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Viral and bacterial minigene products are presented by MHC class I molecules with similar efficiencies

Benjamin J. Wolf, Michael F. Princiotta*

Department of Microbiology and Immunology, SUNY Upstate Medical University, 750 E. Adams St., 2285 Weiskotten Hall Addition, Syracuse, NY 13210, United States

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ABSTRACT

MHC class I molecules present short peptides, usually 8–10 amino acids in length, to CD8$^+$ T cells. These peptides are typically generated from full-length endogenously synthesized proteins degraded by the antigen processing machinery of the target cell. However, exogenous proteins, whether originating from intracellular bacteria or parasites or via phagocytosis during cross-presentation, can also be processed for presentation by MHC class I molecules. It is currently not known whether endogenously synthesized proteins and proteins acquired from exogenous sources follow the same presentation pathway. One clue that the processing pathways followed by endogenous and exogenous proteins may not be identical is the vastly different presentation efficiencies reported for viral versus bacterial antigens. Because class I antigen processing involves multiple steps, we sought to determine where in the processing pathway these differences in efficiency occur. To accomplish this, we expressed identical minimal peptide determinants from viral and bacterial vectors using a minigene expression system and determined the rate of peptide-MHC generation per molecule of minigene product synthesized. We found that peptides expressed from either the viral or bacterial vector were presented with virtually identical efficiencies. These results suggest that differences in the processing pathways followed by endogenous versus exogenous proteins most likely occur at a point prior to where free peptide is liberated from full-length protein.

1. Introduction

CD8$^+$ T cells detect infected or cancerous cells by recognizing specific peptide determinants bound to MHC class I molecules (pMHC) expressed on the cell surface (Yewdell and Haeryfar, 2005). The first step in the generation of pMHC molecules occurs when host cell or pathogen derived full-length proteins are degraded by cellular proteasomes to produce polypeptides ranging in length from a few amino acids to >20 (Rock et al., 2010). The majority of these polypeptides are degraded by cytosolic aminopeptidases to generate single amino acid residues, which are utilized by the cell for *de novo* protein synthesis. However, some peptides survive to enter the endoplasmic reticulum (ER) via the transporter associated with antigen processing TAP (Rock et al., 2010), where they will be available to load nascent MHC class I molecules. ER resident aminopeptidases (ERAAP or ERAP1/2) further trim peptides to the appropriate 8–10 residue length necessary for MHC class I binding (Rock et al., 2010) and subsequent presentation on the surface of the infected or cancerous cell.

Viral and bacterial vectors are commonly used to express recombinant protein antigens targeted to the MHC class I processing pathway. These vectors can be used either to develop vaccines or as tools to study MHC class I processing and presentation and the subsequent CD8$^+$ T cell response to those antigens. Of the numerous bacterial vectors used to introduce recombinant proteins to the MHC class I antigen processing pathway, *Listeria monocytogenes* is probably the most extensively characterized (Brockstedt and Dubensky, 2008; Pamer et al., 1997). To date, recombinant protein expression from Listeria has involved introducing DNA sequences encoding either the full-length protein or large polypeptides containing the antigenic sequence(s) of interest (Brockstedt and Dubensky, 2008). In instances where the expression of multiple peptide determinants from heterologous sources was desired, they have been expressed as a single polypeptide construct containing the peptides of interest separated by amino acid linkers of varying length (Lauer et al., 2008). In all cases, these protein and polypeptide constructs require proteasomal activity for the production of MHC class I binding peptide determinants.

In viral and DNA vector systems, the introduction of so-called "minigene" sequences encoding minimal peptide determinants to generate peptides for presentation by MHC class I molecules can circumvent the requirement for proteasomal activity. These minigenes can be expressed as (1) a methionine-peptide (Met-Peptide), where the methionine is encoded by the ATG translation start-site, or (2) with the peptide immediately following a ubiquitin moiety (Ub-Peptide) (Fruci et al., 2003; Princiotta et al.,

* Corresponding author. Tel.: +1 315 464 7683; fax: +1 315 464 4417.
E-mail address: princiom@upstate.edu (M.F. Princiotta).

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Table 1
Primers used to generate recombinant Listeria strains.

1. ActA-F NotI
AAA*GCGGCCGC*TGAAGCTTGGGAAGCAGTTGGGGTTAACTG
2. ActA-R SOE Venus
CTTGCTCACCATAAGCATTGGCGTCTCTGGCAAAGCAAGTCT
3. Venus-F SOE ActA
GAGACGCCAATGCTTATGGTGAGCAAGGGCGAGGAGCTGTTCACC
4. Ub-SIIN-R BamHI 3stops
TCA*GGATCC*CTATTATTATCATAGCTTTTCGAAGTTGATGATCGAACC
5. ActA 100R SOE Ub
CGAAGATCTGCATACCTTTCTCTGCTTTTGCTTTCAACATTGCTATTAG
6. Ub-F SOEActA1-100
AAAGCAGAGAAAGGTATGCAGATCTTCGTGAAGACGTTAACCGGTAAA
7. Ub-SIIN-R 2stops, NotI
*GCGGCCGC*TTATCATAGCTTTTCGAAGTTGATGATCGAACCACCTCTTAGTCTTAAG
8. ActA-F KpnI
TTTAA*GGTACC*TGAAGCTTGGGAAGCAGTTGGGGTTAACTGATT
9. Ub-R SOE NP
CCTTGGGACGCCATACCACCTCTTAGTCTTAAGACAAGATGTAAGGTC
10. NP-F SOE Ub
CTTGTCTTAAGACTAAGAGGTGGTATGGCGTCCCAAGGCACCAAACGG
11. SIINFEKL-RwithARDG linker
TCCATCCCGGGCTAGCTTTTCGAAGTTGATGATCGA
12. FLAG-F with ARDG linker
CTAGCCCGGGATGGAGACTACAAAGACCATGACG
13. FLAG-R with stops, NotI
TG*GCGGCCGC*TCATTACTTGTCATCGTCATCCTTG
14. mCherry+RBS-F BamHI
CG*GGATCC*GAAAGGAGGTTTATTAAAATGGTGAGCAAG
GGCGAGGAGGATAACATGGCC
15. mCherry-R SphI
TTA*GCATGC*TCACTATTACTTGTACAGCTCGTCCATGCCGCCGGTGGA

2003). In the first construct, the amino-terminal methionine of the Met-Peptide is rapidly removed through the action of cytosolic methionyl aminopeptidases (Princiotta et al., 2003). In the second, peptide liberation from the Ub-Peptide construct occurs in a virtual co-translational manner through the activity of cellular ubiquitin hydrolases (Fruci et al., 2003). Both methods generate free MHC class I binding peptide independently of proteasomal activity.

Expression of peptides from minigenes can be used to study peptide presentation independent of the antigen processing system. Because it also produces significantly higher numbers of surface pMHC than does the degradation of full-length proteins (Porgador et al., 1997; Princiotta et al., 2003), it can be useful in designing vaccines where increasing the level of pMHC expression are desired. This high level of pMHC production from minigene constructs is the result of two factors. First, because minigenes are encoded by relatively short lengths of DNA, more peptide will be synthesized per unit time compared to full-length protein. Second, virtually every minigene translated results in the production of a free peptide, but only a fraction of the full-length proteins synthesized will be degraded to produce a specific peptide determinant (Cascio et al., 2001).

Bacterial vectors are currently in development for use as vaccines. Using bacteria expressing antigenic peptide as minigene constructs to rapidly generate high levels of pMHC could significantly extend the utility of these vectors. Although minigenes are commonly expressed using viral and DNA vectors, they have not been developed for use in a bacterial system. A primary limitation to expressing minigene products in a bacterial system is the fact that recombinant translation products must be secreted by the bacteria to access the host cell antigen presentation pathway. This precludes the use of methods such as the minimal Met-Peptide construct, which would require processing by the host cell proteasome if a secretion signal were appended to the amino-terminus of the peptide. This is not the case, however, for Ub-Peptide motifs. Appending a bacterial secretion signal to the amino-terminus of a Ub-Peptide sequence enables the Ub-Peptide construct to gain access to the host cell cytosol, where the peptide is then liberated through the action of cellular ubiquitin hydrolases.

We report here the development of a recombinant Listeria minigene expression system that produces high levels of pMHC on the surface of infected cells. We use this system to quantify the expression of surface MHC class I molecules presenting secreted bacterial peptide and to determine the efficiency with which the recombinant peptide is presented. We also demonstrate that expression of a minigene encoded minimal peptide determinant from Listeria can be used to generate a robust $CD8^+$ T cell response *in vivo*.

2. Materials and methods

2.1. Mice and cell line cultures

C57BL/6 mice were purchased from Jackson Laboratories, housed in a pathogen-free facility and used between 8 and 10 weeks of age. All experimental protocols involving mice were used in accordance with Institutional Animal Care and Use Committee guidelines and were approved by the SUNY Upstate Committee on the Humane Use of Animals. The BMA 3.1A7 (BMA3) macrophage-like cell line (kindly provided by Ken Rock) was grown in IMDM (Hyclone) supplemented with 10% heat inactivated fetal bovine serum (FBS) (Cellgro) and 1× Glutamax (Invitrogen). BMA3 cells were maintained at 37 °C in a 7% CO_2 atmosphere.

2.2. Recombinant Listeria strains

All primers used to generate minigene constructs are shown in Table 1. All Listeria strains used are shown in Table 2. The *ActA* promoter and sequence encoding the first 300 amino acids of the ActA protein were amplified from Listeria strain DP-L4056 using primers 1 and 2 (kindly provided by Richard Calendar). DNA encoding Venus-Ub-SIINFEKL was amplified from a plasmid kindly provided by Jack Bennink and John Yewdell using primers 3 and 4. DNA encoding the *ActA* promoter and first 300 amino acids of

Table 2
Listeria strains used in this study.

Name	Description	Source
DP-L4029	ActA-Deficient Listeria, parent strain for AVS, AUS, AU-NP-S-FLAG	Camilli et al. (1993)
AVUS	Venus-tagged SIINFEKL minigene in DP-L4029	This paper
AUS	Smaller, non-fluorescent SIINFEKL minigene in DP-L4029	This paper
AVUS pNF8-mC	AVUS with mCherry-labeled bacterium	This paper
AUS pNF8-mC	AUS with mCherry-labeled bacterium	This paper
AUS pNF8-GFP	AUS with GFP-labeled bacterium	This paper
AU-NP-S-FLAG pNF8-GFP	SIINFEKL embedded between influenza Nucleoprotein (NP) and a 3x-FLAG tag in DP-L4029 with GFP-labeled bacterium	This paper