSF, 10 ng/ml IL-4 and 10% fetal calf serum (FCS) to induce dendritic cell (DC) generation. At day 6 the differentiated DCs were washed and resuspended in culture media and then cells were loaded with yeastolate (100 μg/ml), Strp9 (20 μg/ml) or YEA9 (20 μg/ml) were loaded onto DCs for 3 h. Pulsed DCs were washed and 50 μl ($5–7.5 \times 10^5$ cells) injected intradermally in mice into each footpad. After 14 days mice were boosted (with pulsed DCs) and 14 days later mouse splenocytes were isolated and assessed for IFNγ production by ELISpot assay. Yeastolate is present in Insect Xpress® media (Cambrex, USA) at a concentration of 4 g/l and the peptide YEA9 from yeastolate binds to $H2K^b$ with high affinity. Yeastolate as an ultrafiltrate powder (Cellgro, Mediatech, Inc.) was used to pulse DCs as described. All animal experimentations were approved by Austin Health Animal Ethics Committee, Australia.

To assess IFNγ production by $CD8^+$ T cells, splenocytes from immunized mice were used in IFNγ ELISpot assays. Freshly isolated spleen cells were incubated with titrating concentrations (10^{-6} to 10^{-10} M) of either Strp9 or YEA9 peptide, irrelevant peptide (SEV9; negative control) or concanavalin A (ConA; internal positive control) for 18 h at 37 °C on nitrocellulose plates [pre-coated with an anti-murine IFNγ monoclonal antibody (Mab)]. Following incubation cells were washed from the plates, and plates were incubated with biotin conjugated Mab to murine IFNγ followed by streptavidin-alkaline phosphatase conjugate. Spots of alkaline phosphatase activity were detected using colorimetric AP detection and counted on an ELISpot reader. The data are presented as spot forming units (SFU) per 0.5 million cells. Experiments were performed at least 2–3 times.

2.1.3. Production of soluble $H2K^b$ and affinity measurements

The soluble extracellular domains of $H2K^b$ (heavy chain residues 1–274 with C-terminal His-tag and β_2-microglobulin residues 1–99) were expressed in *Drosophila melanogaster* cells, under the control of a metallothione promoter as previously described (Fremont et al., 1992; Jackson et al., 1992; Lazoura et al., 2006; Saito et al., 1993; Stura et al., 1992). *D. melanogaster* cells were expanded to large scale (up to 6 l) in serum free Insect Xpress® media (Cambrex, USA). $CuSO_4$ (625 μM final concentration) was added 3–5 days prior to cell harvesting to induce expression of $H2K^b$. The supernatant was concentrated to a volume of less than 600 ml using a CENTRAMATE tangential flow concentrator (PALL, USA) with a 10 kDa MWCO membrane (PALL, USA). The concentrated supernatant was loaded onto a Ni-NTA column and eluted using a 10–250 mM imidazole buffer gradient, pH 7.5. Further purification was achieved using a Mono-Q column (Pharmacia Biotech; elution using a 25–500 mM NaCl gradient, in Tris–HCl buffer, pH 8.0). The final sample was dialyzed overnight against double distilled water and further concentrated using a prewashed Nanosep 10 kDa MWCO concentrator (PALL, USA) to final concentrations of >10 mg/ml. The final concentration was determined using the NanoDrop spectrophotometer (NanoDrop Technologies, USA).

Affinity measurements for binding of Strp9 and YEA9 peptides to soluble $H2K^b$ were performed as previously described (Lazoura et al., 2006; Matsumura et al., 1992b; Saito et al., 1993). Briefly, VSV8 peptide was labeled with ^{125}I using the Iodogen method. The ^{125}I-VSV8 peptide was purified using a Sep-Pak column (Waters, Milford, MA). Competition assays were performed at 23 °C with a few modifications. The binding studies were carried out in phosphate buffered saline (PBS) supplemented with 1% FCS and the free peptide was removed by gel filtration on Sephadex columns (NAP-5, Pharmacia Biotech). The dissociation constants for unlabelled peptides were determined from the molar concentrations of