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Strategy for isolating and sequencing biologically derived MHC class I peptides

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Abstract

The presentation of MHC class I peptides at cell surfaces and the subsequent cytolytic T-lymphocyte response are critical components of the mammalian immune response. However, the identification and sequencing of such peptides present a considerable analytical challenge since >10 000 peptides at 10^{-15}–10^{-18} *M* concentrations are often present in the mixture. We describe a two-dimensional chromatography approach in conjunction with tandem mass spectrometry to sequence and identify such peptides. After immunoaffinity concentration, and subsequent acetic acid release of MHC class I peptides from MHC protein complex, the peptides are subjected to reversed phase HPLC, where they are separated based on their hydrophilic–hydrophobic character. These coarse fractions are then loaded onto a specially designed membrane preconcentration-capillary electrophoresis cartridge (mPC-CE) and subsequently subjected to on-line mPC-CE–MS analysis. The second dimension of chromatography by CE separation affords resolution of peptides based on their charge/mass (to a first approximation) ratio. Ultimately peptides are sequenced using mPC-CE–tandem mass spectrometry (mPC-CE–MS–MS). We describe the strategy for sequencing <60 femtomoles of a peptide obtained from $3 \cdot 10^9$ K^b-derived EL-4 cells.

Keywords: Peptides

1. Introduction

Major histocompatibility complex (MHC) proteins are essential components of the immune system. One of their roles is to bind and present small peptides (8–10 amino acids — MHC class I peptides) at the cell surface [1,2]. Certain exogenous (non-self) peptides presented elicit a cytolytic T-lymphocyte (CTL) response that results in cell lysis and ultimately death of the infected cell [2]. However, identification and sequencing of such peptides present a formidable analytical challenge. This is due to the fact that MHC class I proteins can bind and present >10 000 cellularly derived peptides that are present at very low concentrations (10^{-15}–10^{-18} molar amounts) [3,4].

In the pioneering work of Hunt and co-workers [3,5,6] a microcapillary HPLC–MS–MS method to sequence both MHC class I and class II peptides was developed. Recently, their efforts culminated in the sequencing of a melanoma-associated antigen using a two-dimensional HPLC–MS–MS approach [4,7]. King et al., have applied the same microcapillary HPLC–MS–MS approach to sequence MHC class II peptides, but in conjunction with a database search to determine the protein of origin from which the peptide was derived [8]. More recently Castoro and Wilkins have advocated the use of MALDI/FTMS as an alternative approach in the sequencing of MHC-derived peptides [9]. In the present work we

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describe a new two dimensional chromatographic approach employing reversed phase HPLC and on-line capillary electrophoresis–mass spectrometry (CE–MS) to separate and sequence MHC class I peptides.

2. Experimental

2.1. Isolation of MHC class I peptides

EL-4 cells ($3 \cdot 10^9$) were lysed with N,N-dimethyl-N-(3-sulfopropyl)-3-[[(3α,5β,7α,12α)-3,7,12-trihydroxy-24-oxocholan-24-yl]-amino]-1-propanaminium hydroxide (CHAPS). The nuclei and membranes were pelleted and the supernatant lysate filtered to remove lipids. The lysate was sequentially passed over sepharose columns containing a) normal mouse serum; b) Y-3 which is an anti-K^b monoclonal antibody. Both columns were washed with 45 column volumes of progressively lower molarity salt solutions. The beads were then treated with acetic acid to release antigen–antibody complexes and the complex was denatured by boiling in 10% acetic acid. The mixture was filtered through a 3 kDa pore-size membrane and the filtrate containing MHC class I peptides subjected to reversed-phase HPLC.

2.2. HPLC

Separations were performed on a Shimadzu HPLC instrument. A 50 μl aliquot was injected (in water–acetonitrile 98:2 v/v) via a Rheodyne injector (Cotati, CA) onto a Vydac analytical column (4.6 cm×250 mm) containing C_{18} packing material (300 Å, 5 μm). Separations were achieved using a mobile phase of A) 0.06% TFA and B) 0.052% TFA in CH_3CN. A solvent gradient of 2–37.5% B (0–60 min); 37.5–75% B (60–90 min) and 75–98% B (90–105 min) was used at a flow-rate of 500 μl/min. Fractions were collected based on their UV response at 214 nm.

2.3. Membrane preconcentration-capillary electrophoresis–tandem mass spectrometry (mPC-CE–MS–MS)

Optimal conditions and a detailed description of sequencing peptides by mPC-CE–MS–MS are described in detail elsewhere [10]. Briefly, mPC-CE–MS–MS analyses were performed using a Beckman P/ACE 2100 CE instrument (Fullerton, CA) coupled to a Finnigan MAT 95Q (Bremen, Germany) mass spectrometer. A Finnigan MAT electrospray ion source was used in positive ion mode. MS–MS conditions consisted of xenon in the r.f.-only octapole collision cell at a gas pressure of $1.2 \cdot 10^{-5}$ mbar. A collision energy of ~24 eV on the MH_2^{2+} precursor ions was used.

The HPLC fraction was pressure injected onto the mPC-CE cartridge after CH_3CN had been removed and the sample diluted to ~150 μl with CE separation buffer (2 m*M* NH_4OAc:1% acetic acid, pH 2.9). Approximately 50 μl was loaded for mPC-CE–MS analysis and ~80–100 μl for mPC-CE–MS–MS. Peptides were eluted from the styrene divinylbenzene (SDB) membrane with ~80 nl of MeOH–H_2O (80:20) sandwiched between a leading stacking buffer (LSB) of 0.1% NH_4OH and a trailing stacking buffer (TSB) of 1% AcOH. This arrangement ensures efficient stacking/focusing of the analyte zone on application of the CE voltage (25 kV). CE separation was carried out in a bare-fused silica capillary (75 cm×25 μm).

3. Results and discussion

3.1. General strategy

The complexity of MHC class I peptide mixtures, as well as the similarity of their amino acid sequences requires a number of factors to be considered for specific peptide structural characterization. In particular it is important that cell lysis and the subsequent purification of the peptides utilizes reagents that will not decrease sensitivity limits of the MS–MS analysis. Furthermore, it is also important to develop a two-dimensional chromatography approach that employs different physical properties of the peptides in the mixture. This affords optimal opportunity to separate complex mixtures of structurally similar peptides. The strategy we have developed for sequencing MHC class I peptides is summarized in Fig. 1.

The analytical challenges to sequence MHC class I peptides are formidable and warrant several stages of prefractionation and purification [3,7]. However, it

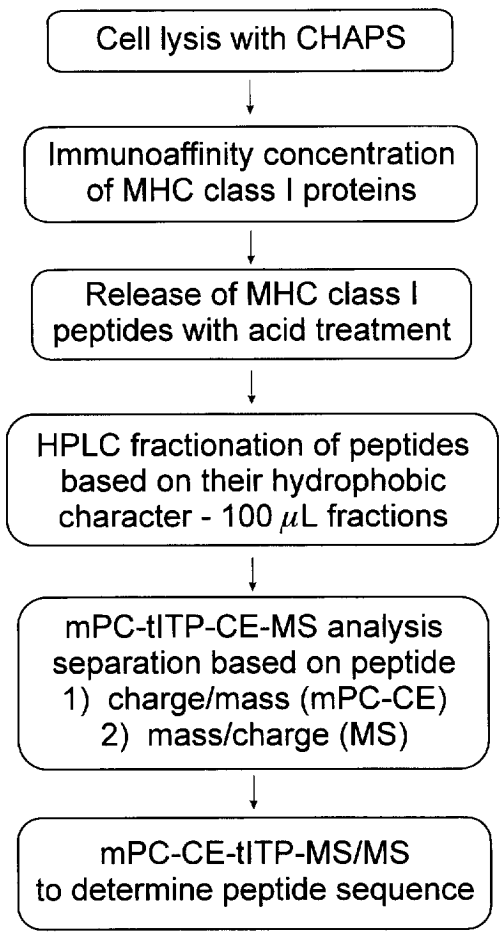

Fig. 1. Strategy for isolation, purification, and structural characterization of MHC class I peptides using two-dimensional chromatography in conjunction with MS.

appears that the choice of detergent to lyse cells is important. Both cationic and anionic compounds can adversely affect electrospray ionization-MS (ESI-MS) performance. Such detergents are extremely difficult to remove from peptide mixtures, even after many stages of purification. Hence, they can still be present in the final MS analysis step, and this results in significant suppression of ESI-MS peptide ion current [11]. However, we have found that using a zwitterionic detergent results in no deleterious effects on the ESI-MS analysis of MHC class I peptides. The use of CHAPS in the present study resulted in efficient cell lysis and subsequent MHC protein recovery with no discernible compromise in CE–MS and CE–MS–MS MHC class I peptide analysis performance.

After immunoaffinity concentration of MHC proteins containing MHC class I peptides, and subsequent acetic acid release of the peptides, the latter were subjected to reversed-phase HPLC. In this first dimension of chromatography, the MHC class I peptides are separated based on their hydrophobic/hydrophilic properties. This initial chromatographic step affords a course fractionation of the complex peptide mixture into ~100 μl aliquots. Such fractions have been shown to contain a large number (~5–50) of peptides [4,12]. Hence a complimentary second stage of high resolution chromatography is necessary to ensure optimal resolution of individual peptides.

The second dimension of chromatography using the enhanced resolving capability of CE to separate complex peptide mixtures appears to be appropriate since analytes are separated (to a first approximation) on their charge-to-mass ratio [13]. The potential of on-line CE–MS to effect the separation of MHC class I peptide mixtures has been reported previously [11,14,15]. However, it has been noted that this approach can be problematical due to the limited sample volume loading capacity of conventional CE capillaries [10,11]. Ultimately, this leads to poor concentration limits of detection (CLOD), and an inability to handle dilute, complex analyte mixtures. In order to overcome this problem we have developed technology which we term membrane preconcentration-CE–MS (mPC-CE) [10,16–21] and in conjunction with MS (mPC-CE–MS) it consists of an impregnated adsorptive membrane in a Teflon cartridge installed at the inlet of the conventional CE capillary. This arrangement facilitates the ready removal of the cartridge to allow CE capillary cleaning/conditioning and activation of the adsorptive membrane. Using this approach, it is possible to undertake on-line analyte loading onto the membrane of in excess of 100 μl solution volumes. Furthermore, on-line sample clean-up prior to CE–MS analysis is possible, as this is particularly important for in vivo derived samples such as cell culture lysates.

The use of mPC-CE–MS in the analysis of dilute synthetic peptide mixtures has recently been reported [10,20]. However, in order to remove peptides from the adsorptive membrane, an elution solvent that

contains some water [e.g., methanol–H_2O (80:20, v/v)] is necessary. This ensures that efficient removal of analytes from the membrane occurs. Furthermore, optimal peptide recovery is achieved only when >50 nl of such a solvent mixture is used. This relatively large volume of elution solvent, along with the inefficient analyte stacking that occurs, results in some peak broadening and loss of analyte resolution. Therefore, the use of moving boundary tITP conditions are used to focus analyte zones and also aid in the dispersion of the organic elution solvent [10,21]. It is carried out by eluting peptides from the membrane between zones of a leading stacking buffer (LSB), typically 0.1–5% NH_4OH in water, and a trailing stacking buffer (TSB), typically 1% acetic acid in water or CE separation buffer. We have also demonstrated that variation of the ratio of concentration and volume of LSB, elution solvent, and TSB can be used to manipulate the analyte migration time, peak width, and resolution [21].

3.2. Sequencing of K^b-derived MHC class I peptides

It has become clear in recent years that MHC protein molecules are regarded as peptide transporters [22]. T-cell recognition of MHC antigens is strongly influenced by the associated peptide in both positive and negative ways. Thus certain peptide/MHC protein combinations will activate T-cells while others termed TCR antagonists will block T-cell stimulation [23,24]. TCR antagonists are also capable of inducing a critical stage of T-cell development in the thymus, called positive selection [24,25]. In the present study, we utilize a TCR transgenic model system in which the TCR is specific for an ovalbumin peptide (SIINFEKL) in the context of the mouse MHC class I molecule K^b [25].

Specifically, in the present study a K^b fraction of $3 \cdot 10^9$ mouse derived EL-4 cells were subjected to the protocols outlined in Fig. 1. HPLC fractions (~100 μl) were collected based on their UV absorbance, and this is shown in Fig. 2. In order to undertake mPC-CE–MS and mPC-CE–MS–MS analysis, the CH_3CN was removed and the resulting aqueous fractions (~40–70 μl) were *diluted* with CE separation buffer solution. This was done in order to maximize recovery of the MHC class I peptides

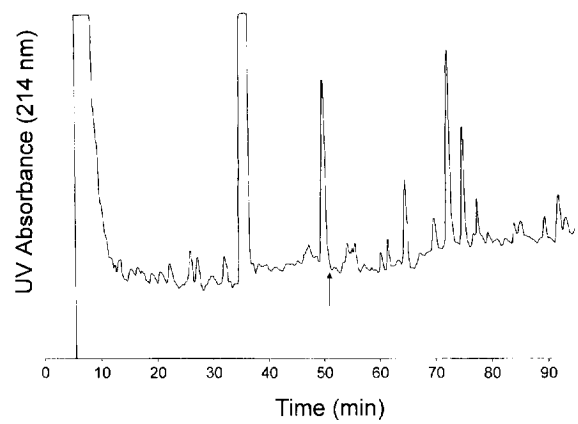

Fig. 2. Reversed-phase HPLC analysis of $3 \cdot 10^9$ K^b derived EL-4 MHC class I peptides. UV detection at 214 nm. Arrow denotes the ~100 μl fraction subjected to further mPC-CE–MS and MS–MS analyses.

present in each fraction. Subsequently an aliquot of this solution (50 μl) was loaded off-line onto the mPC-CE cartridge. This off-line loading and sample clean-up method (with CE separation buffer) was used, since the flow-rate in the on-line 25 μ I.D. mPC-CE capillary was only 120–150 nl/min. Hence on-line loading of a 50 μl of sample would take up to 5.5 h. However, the flow-rate in an mPC cartridge alone can be much higher off-line, since these devices can withstand relatively high pressures (~60 p.s.i.; 1 p.s.i.=6894.76 Pa). Furthermore, system back pressure is also reduced and up to 100 μl of sample can often be loaded off-line in <5 min, significantly reducing analysis time. Also, since the flow in an mPC cartridge is bidirectional, sample loaded with a reverse flow followed by clean-up in the forward direction leads to flushing of sample-derived particulate matter from the mPC cartridge prior to assembly of the mPC-CE capillary. This improves the reproducibility of MPC-CE–MS by reducing the tendency for clogging of the cartridge.

The mPC-CE–MS ion electropherogram revealed several major ion responses (Fig. 3A). A minor ion at a migration time at ~14 min afforded a doubly charged ion at m/z 503.6 (MH_2^{2+}) (Fig. 3B). Approximately 80 μl of the remaining diluted HPLC fraction was subsequently subjected to mPC-CE–MS–MS. The doubly charged precursor ion at m/z 503.6 was subjected to collision induced dissociation

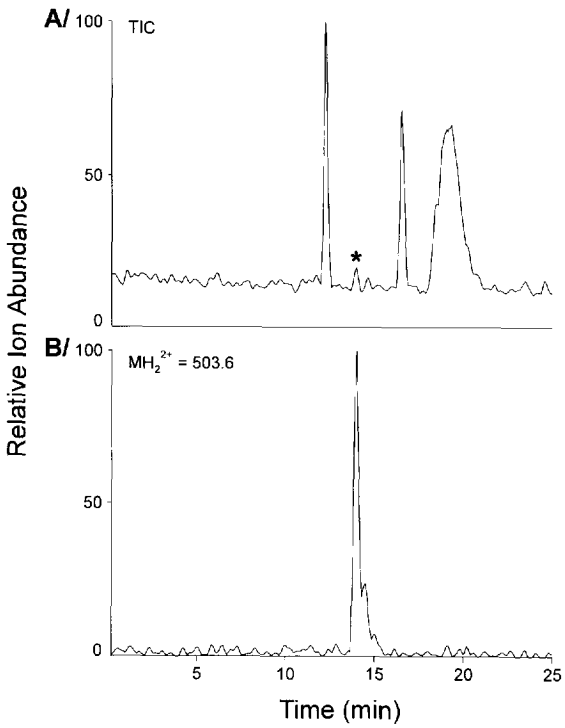

Fig. 3. mPC-CE–MS analysis of HPLC peptide fraction. A/: total ion current (TIC) of the analysis of ~50 μl aliquot of the diluted HPLC fraction. B/: ion electropherogram of response marked with asterisk (*), shown to be doubly charged, corresponding to MH_2^{2+}=503.6.

using xenon as the collision gas. The resulting product ion spectrum (Fig. 4) revealed a well defined series of y and b series ions. Interpretation of these two series indicated a sequence of XSFKFDHX (where X is either I or L). The spectral data was also

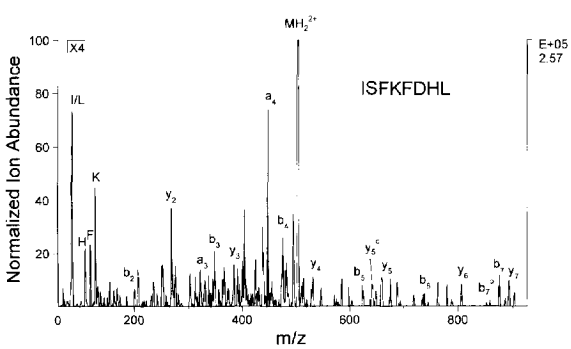

Fig. 4. Product ion spectrum of precursor ion (MH_2^{2+}) after mPC-CE–MS–MS analysis.

searched and interpreted using the SeaQuest database routine developed by Yates et al. [26], The search revealed that the peptide was derived from F-actin and found to be of self origin. From this information, the peptide sequence was determined to be ISFKFDHL

4. Conclusions

In the present study, we describe a strategy for sequencing MHC class I peptides. We employ a two-dimensional chromatography that consists of HPLC fractionation and on-line mPC-CE–MS. The use of mPC-CE–MS allows loading and on-line sample clean-up for >100 μl solutions containing analyte peptides and utilizes an impregnated membrane adsorptive phase contained in a cartridge placed at the inlet of the CE capillary. We show that the mPC-CE cartridge has no adverse effects on overall CE–MS performance. Ultimately we use this approach to sequence peptides derived from EL-4/K^b immunoprecipitated MHC class I molecules and show for the first time, to our knowledge, a peptide sequence derived from MS–MS analysis of K^b.

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