Molecular Immunology xxx (2015) xxx–xxx

Contents lists available at ScienceDirect

Molecular Immunology

journal homepage: www.elsevier.com/locate/molimm

Review

Quantifying epitope presentation using mass spectrometry

Nathan P. Croft[a,*], Anthony W. Purcell[a], David C. Tscharke[b,c]

[a] Department of Biochemistry and Molecular Biology, Monash University, Clayton, VIC 3141, Australia
[b] Research School of Biology, The Australian National University, Canberra, ACT 2601, Australia
[c] The John Curtin School of Medical Research, The Australian National University, Canberra, ACT 2601, Australia

ARTICLE INFO

Article history:
Received 16 April 2015
Received in revised form 1 June 2015
Accepted 4 June 2015
Available online xxx

Keywords:
Mass spectrometry
Antigen processing and presentation
MHC
Epitope

ABSTRACT

Understanding the absolute quantities of MHC-bound epitopes (pMHC) presented on the surface of cells has long been a critical missing element in our knowledge of antigen presentation to T cells. Until recently, attaining such information has been restricted to the use of pMHC complex-specific monoclonal antibodies or T cell assays probing fractionated peptides eluted from cells. Although successful in a variety of cases, such approaches are limited in their scope and feasibility due to the nature of the reagents they are reliant upon. Here we report on the advancement of targeted mass spectrometry techniques to provide simultaneous and direct measurements of the relative and absolute levels of pMHC molecules and its potential for impact upon the field of antigen processing and presentation.

1. Introduction

The sampling and presentation of proteomes by MHC molecules provides an elegant means for the immune system to survey the products of translation in most vertebrate species. This is achieved through degradation of proteins into peptides that are then presented in the groove of MHC class I or class II molecules on the surface of cells for scrutiny by $CD8^+$ or $CD4^+$ T cells, respectively. The MHC class I and II antigen presentation pathways are now understood in great detail, from the initial degradation of proteins through to the molecular architecture of a peptide epitope bound within a MHC groove and subsequent T cell receptor (TCR) mediated recognition (reviewed in Gras et al., 2012; Neefjes et al., 2011). Furthermore, there have been countless studies assessing the immunogenicity of MHC-bound epitopes (pMHC) from many different sources, including pathogens such as viruses and bacteria, and pathologies such as cancer and autoimmune diseases. However, the factors that drive an epitope to be immunogenic or not, and the fine tuning of that immunogenicity to be dominant or subdominant, are still not fully understood. This knowledge remains critical for the generation of efficacious vaccines and immunotherapies.

An area of MHC presentation that has seen a shortage of analysis is epitope abundance, particularly quantification of multiple pMHC on the same population of cells. This can be considered in absolute terms or as a fraction of the number of MHC molecules available for presentation on a given cell type (Granados et al., 2014). Further, these values may be taken as a snapshot in time, or (perhaps more usefully) studied as the temporal landscape following cellular changes such as infection.

There are a variety of techniques that can be used to detect peptide epitopes and provide measures of abundance, either as intact pMHC surface complexes or free peptide following liberation from MHC. For example, antibodies and flow cytometry can be utilized to stain and detect MHC molecules, whereby fluorescence intensity is used to infer the copy number of pMHC. This has been achieved at a global level, with readily available quantitative immunofluorescence assays to profile individual alleles (Smith and Ellis, 1999), although this readout gives no information on a specific pMHC itself. Such measurements are possible, but require antibodies specific to the pMHC complex of interest. For example, the elegant works of Porgador et al. and Princiotta et al. detailed the fluorescent quantification of murine K^b-SIINFEKL epitope levels using monoclonal antibodies (Princiotta et al., 2003; Porgador et al., 1997), and Dolan has reviewed the application of multivalent TCRs to achieve a similar goal (Dolan, 2013).

A second method of measuring abundance is the use of epitope-specific T cell clones or hybridomas. These can be probed against peptides dissociated from MHC molecules and separated by liquid chromatography (typically reversed phase HPLC, RP-HPLC–see

* Corresponding author.
E-mail addresses: nathan.croft@monash.edu (N.P. Croft), david.tscharke@anu.edu.au (D.C. Tscharke).

http://dx.doi.org/10.1016/j.molimm.2015.06.010
0161-5890/

below) and the T cell response gauged against that of precisely titrated amounts of cognate synthetic peptide in order to arrive at an estimate of presented copies per cell (Anton et al., 1997; Chen et al., 2000).

The above approaches, despite their successes, have a number of drawbacks. Firstly, they may be considered, to different degrees, to be somewhat indirect measurements, i.e. the readout is not directly that of peptide but the reagent(s) used to detect the peptide and/or MHC; secondly, the reagents themselves may be difficult and costly to generate and maintain; lastly, these approaches do not scale easily as they require specific reagents for each pMHC of interest, which further complicates comparisons when considering the potential variance of affinities and specificities inherent to T cells and antibodies.

There is therefore a requirement to be able to measure epitope levels in a more direct manner and that is readily scalable. Mass spectrometry (MS) lends itself well to this ideal: such instrumentation has a long history in being used to detect and measure peptides and offers means to achieve absolute quantitation. MS has successfully been used as a tool to identify the peptides presented on MHC and a variety of quantitative approaches have also been published, but historically, sensitivity has been a major problem. Recent advances in techniques and instrumentation are overcoming this hurdle and improving quantitative approaches and these form the focus of this review.

2. Mass spectrometry techniques for targeted epitope quantitation

MS exists in many different forms, but its basis relies upon separating and detecting ionized compounds according to their mass-to-charge ratio. Proteins and peptides are highly suited to MS due to the propensity for amino acids to become ionized. Further, they can be readily separated by upstream chromatography. This is typically reverse phase high performance liquid chromatography (RP-HPLC), the chemistry of which relies upon the hydrophobic interactions of the analyte with long alkyl chains that are bound to a solid support. Given the complexity of a cellular proteome, such separation is often vital to achieve the deep coverage that is desired in modern proteomics experiments.

Applying existing MS methods to the detection of MHC peptide epitopes is therefore, in principle, a case of modifying the upstream techniques to arrive at a sample of purified peptides. It is important to appreciate that the MS has no discrimination as to the origin of these peptides, and so there are implications when using search algorithm-based sequencing of unknown peptides in this context. There are also considerations and technical challenges to overcome when purifying MHC-bound peptides from cells or tissues (Fig. 1). Firstly, peptides must be liberated from the MHC to which they are bound, either directly from the cell surface or following an isolation step. A direct elution from the cell surface is advantageous because it represents extraction of only the pMHC that are being displayed on the cell at the time of analysis. In practice this is done by using mild acid to strip the pMHC from intact cells (Storkus et al., 1993; Herr et al., 1999; Fortier et al., 2008), which is relatively inefficient in its yield. A method that has seen more widespread use is that of mild cell lysis and enrichment of MHC complexes by immunoaffinity capture using MHC-specific antibodies (the protocols for which have been recently described (Dudek et al., 2015)). This is followed by the addition of acid to dissociate the antibody-pMHC complex, generating a free pool of peptides that can then be analysed by MS. Downstream fractionation by RP-HPLC can also be employed to reduce sample complexity further (such as removal of free MHC heavy chain molecules and β2-microglobulin in the case of MHC class I), in order to maximize detection.

In general, the type of MS device and approach used can lead to either the identification of MHC-bound peptides, or provide relative and absolute quantitation of each peptide of interest. The former has been studied and discussed elsewhere (for review see (Granados et al., 2014)), whilst the latter has seen limited investigation and is only recently seeing a surge in interest (e.g. (Crotzer et al., 2000; Croft et al., 2013; Tan et al., 2011; Keskin, 2015; Testa et al., 2012; Dudek et al., 2012; Hassan et al., 2013; Ternette, 2015)). In order to attain quantification, peptides must be measured in a manner that records signal data with sufficient integrity across multiple data points within the sample. The benchmark method to achieve this is termed multiple reaction monitoring (MRM; also selected reaction monitoring, SRM) (Croft et al., 2013) (Fig. 1). Here, the MS employs three quadrupoles for ion selection: in the first quadrupole (Q1), precursor ions are selected for the peptide of interest; Q2 fragments the selected precursor; and Q3 selects specific fragmentation ions derived from that precursor. This ensures selectivity and specificity and allows extremely sensitive detection of peptides down to attomole levels. Or in the context of pMHC, less than a single copy per cell if starting with 10^8 cells. The major limitation with this approach is that each peptide of interest must already be known in sequence so that the instrument parameters can be configured for detection. However, knowledge from prior literature, predictive MHC epitope binding and/or (preferably) prior MS-based discovery can be used to provide this sequence information. Ideally, synthetic peptides corresponding to each epitope of interest can then be used to fine-tune the MRM parameters and also provide liquid chromatographic retention time data. These also combine to validate the detection of native peptides from biological samples.

The gold standard for quantifying any form of peptide by MS is to use an internal standard in the form of a stable isotopically labeled equivalent of the peptide of interest (AQUA peptide (Gerber et al., 2003)). This approach is applicable to quantification of pMHC (Tan et al., 2011) (Fig. 1). Such an internal standard generates a sufficient mass shift between the light (i.e. native) and heavy (i.e. synthetic isotopically labeled) peptides that they are detected as distinct ions by the MS yet share otherwise identical properties. The most salient of these properties are their chromatographic retention time and propensity to be ionized by the MS. Therefore if a known amount of heavy peptide has been introduced into a sample (at the earliest possible step, the point of epitope elution), the signal it generates can be used as a standard to allow the amount of the native peptide being detected in the same sample to be calculated. This process also automatically accounts for any sample loss during processing, as any losses are reflected into the isotopic standard levels. In the context of pMHC quantitation, the starting number of cells can be incorporated into these equations in order to determine the resultant epitope copy number per cell (Croft et al., 2013; Tan et al., 2011; Keskin, 2015) (Fig. 1).

An important benefit of MRM methods is that they are easily multiplexed, such that a multitude of peptides can be analyzed within the same experiment, thus achieving high throughput and minimizing sample material required for analysis. Such an approach is ideal for systems biology, where it is desirable to profile as many parameters as possible in each experiment. Whilst the use of isotopically labeled internal peptide standards ensures that any downstream sample loss, however minimal (typically <5% signal, NPC unpublished), is automatically accounted for, as noted above these peptides can only be added at the step of acid elution. Hassan et al. have recently proposed the inclusion of isotopically labeled MHC molecules into the experimental procedure in order to quantify sample loss at every step of purification (Hassan et al., 2014). However, the use of saturating amounts of antibody should ensure total capture of pMHC, therefore making the inclusion of isotopic MHC debatable, not to mention expensive.

Please cite this article in press as: Croft, N.P., et al., Quantifying epitope presentation using mass spectrometry. Mol. Immunol. (2015), http://dx.doi.org/10.1016/j.molimm.2015.06.010