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A membrane penetrating multiple antigen peptide (MAP) incorporating ovalbumin CD8 epitope induces potent immune responses in mice

Nicole A. Brooks [a,b], Dodie S. Pouniotis [b], Kuo-Ching Sheng [c], Vasso Apostolopoulos [c], Geoffrey A. Pietersz [a,*]

[a] Bio-organic and Medicinal Chemistry, Burnet Institute, Melbourne, VIC, Australia
[b] School of Medical Sciences, RMIT University, Melbourne, VIC, Australia
[c] Immunology and Vaccine Laboratory, Burnet Institute, Melbourne, Australia

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ABSTRACT

Cell penetrating peptides (CPP) represent a novel approach to facilitate cytoplasmic delivery of macromolecules. The DNA binding domain of *Drosophila Antennapedia* contains 60 amino acids and consists of 3 α-helices, with internalizing activity mapped to a 16-amino acid peptide penetratin (Antp) within the third α-helix. Here, we report on the use of penetratin to deliver a multiple antigen peptide (MAP) incorporating the immunodominant CD8 epitope of ovalbumin, SIINFEKL (MAPOVACD8). We demonstrate that penetratin linked to the MAPOVACD8 construct either by a disulfide (SS) or thioether (SC) linkage promotes the uptake, cross presentation and subsequent *in vivo* proliferation and generation of OVACD8 (SIINFEKL)-specific T cells. The MAPOVACD8 construct without penetratin is not presented by MHC class I molecules nor does it generate an *in vivo* IFN-γ response in C57BL/6 mice. Moreover, we clearly define the uptake and intracellular processing pathways of AntpMAPOVACD8 SS and SC revealing the majority of AntpMAPOVACD8 is taken up by DC via an endocytic, proteasome and tapasin independent mechanism. We also show that the uptake mechanism of AntpMAPOVACD8 is dose dependent and uptake or intracellular processing is not altered by the type of chemical linkage.

1. Introduction

Fundamental for an effective vaccine is the delivery of immunogenic antigens to antigen-presenting cells (APC), ensuing processing and presentation, and induction of an immune response [1,2]. Vaccination with peptides incorporating cytotoxic T lymphocyte (CTL) epitopes has proven limited due to the failure for exogenous antigens to be presented efficiently to T cells [2]. There are now several strategies to promote the delivery of antigens to APC, with one such method utilizing the unique translocating properties of cell penetrating peptides (CPP) [3–7].

CPP offer a unique approach for the transport of peptides and proteins into the cytoplasm of cells. The TAT protein from human immunodeficiency virus, penetratin (Antp) from *Drosophila Antennapedia* and the VP22 protein from herpes simplex virus are some of the CPP which have been investigated in vaccine studies for delivery of tumor associated antigens to APC and for the use as a non-viral gene delivery vehicle in DNA vaccines, producing promising results [4,8–10].

It is now clear to overcome some of the limitations of previous peptide vaccines it is necessary to firstly, incorporate both CD8 T cytotoxic (Tc) and CD4 T helper (Th) epitopes to promote the generation of long term immunity and secondly major histocompatibility complex (MHC) restriction must be considered to allow application to the wider population [11,12].

Lu et al. utilized a recombinant protein incorporating the 9-mer TAT peptide linked to multiple epitopes from the protein ovalbumin (OVA) to demonstrate that immunization with multiepitope vaccines incorporating TAT-OVA CD8-CD4 T cell epitopes and the adjuvant CpG results in strong CTL and Th responses and generates an anti-tumor response [13]. Similarly, Dakappagari et al. (2005) utilized a multiepitope peptide incorporating the CPP Pep-1 with three HLA-A2 restricted epitopes from Her-2/neu to prime CTL responses in mice [14]. However in both cases either furin sensitive (RVKR) sequences or arginine spacers were required to facilitate cleavage for processing and presentation [13,14].

Due to chemical synthesis restraints on the length of linear peptides, the number of Tc and Th epitopes that can be incorporated is limited. Branched multiple antigen peptides (MAP) may help overcome such limitations by allowing the delivery of numerous tumor antigens. Yet there is now evidence that as a result of varying antigen uptake mechanisms and/or differential resistance to the

Abbreviations: Antp, penetratin 16-mer peptide-RQIKIWFQNRRMKWKK; CPP, cell penetrating peptide; CTL, cytotoxic T lymphocyte; OVACD8, immunodominant CD8 peptide from ovalbumin, SIINFEKL; SS, disulfide linkage; SC, thioether linkage

* Corresponding author. Bio-Organic and Medicinal Chemistry Laboratory, Burnet Institute, 85 Commercial Rd, Melbourne, 3004, Victoria, Australia. Tel.: +61 3 9282 2111; fax: +61 3 9282 2100.
E-mail address: gpietersz@burnet.edu.au (G.A. Pietersz).

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proteases involved in antigen processing, peptides generated from branched constructs presented to T cells are processed differently to those derived from linear peptides [11,15]. Thus far there have been no reports on the use of CPP to transport branched MAP incorporating CTL epitopes into APC.

Herein we report on the uptake, processing and presentation pathways of a novel asymmetric 4 branched MAP incorporating the H-2K^b CD8 eight-mer epitope SIINFEKL, from the ovalbumin protein. We reveal that linkage of a MAPOVACD8 construct to Antp via a disulfide (SS) or thioether (SC) linkage is endocytosed via phagocytosis or macropinocytosis by dendritic cells in an ATP-dependent manner and processed in a proteasome and tapasin (TAP) independent pathway. Moreover AntpMAPOVACD8 constructs generate potent *in vivo* proliferation and killing and induce antigen-specific IFN-γ secreting T cell responses superior to MAPOVACD8 construct alone.

2. Materials and methods

2.1. Mice and immunizations

C57BL/6, OT-I and OT-II mice, aged 6–10 weeks, were obtained from Walter and Eliza Hall Institute (Vic., Australia) and housed in the animal facilities of the Burnet Institute or RMIT University (Victoria, Australia). For immunization experiments mice were injected three times on days 0, 10 and 17 intradermally (i.d.) at the base of tail with 100 μg peptide. CpG-ODN 1668 (5′-TCC ATG ACG TTC CTG ATG CT-3′) with phosphorothioate linkages was synthesized by Geneworks (Adelaide, Australia) and dissolved in sterile PBS and stored at −20 °C. For tumor protection experiments mice were immunized with 50 μg CpG i.d.

2.2. Peptides

Peptides were synthesized by Genescript Corporation (San Francisco, USA) and purity determined by mass spectrometry. OVACD8 (SIINFEKL) is the ovalbumin H-2K^b CTL epitope 8-mer peptide and penetratin (Antp) is a 16 amino acid (RQIKIWFQNRRMKWKK) *Antennapedia* peptide. MAPOVACD8 is a 4 asymmetric branched (C-Terminal) MAP synthesized using the $(Fmoc)_4Lys_2LysCys\beta Ala$ (Free Cysteine) resin with SIINFEKLKGKGKGKGKG on each branch. The conjugation of MAPOVACD8 to Antp was performed using a disulfide (SS) bond or a thioether (SCH2CO (SC)) bond (Fig. 1) as described below.

For synthesis of the disulphide-linked MAP cysteine modified MAPOVACD8 (0.5 ml, 6.2 mg/ml) in water was reacted with 5,5′-dithiobis-(2-nitrobenzoic acid, DTNB) (0.2 ml, 7.5 mg/ml) in 0.1 M phosphate buffer, pH 7.5 for 3–4 h. The yellow solution was dialysed into PBS overnight. The MAPOVACD8 incorporating the activated disulphide was reacted with Antp peptide (0.2 ml, 5 mg/ml) incorporating a Cysteine at the C-terminal. After 3–4 h the mixture was dialysed into PBS overnight. The concentration of MAPOVACD8 was determined spectrometrically based on the DTNB anion released ($\varepsilon_{412} = 13{,}600\ M^{-1}\ cm^{-1}$). The thioether conjugated MAP was synthesized by reacting MAPOVACD8 (0.5 ml, 6.2 mg/ml) in 0.1 M phosphate buffer, pH 7.5 with the N-terminal bromoacetyl-modified Antp (0.2 ml, 5 mg/ml) for 16 h. The mixture was dialysed overnight to remove free Antp.

The cytotoxicity of the complexes was measured using the lactate dehydrogenase assay [16]. Antp complexes showed no significant cytotoxicity at concentrations of up to 25 μM (not shown).

2.3. Generation of bone marrow derived dendritic cells (BMDC)

Bone marrow cells from C57BL/6 female mice were collected by flushing the tibias of hind legs and treated with ACK lysis buffer (0.15 M NH_4Cl, 1 mM $KHCO_3$ and 0.1 mM Na_2EDTA) to lyse erythrocytes. Cells were washed and cultured at 5×10^5 cells/ml in 24 well plates with complete RPMI-1640 medium 10% (v/v) heat inactivated fetal calf serum, 4 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin sulphate and 100 μM β-mercaptoethanol) with 10 ng/ml each of recombinant mouse granulocyte macrophage colony-stimulating factor (GM-CSF) and interleukin- 4 (IL-4) (Pharmingen, San Diego, CA). At day 6 cells were >80% CD11c$^+$ which was confirmed by flow cytometry (data not shown).

2.4. Stimulation of lacZ-inducible ovalbumin-specific T cell hybrid

The B3Z mouse T cell hybridoma line contains a gene construct of *Escherichia coli* lacZ reporter gene linked to the nuclear factor of activated T cells. Recognition of the OVACD8 peptide in the context of class I by the T cell receptor (TCR) results in activation of the enzyme and conversion of the chromogenic substrate that can be measured by absorbance spectrophotometry [17]. DC, EL-4 and RMA-S cells (2×10^5 cells) were pulsed with peptides at 5, 25 and 50 μM in 96-well microtitre plates (Falcon, BD Biosciences, North Ryde, Australia) for 24 h at 37 °C. Cells were then washed and 10^5 B3Z cells were added for 18 h at 37 °C. The next day cells were washed with sterile PBS and incubated with chlorophenol red-β-galactoside (Calbiochem, San Diego, CA) (100 μM 2-ME, 9 mM $MgCl_2$, 0.125% NP40, 0.15 mM chlorophenol red-β-galactoside). After 4 h incubation at 37 °C the absorbance was read at 560 nm.

2.5. Enzyme-linked immunosorbent spot-forming cell assay (ELISpot)

Splenocytes from immunized C57BL/6 mice were isolated and assessed by ELISpot for antigen-specific IFN-γ secretion. MultiScreen filter plates (Millipore, Billerica, MA) were coated with 5 μg/mL of anti-mouse IFN-γ antibody (AN18) (Mabtech, Stockholm, Sweden) overnight at 4 °C. Plates were washed six times with sterile PBS and blocked with 200 μl complete RPMI media for 2 h at 37 °C. Spleen cells (5×10^5/well) from immunized mice were added to wells in 100 μl of complete medium and incubated with 20 μg/ml recall antigens for 18 h. Concanavalin A (1 μg/ml) or cells alone were used as positive and negative controls, respectively. Triplicate wells were set up for each condition. Cells were discarded after washing (PBS) and 1 μg/mL biotinylated anti-mouse IFN-γ antibody (Mabtech) was added for 2 h at room temperature. The plates were washed with PBS and 1 μg/ml streptavidin–alkaline phosphatase (Mabtech) added at room temperature for 2 h. Spots of activity were detected using a colorimetric AP-conjugate substrate kit (Bio-Rad Laboratories, Foster City, CA). Cytokine spots were counted with an AID ELISpot Reader system (Autoimmun Diagnostika GmbH, Strassberg, Germany). Data is presented as mean spot-forming units (SFU) per 5×10^5 cells ± standard error of the mean (SEM).

2.6. Tumor protection

Groups of C57BL/6 mice ($n=8$) were immunized i.d. with PBS, AntpMAPOVACD8 SC or AntpMAPOVACD8 SC + CpG on days 0, 10 and 17. Ten days later, mice were challenged with a subcutaneous dose of 2×10^5 B16-OVA cells resuspended in 100 μl sterile PBS. The expression of OVA in B16-OVA tumor cells was confirmed by flow cytometry (data not shown). The subcutaneous growth of the tumor was monitored by measuring the two perpendicular diameters using calipers and the results are expressed as the product of the two perpendicular diameters.

2.7. In vivo maturation

C57BL/6 mice were injected i.d. in the footpad with peptides or LPS (positive control). 18 h later popliteal lymph nodes were isolated and pooled ($n=4$) and stained with CD11c-APC and maturation markers