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Comparison of peptide mass mapping and electron capture dissociation as assays for histone posttranslational modifications

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Abstract

Posttranslational modifications of core histones play a critical role in the structure of chromatin and the regulation of gene activities. Improved techniques for determining these modification sites may lead to a better understanding of histone regulation at the molecular level. LC–MS peptide mass mapping was performed on pepsin, trypsin and Glu-C digests of bovine thymus H4 using a QqTOF instrument. The well established modification sites of H4 (acetylation of K8, 12, 16 and methylation of K20) were observed in addition to several recently discovered modifications including: methylation of K31, 44, 59 and acetylation of K20, 77, 79. For comparison, electron capture dissociation (ECD) was performed on intact H4 along with several peptides from enzymatic digestion. The results from the ECD experiments of histone H4 indicated the acetylation of K5, 12, 16, 31, 91 and the methylation of K20 and 59 in good agreement with the result from peptide mapping. The work is dedicated to Alan G. Marshall on his 60th birthday. His endeavors in the advancement of FT-ICR facilitated experiments reported herein.

Keywords: Histone posttranslational modifications; LC–MS peptide mapping; Electron captured dissociation (ECD)

1. Introduction

Histones play an important role in the assembly of chromatin and regulation of gene activities such as transcription, DNA replication, DNA repair and recombination. The posttranslational modifications (acetylation, methylation, phosphorylation, ADP-ribosylation and ubiquitination) of specific residues on the core histones have been demonstrated to be critical to these regulatory functions [1–6]. Traditional methods for the study of histone posttranslational modifications include micro-sequencing and immunoassay based methods [7,8]. Micro-sequencing can provide identity and location of N-terminal posttranslational modifications. However, this technique is extremely tedious and requires relatively large amount of purified sample for analysis of the entire protein sequence. Immunoassays are very sensitive, but rely on antibodies that recognize the different modifications. At present antibodies are limited to a few well characterized sites of modification (primarily on the N-terminal tail). The lack of a comprehensive set of antibodies has limited the systematic analysis of histone modifications. In addition, the simultaneous presence of other modifications on the same histone may impact the specificity of antibodies directed at a specific individual modification. For example, antibodies for the acetylated lysine 14 (K14) of histone H3 are blocked by the presence of a phosphorylated serine 10 (S10) [9,10].

Posttranslational modifications result in an increase or decrease in the molecular weight of a protein as compared with its unmodified form. These mass shifts provide information regarding the identity of the modifications. Mass spectrometry (MS) is well suited to the task of determining these mass shifts and subsequently the identity of the posttranslational modifications. MS-based modification assays do not suffer the same limitation as antibodies. For example, the simultaneous presence of multiple modifications does not impact specificity as with antibodies and thus provides a better picture of the modification status in vivo. More detailed information related to the location of a specific modification can be achieved using peptide mass mapping and/or tandem mass spectrometry.

Both “bottom-up” and “top-down” sequencing approaches are widely used for the investigation of the identification of proteins and their posttranslational mod-

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ifications [11,12]. In the "bottom-up" approach (peptide mapping, or peptide mass fingerprinting), the target protein is enzymatically digested. The molecular weight of each peptide fragment is measured by mass spectrometry and then compared with the theoretical molecular weight of the unmodified sequence. The mass shift between the measured mass and theoretical mass gives the identity of the modification. In recent years, "top-down" approaches that use collision-induced dissociation (CID), infrared multi-photon dissociation (IRMPD) and/or electron captured dissociation (ECD) have been employed to determine primary structural information from intact proteins [13–16]. In this approach, precursor ions of the intact protein are isolated and fragmented in vacuo followed by the mass analysis of the product ions. Based on the mass of the product ions, one can piece together the protein sequence and more importantly identify and localize the posttranslational modifications. Among these tandem mass spectrometric techniques, ECD is increasingly used for the structural and posttranslational modification studies of protein and peptide. As a nonergeodic technique, ECD primarily cleaves the protein backbone preserving posttranslational modification labile to dissociation by other fragmentation techniques [17,18]. Therefore, the information regarding posttranslational modifications can be directly assigned from the ECD spectrum.

As a component of evaluating potential chemopreventative therapies using histone deacetylase (HDAC) inhibitors, there is a need to develop assays for histone posttranslational modifications from preclinical in vitro experiments and from patients involved in clinical trial of the new therapies [19–26]. Previously we reported the application of peptide mass mapping by use of 7 T Fourier transform ion cyclotron resonance mass spectrometry (FT-ICR MS) to determine the posttranslational modifications of bovine thymus core histones [27]. The results showed that in addition to the well established N-terminal modification sites, several novel modification sites were also observed. These included the acetylation of K20, 77, 79, 31 and the methylation of K16, 31, 44 and 59 for histone H4. Unfortunately, the accessibility of FT-ICR MS instrumentation in clinical settings is not widely available. Thus, we are interested in methods using readily available instrumentation for the development of these histone assays. Liquid chromatography mass spectrometry (LC–MS) has greatly facilitated the determination of the molecular weight of proteins from complicated mixtures [28–30]. Furthermore, LC–MS instrumentation is widely available and has been adapted to the analysis of global modification patterns of histones [31–33]. Thus, we sought to evaluate LC–MS of proteolytic fragments of H4 and ECD of intact H4 as potential histone assays. The resulting sequence data from the two techniques were compared and evaluated for their effective translation to a clinical assay. Both techniques revealed the abundant N-terminal modifications and also several of the core and C-terminal modifications previously reported by our group.

2. Experimental

2.1. Extraction and purification of H4

Core histones were extracted from bovine calf thymus (Worthington Biochemical Corp., Lakewood, NJ, USA) using an acidic extraction procedure [34]. Frozen calf thymus was thawed in 10 volumes of buffer A (0.01 M $MgCl_2$, 0.025 M KCl, 0.075 M Tris–HCl, pH 7.5, 0.05 M $NaHSO_3$ and 0.25 M sucrose, all purchased from Sigma, St. Louis, MO, USA) before homogenization using a Polytron Homogenizer (Brinkmann Instruments Inc., Westbury, NY, USA). The homogenized mixture was centrifuged for 5 min at 1000 × *g*. The pellet was then resuspended 2–3 times in buffer A and centrifuged as described above followed by five washings with 0.14 M NaCl containing 0.05 M $NaHSO_3$. The pellet was resuspended in 10 volumes of 0.25 M HCl and stirred for 2 h in a cold room at 4 °C. The mixture was centrifuged at 27,000 × *g* for 10 min. The supernatant was dialyzed against 300 volumes of 0.025 M HCl for 12 h in a cold room at 4 °C. Core histones were then precipitated using acetone, separated and fractionated by reverse phase high performance liquid chromatography (RP-HPLC) with a variable wavelength UV detector (HP 1100 system, Agilent Technologies, USA). H4 fractions were collected and subsequently used for the LC–MS peptide mapping and ECD experiments.

2.2. LC–MS of histone H4 proteolytic digests

A 1.0 mM aqueous stock solution of the purified H4 fraction was diluted into a buffer solution to a final concentration of 100 μM. Enzymes were added to the corresponding buffer solutions: (1) 100 mM phosphate buffer (pH 7.8) for trypsin and Glu-C digestions and (2) 0.5% formic acid solution (pH = 2.1) for pepsin digestion. The enzyme:substrate ratio was 1:100 for pepsin and trypsin and 1:40 for Glu-C. The mixture was incubated at 37 °C for different reaction periods (trypsin 2 h, Glu-C 4 h and pepsin 10 min). The reactions were quenched by addition of 0.1% TFA to the tryptic and Glu-C digests or by addition of phosphate buffer (pH 8) to the peptic digest.

The digest mixtures of histone H4 prepared as described above were separated by RP-HPLC. HPLC grade water with 0.1% trifluoroacetic acid and acetonitrile with 0.1% trifluoroacetic acid (VWR Scientific, Bridgeport, NJ, USA) were used as mobile phases A and B. A 20 μl aliquot of a H4 digest (100 μM) was injected and separated on a C18 column (Supelco LC-C18-DB, 4.6 mm × 250 mm). The composition of mobile phase B was increased linearly from 5 to 15% in 5 min, 15–40% from 5 to 30 min and then 40–50% from 30 to 35 min. The gradient was maintained at 50% B until 40 min. Composition of B was then increased from 50 to 100% from 40 to 45 min followed by column washing at 100% B for 1 min. The gradient was then re-

turned to 5% B. Finally, the column was equilibrated at 5% B for 8 min prior to the next injection. The flow rate was 1.0 ml min^{-1}. Peptide fractions eluted from the column were detected with a UV detector at the wavelength of 214 nm. A Micromass Q-Tof™ II (Micromass, Wythenshawe, UK) mass spectrometer with an orthogonal electrospray source (Z-spray) was coupled to the outlet of the HPLC using a T-splitter. Histones were infused into the electrospray source at the flow rate of about 20 μl min^{-1} (split ratio 50:1). For the optimal ESI conditions, the capillary voltage was 3 kV, source temperature was 100 °C and cone voltage was 50 V. Data were acquired in continuum mode at the rate of 1 scan s^{-1} and deconvoluted using the MaxEnt method. All spectra were obtained in the positive ion mode. NaI was used for external mass calibration over the *m*/*z* range 500–2500.

2.3. Electron capture dissociation

ECD was performed on intact H4 and its proteolytic fractions purified by RP-HPLC. The H4 tryptic digest fractions were separated under the same chromatographic conditions described above. After separation, each fraction was collected and concentrated prior to ECD. Experiments were performed on a home-built 9.4 T FT-ICR mass spectrometer (National High Magnetic Field Laboratory, Florida State University, Tallahassee, FL, USA) [35,36] or a Bruker Apex II7e 7T FT-ICR. Samples were directly infused into the instrument through a fused silica capillary (i.d. 50 μm) at a flow rate of 100 nl min^{-1}. A voltage of 2.2 kV was applied to the capillary to obtain the electrospray. The ionized sample was accumulated in an external octapole prior to transfer to the ICR cell (2 s for the 9.4 T and 0.8 s for the 7 T).

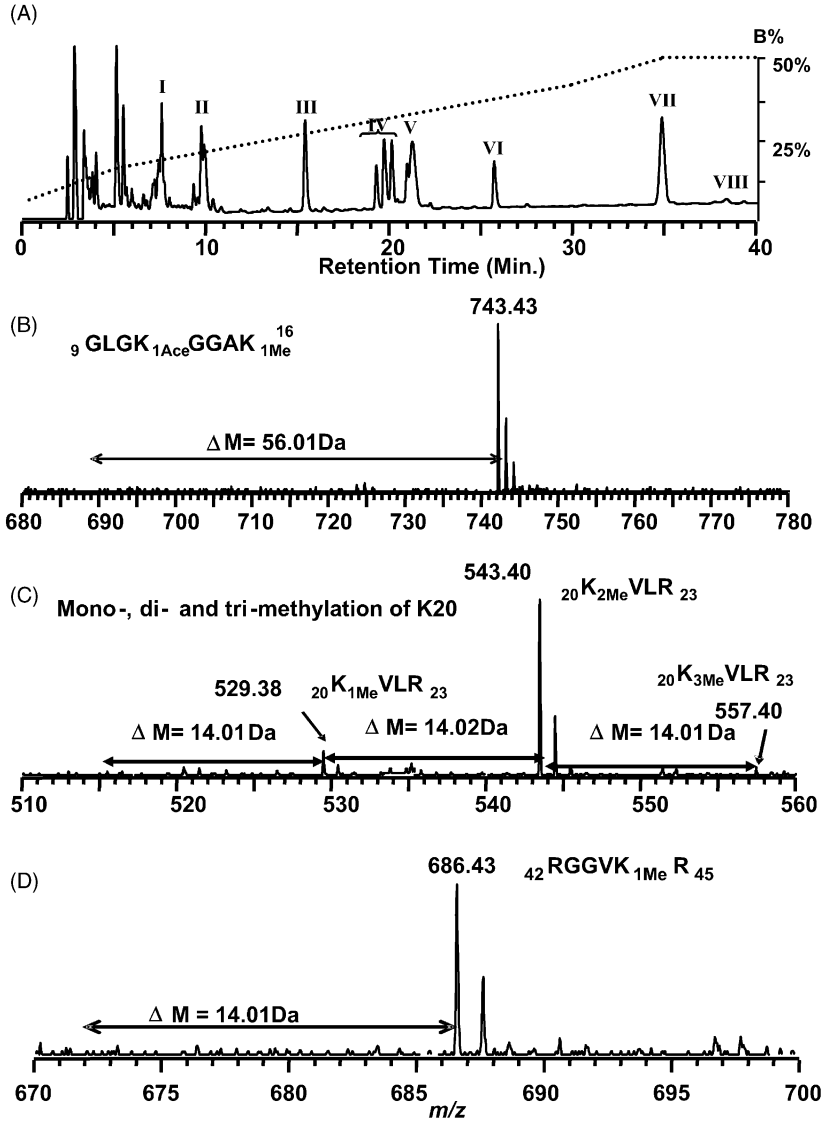

Fig. 1. LC–MS peptide mapping of histone H4 after digestion using trypsin. (A) The tryptic digestion elution profile. (B) The mono-methylation of K16 in H4. (C) The mono-, di- and tri-methylation of K20. (D) The mono-methylation of K44.

Table 1
LC–MS peptide mapping of H4 trypsin digest

Fraction	*m/z* (measured)	*m/z* (theoretical)	Δ*M*	Error (1‰)	Fragment	Modification
I	515.35	515.34		0.118	^{37}LARR^{40}/^{36}RLAR^{39}	
II	529.38	515.37	14.01	−0.086	^{20}K$_{1Me}$VLR^{23}	1 Me
	543.40	515.37	28.03	−0.035	^{20}K$_{2Me}$VLR^{23}	2 Me
	557.40	515.37	42.03	−0.305	^{20}K$_{3Me}$VLR^{23}/^{20}K$_{1Ac}$VLR^{23}	1 Ac or 3 Me
	1134.54	1134.54		−0.003	^{68}DAVTYTEHAK^{77}	
III	1325.75	1325.75		0.002	^{24}DNIQGITKPAIR^{35}	
	1347.87	1277.80	70.07	0.197	^{13}GGAK$_{1Ac}$RHRK$_{2Me}$VLR^{23}/^{13}GGAK$_{2Me}$RHRK$_{1Ac}$VLR^{23}	1 Ac, 2 Me
	1378.79	1378.81		−0.148	^{6}GGKGLGKGGAKRHR^{19}	
	1400.84	1386.84	14.00	−0.166	^{56}GVLK$_{1Me}$VFLENVIR^{67}	1 Me
IV	714.34	714.35		−0.077	^{96}TLYGFGG^{102}	
	850.54	808.53	42.01	−0.020	^{18}HRK$_{1Ac}$VLR^{23}	1 Ac
	923.54	909.55	13.99	−0.259	^{13}GGAK$_{0\text{-}1Me}$RHRK$_{0\text{-}1Me}$^{20}	1 Me
	927.52	843.52	84.00	−0.173	^{9}GLGK$_{1Ac}$GGAK$_{1Ac}$R^{17}	2 Ac
	1180.62	1180.62		−0.012	^{46}ISGLIYEETR^{55}	
V	686.43	672.43	14.00	−0.130	^{40}RGGVK$_{1Me}$R^{45}	1 Me
	850.54	808.53	42.01	−0.02	^{18}HRK$_{1Ac}$VLR^{23}	1 Ac
	1180.63	1180.621		0.057	^{46}ISGLIYEETR^{55}	
VI	630.37	616.38	13.99	−0.294	^{6}GGK$_{0\text{-}1Me}$GLGK$_{0\text{-}1Me}$^{12}	1 Me
	743.43	687.42	56.01	−0.173	^{9}GLGK$_{1Ac}$GGAK$_{1Me}$^{16}	1 Ac, 1 Me
	989.56	989.58		−0.134	^{60}VFLENVIR^{67}	
	1027.57	929.55	98.02	−0.212	^{6}GGK$_{1Ac}$GLGK$_{1Ac}$GGAK$_{1Me}$^{16}	2 Ac, 1 Me
VII	966.08^{2+}	938.08^{2+}	56.00	−0.137	^{6}GGKGLGKGGAKRHRKVLR^{23}	1 Ac, 1 Me
VIII	1136.18^{2+}	1136.18^{2+}		0.025	^{18}HRKVLRDNIQGITKPAIRR^{36}/^{17}RHRKVLRDNIQGITKPAIR^{35}	

Ions were transferred to the ICR cell and trapped by applying trapping potentials of 2–10 V. The precursor ions of interest were isolated by application of SWIFT excitation to the excite electrodes. Electrons were then gated into the cell by floating the bias of the dispenser cathode negative with respect to the adjacent trapping electrode. Optimal ECD efficiency was obtained by gating the electron gun from 10 to −10 V for 5 ms with the trapping potentials at 10 V. All experiments were controlled by a MIDAS data station [37,38]. Individual scans (512 K-2MWord), were coadded prior to baseline correction, Hanning apodization, Fourier transform and magnitude calculation. Product ion mass and charge were determined using the THRASH algorithm integrated into the MIDAS analysis software [39].

3. Results and discussion

3.1. LC–MS peptide mapping of H4 tryptic digestion

The H4 tryptic peptide elution profile detected at 214 nm is shown in Fig. 1A. These peptide fragments were infused into the mass spectrometer with a split ratio of about 50:1. All the peptide fragments detected are listed in Table 1. The sequence coverage of H4 digested by trypsin was about 76% (residues 6–77 and 96–102). Most of the detected modifications occurred on the N-terminal tail of the protein, such as the acetylation of K8, 12, 16, 20, and methylation of K16 and 20. In addition, modifications in the core domain of the protein were also observed including methylation of K44 and K59.

The N-terminal tails of histones are known for their high degree of acetylation. The mono-, di-, tri- and tetra-acetylated forms of histone H4 are well established [40,41]. The most likely sites of modifications are believed to occur on the N-terminal tail and lysine residues 5, 8, 12 and 16 [42]. In our experiments, no tryptic fragments were observed that covered K5 and the N-terminal serine residue. Thus, these two sites were not considered. However, acetylation and possible methylations were observed for lysine 8, 12 and 16. A mass shift (84.00 Da) corresponding to di-acetylation was observed at 927.52^{+} *m/z* for fragment 9-17 (GLGKGGAKR) in the eluted fraction IV. Since this peptide only contains two lysine residues, these acetylations most likely occur at K12 and 16. Meanwhile, a peak at 743.43^{+} *m/z* was shifted by 56.01 Da compared with the unmodified form of fragment 9-16 (GLGKGGAK) in fraction VI, suggesting a combination of a single acetylation and methylation on this fragment (Fig. 1B). Trypsin is a serine protease, and its activity is inhibited at lysine residues that have been acetylated [27,41,43,44]. Therefore, the acetylation was assigned to K12 and 16 were assigned as the most likely methylation site. In fraction VI, a peak was observed as the di-acetyl, mono-methyl isoform of fragment 6-16 (GGKGLGKGGAK) at 1027.59^{+} *m/z*. Similarly, this mass shift was assigned as the acetylation of

K8 and 12 and methylation K16. Lysine residue 8, 12, 16 and 20 were also assigned as possible methylation sites because a mass shift corresponding to mono-methylation was observed in fragment 6-12 (GGKGLGK) at 630.37^{+} *m*/*z* (fraction VI) and fragment 13-20 (GGAKRHRK) at 923.54^{+} *m*/*z* (fraction IV).

K20 had been shown to exist as mono-, di- and tri-methylated forms [32,41,45]. Ions corresponding to the different methylated isoforms of the fragment 20-23 (KVLR) at 529.38^{+} *m*/*z*, 543.40^{+} *m*/*z* and 557.40^{+} *m*/*z* (all in fraction II) were observed. Direct comparison of the relative abundances of these isoforms allows for a semi-quantitative estimation of the relative levels of methylation on K20. Sarg et al. reported a similar distribution of K20 methylation determined by the use of hydrophilic interaction chromatography [45]. In both cases, the di-methylated isoform was present in the highest abundance (Fig. 1C). The methylation pattern of K20 can be used to help interpret other multiply modified isoforms detected. For example, a mass shift (70.07 Da) corresponding to the combination of a mono-acetylation and di-methylation was observed for fragment 13-23 (GGAKRHRKVLR, 1347.87^{+} *m*/*z*) in fraction III. Since K20 is methylated, the acetylation was assumed to reside on K16. However, it is important to note that K20 might also be acetylated. This concern stems from the presence of a peptide with a mass shift of 42.01 Da that corresponds to fragment 18-23 (HRKVLR). The mass accuracy in Q-TOF is not sufficient to distinguish tri-methylation from mono-acetylation (42.05 Da versus 42.01 Da). Mono-acetylation of K20 in the same fragment has been observed [27] and the presence of tri-methylation is believed to be low [45]. Thus, this modification may likely be due to mono-acetylation of K20.

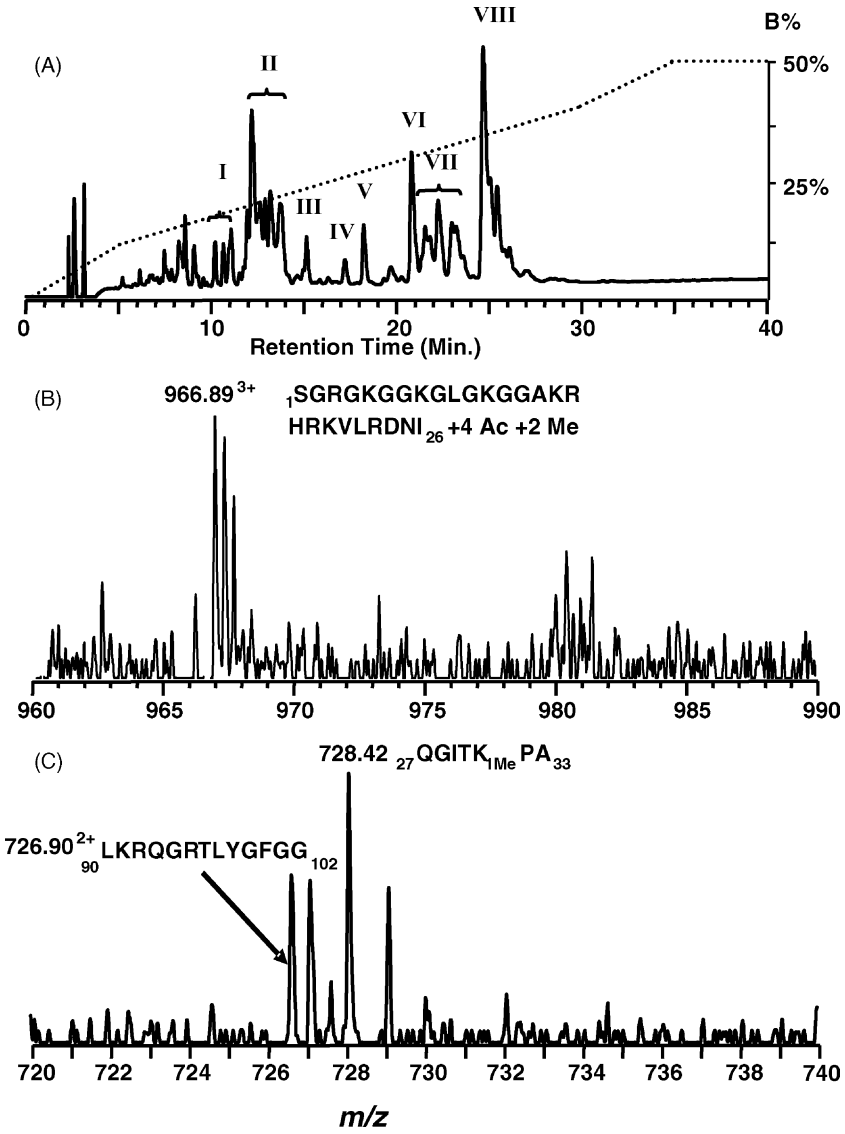

Fig. 2. LC–MS peptide mapping of histone H4 after digestion using pepsin. (A) The elution profile. (B) N-terminus modifications. (C) Mono-methylation of K31.

Table 2
LC–MS peptide mapping of H4 pepsin digest

Fraction	*m/z* (measured)	*m/z* (theoretical)	Δ*M*	Error (%)	Fragment	Modification
IV	1012.65	1012.65		−0.019	$_{38}$ARRGGVKRI$_{46}$	
	927.51^{2+}	873.46^{2+}	108.10	0.232	$_{52}$EET1PO$_4$RGVLK$_{2Me}$VFLENVI$_{66}$	1 PO$_4$, 2 Me
	803.53	803.51		0.273	$_{77}$KRKTVTA$_{83}$	
V	887.51	803.51	84.00	−0.180	$_{77}$K$_{1Ac}$RK$_{1Ac}$TVTA$_{83}$	2 Ac
	728.42	714.51	13.91	−0.113	$_{27}$QGITK$_{1Me}$PA$_{33}$	1 Me
	726.90^{2+}	705.89^{2+}	42.03	0.114	$_{77}$K$_{0\text{-}1Ac}$RK$_{0\text{-}1Ac}$TVTAMDVVY$_{88}$	1 Ac
	726.90^{2+}	726.90^{2+}		−0.018	$_{90}$LKRQGRTLYGFGG$_{102}$	
VIII	966.48	966.49		−0.068	$_{51}$YEETRGVL$_{58}$	
	1079.59	1079.57		0.128	$_{50}$IYEETRGVL$_{58}$	
VIII	773.46^{2+}	773.47^{2+}		−0.085	$_{38}$ARRGGVKRISGLIY$_{51}$	
	766.87^{2+}	705.89^{2+}	121.97	−0.072	$_{77}$K$_{0\text{-}1Ac}$RK$_{0\text{-}1Ac}$T$_{0-1PO_4}$VT$_{0-1PO_4}$AMDVVY$_{88}$	1 Ac, 1 PO$_4$
	726.90^{2+}	705.89^{2+}	42.03	0.114	$_{77}$K$_{0\text{-}1Ac}$RK$_{0\text{-}1Ac}$TVTAMDVVY$_{88}$	1 Ac
	726.90^{2+}	726.90^{2+}		−0.018	$_{90}$LKRQGRTLYGFGG$_{102}$	
	506.33	506.33		−0.075	$_{59}$KVFL$_{62}$	
	762.42^{2+}	741.41^{2+}	42.02	0.085	$_{77}$K$_{0\text{-}1Ac}$RK$_{0\text{-}1Ac}$TVTAMDVVYA$_{89}$	1 Ac
	781.42^{2+}	741.41^{2+}	80.01	0.291	$_{77}$KRK T$_{0-1PO_4}$VT$_{0-1PO_4}$AMDVVYA$_{89}$	1 PO$_4$
VIIII	838.81^{3+}	834.16^{3+}	13.97	−0.186	$_{11}$GK$_{0\text{-}1Me}$GGAK$_{0\text{-}1Me}$RHRK$_{0\text{-}1Me}$VLRDNIQGITK$_{0\text{-}1Me}$PA$_{33}$	1 Me
	762.41^{2+}	762.42^{2+}		−0.104	$_{89}$ALKRQGRTLYGFGG$_{102}$	
	762.41^{2+}	741.41^{2+}	42.01	0.021	$_{77}$K$_{0\text{-}1Ac}$RK$_{0\text{-}1Ac}$TVTAMDVVYA$_{89}$	1 Ac
	773.48^{3+}	773.49^{3+}		−0.088	$_{30}$TKPAIRRLARRGGVKRISGLI$_{50}$	
	775.72^{3+}	761.72^{3+}	42.00	−0.065	$_{70}$VTYTEHAK$_{0\text{-}1Ac}$RK$_{0\text{-}1Ac}$TVTAMDVVYA$_{89}$	1 Ac
	966.89^{3+}	901.53^{3+}	196.08	−0.025	$_{1}$SGRGKGGKGLGKGGAKRHRKVLRDNI$_{26}$	4 Ac, 2 Me
	1147.66^{2+}	1065.64^{2+}	164.04	0.022	$_{16}$K$_{0\text{-}1Ac}$RHRK$_{0\text{-}1Ac}$VLRDNIQGIT$_{0-1PO_4}$K$_{0\text{-}1Ac}$PA$_{33}$	2 Ac, 1 PO$_4$
	809.41^{2+}	809.39^{2+}		0.185	$_{63}$ENVIRDAVTYTEHA$_{76}$	
X	876.51^{3+}	871.85^{3+}	13.98	−0.119	$_{11}$GK$_{0\text{-}1Me}$GGAK$_{0\text{-}1Me}$RHRK$_{0\text{-}1Me}$VLRDNIQGITK$_{0\text{-}1Me}$PAI$_{34}$	1 Me
	835.18^{3+}	835.18^{3+}		−0.018	$_{27}$QGITKPAIRRLARRGGVKRISGL$_{49}$	
	918.22^{3+}	890.21^{3+}	84.02	0.016	$_{16}$K$_{0\text{-}1Ac}$ RHRK$_{0\text{-}1Ac}$VLRDNIQGITK$_{0\text{-}1Ac}$ PAIRRL$_{37}$	2 Ac
	910.88^{3+}	901.53^{3+}	28.05	0.081	$_{1}$SGRGK$_{0\text{-}2Me}$GGK$_{0\text{-}2Me}$GLGK$_{0\text{-}2Me}$GGAK$_{0\text{-}2Me}$RHRK$_{0\text{-}2Me}$VLRDNI$_{26}$	2 Me

Two modifications were detected within the globular domain of histone H4: methylation of K44 and K59. As shown in Fig. 1D, in the elution fraction V, a mass shift of 14.00 Da was seen on fragment 40-45 (RGGVKR, at 686.43^{+} *m/z*), which can be assigned as mono-methylation of the only lysine in this fragment: K44 (or possibly either of the arginine). Similarly, the mass difference of 14.00 Da was seen at another peak with low abundance (1400.84^{+} *m/z* in fraction IV), supporting mono-methylation of K59 in the fragment 56-67 (GVLKVFLENVIR).

3.2. LC–MS peptide mapping of H4 peptic digestion

The histone H4 peptic digest was separated using the same chromatographic condition as for the tryptic digest. The elution profile is shown in Fig. 2A and the fragments are listed in Table 2. Complete sequence coverage was obtained using pepsin for digestion. Assigning modification sites in peptic digest can be quite problematic due to its broad specificity for hydrophobic residues. As a result, there are many putative peptides with the same elemental formula as modified peptides. For the purposes of assigning modifications only peptides with a unique elemental composition and no overlapping isobaric species were considered in assigning peptide identity. Most of the modifications that can be assigned unambiguously were observed on the globular domain of the protein, such as the methylation of K31, 59 and the acetylation of K77 and 79.

Although the N-terminal peptides were detectable in H4 peptic digestion spectra, the relative abundance of these peaks was very low. As shown in Fig. 2B, a triply charged peak with low abundance was observed at 966.89^{3+} *m/z* (fraction VIIII). This peak correlates to the modified form of fragment 1-26 (SGRGKGGKGLGKGGAKRHRKVLRDNI) shifted by a mass of 196.08 Da due to a combination of tetra-acetylation and di-methylation. Since K5, 8, 12 and 16 were reported as acetylation sites and K20 as a methylation site, the four acetylations were assumed to occur at K5, 8, 12 and 16 or the N-terminal serine residue while the di-methylation was assigned to K20. Because of the low abundance of this peak and the lack of other corroborating peaks, this assignment is considered tentative for the purposes of an assay and would require verification by tandem mass spectrometry. An additional peak corresponding to either mono-methylated fragment 11-33 or di-methylated fragment 11-34 (GKGGAKRHRKVLRDNIQGITKPAI) was also observed. This example further illustrates the difficulty when using pepsin as a protease for assigning methylation sites. An additional peak corresponding to methylation of the N terminus was also observed. Frag-

ment 1-26 was observed to be di-methylated. Based on the tryptic data, the location of the methylation is most likely K20. However, because of the size of these fragments and ambiguity in assignment, the modifications are considered tentative pending verification by tandem mass spectrometry.

As observed in the tryptic digest, K20 may be a site of acetylation. Similar observations were obtained in the analysis of peptic digest fragments. For example, a mass shift (84.03 Da) corresponding to di-acetylation was observed for fragment 16-37 (KRHRKVLRDNIQGITKPAIRRL at *m/z* 918.22^{2+} in fraction X), which contains three possible acetylated lysine residues: K16, 20 and 31. Another mass shift corresponding to the combination of phosphorylation and di-acetylation was observed for fragment 16-33 (KRHRKVLRDNIQGITKPA) at 1147.66^{2+} *m/z* in fraction VIIII. These observations indicated that K16, 20 and 31 might undergo acetylation in vivo. However, due to the low abundance of these modified fragments, it would be difficult to perform tandem MS without substantial fractionation.

As described above, K31 is also a possible acetylation site since multiple acetylations were observed for fragments containing K31. Meanwhile, K31 was also found as a methylation site. As shown in Fig. 2C, a mass shift of 13.91 Da was observed for fragment 27-33 (QGITKPA) at 728.42 Da (in fraction V) and can be assigned as mono-methylation to the only possible modification site in this fragment: K31. The methylation of K59 on the globular domain was observed as well. A mass shift (108.10 Da), representing the combination of mono-phosphorylation and di-methylation, was observed for fragment 52-66 (EETRGVLKVFLENVI) at 927.51^{2+} in fraction IV. Since K59 was the only possible modification residue in this peptide fragment, this di-methylation was assigned to K59 and the phosphorylation was assigned to T54.

Interestingly, LC–MS peptide mapping results showed that the C-terminal tail of H4 was also acetylated. For example, in fraction V, a species corresponding to the mass of fragment 77-83 (KRKTVTA) shifted in mass by 83.99 Da (di-acetylation) was observed at 887.52^{+} *m/z* along with the unmodified form at 803.53^{+} *m/z*. Mono-acetylation was observed for a different fragment containing K77 and K79. These fragments included fragment 77-88 (KRKTVTAMDVVY, at 726.72^{2+} *m/z*), 77-89 (KRKTVTAMDVVYA, at 762.42^{2+} *m/z*) and 70-89 (VTYTEHAKRKTVTAMDVVYA, at 775.73^{3+} *m/z*). Because each of these peptide fragments contains both lysine residues: K77 and K79, these fragments are present as a mixture of modified isoforms in vivo. Meanwhile, phosphorylation was observed for fragments 77-88 and 77-89 either as mono-phosphorylation or a combination of mono-acetylation and mono-phosphorylation. Phosphorylation can be assigned to either T80 or T82.

3.3. Mapping posttranslational modification of histone H4 by ECD

The mass spectrum of histone H4 showed that this protein exists in vivo as the isoforms of different modifications. The distribution of ions from the most abundant charge state (15+) at *m/z* region 750–770 was isolated and subsequently fragmented by ECD (Fig. 3). Accurate masses were determined for these peaks. The unmodified form of H4 was not detected. Based on the mass shift between the detected molecular weights and the unmodi-

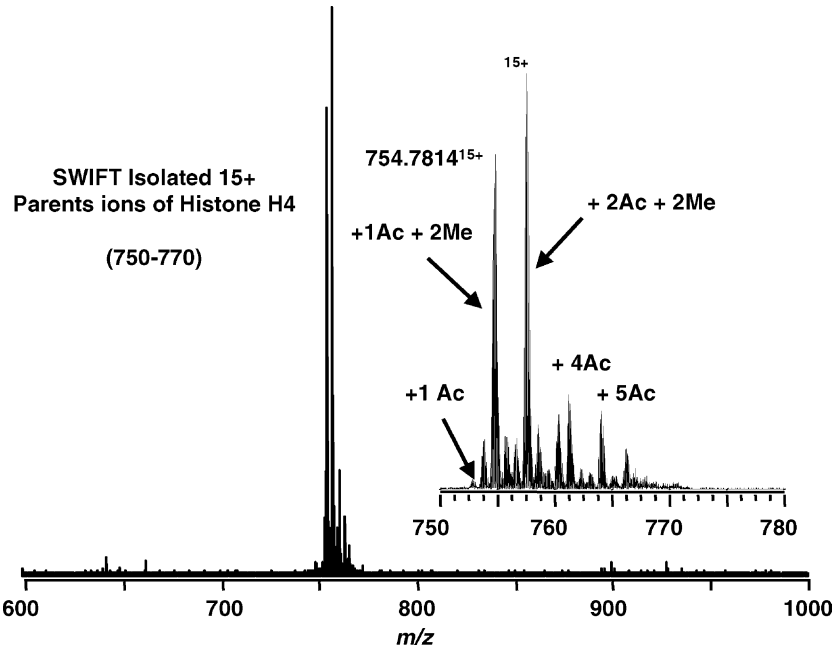

Fig. 3. Isolated precursor ions for the H4 ECD experiment. Precursor ions in the *m/z* range of 750–770 (15+) were isolated by SWIFT excitation and fragmented by ECD. The precursor ions included all the isoforms with different modifications (see inset at right).

fied molecular weight, the detected peaks can be assigned as the following isoforms: mono-acetyl H4 (11,279 Da), mono-acetyl, mono-methyl H4 (11,292 Da), mono-acetyl, di-methyl H4 (11,306 Da), di-acetyl H4 or mono-acetyl, tri-methyl H4 (11,320 Da), di-acetyl, mono-methyl H4 (11,334 Da), di-acetyl, di-methyl H4 (11,348 Da), tri-acetyl H4 or di-acetyl, tri-methyl H4 (11,362 Da), tri-acetyl, mono-methyl H4 (11,377 Da), tri-acetyl, di-methyl H4 (11,391 Da), tetra-acetyl H4 (11,405 Da), tetra-acetyl, mono-methyl H4 (11,419 Da), tetra-acetyl, di-methyl H4 (11,433 Da) and penta-acetyl H4 (11,447 Da). The most abundant peak was the isoform of di-acetyl, di-methyl H4 (11,348 Da) with a mass shift of 112 Da as compared with the unmodified protein.

After SWIFT isolation of the +15 charge state, ECD was performed on the isolated precursor ions. A peak list of the product ions was generated using the computer program THRASH [39,46]. The theoretical molecular weights for $z^{\bullet}$ and c ions resulting from breaking of the N–C_{α} bond of the peptide of H4 were generated by use of Protein Prospector (http://prospector.ucsf.edu) [47]. Mass shifts were calculated from differences between the measured and theoretical *m*/*z*. According to these mass shifts, assignments of acetylation and methylation were made (the majority of which resides on the N-terminus).

Nearly all c ions were observed at higher *m*/*z* than predicted for unmodified H4 (Fig. 4A). A mass shift of 112 Da, representing the combination of a di-acetylation and di-methylation was observed for the c ion series larger than c22. Since the primary modification isoform observed in the protein spectrum was the di-acetyl, di-methyl H4, with a mass shift of 112 Da, it was deduced from the c ion

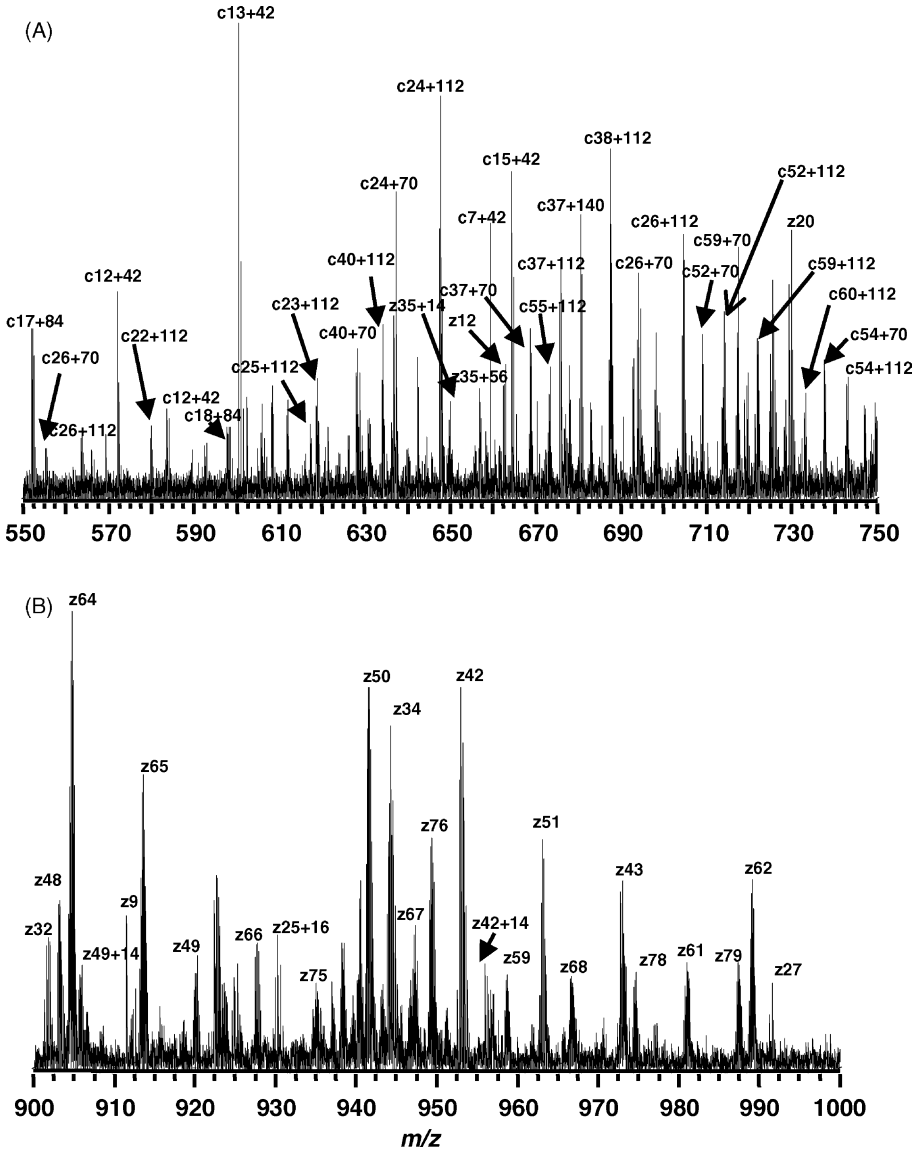

Fig. 4. ECD spectra of H4 product ions. Several c and z ions were observed at lower mass (A) and the higher mass (B), respectively. Mass shifts corresponding to posttranslational modification of the N-terminus were observed for the c ion series.

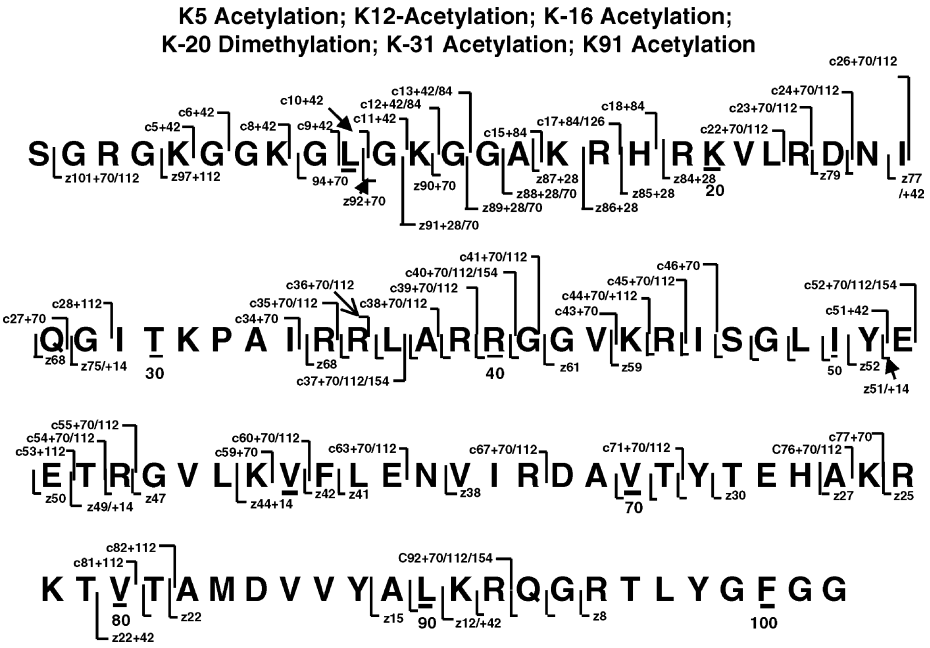

Fig. 5. Assignments of the H4 product ions. Starting from the N-terminus, c ions were labeled as c1-c101, c+42/70/112/156 represent that mass shifts of 42, 70, 112, 156 observed for the c ion series. Similarly, z ions starting from the C-terminus are labeled z1-101. The z+14, 28, 42, 70, 112 ions represent the correlated mass shifts observed for these ions.

series that the majority of abundant modifications on H4 occurred on the N-terminus, residues 1–22.

According to the observed c ions series, the N-terminal modifications can be assigned as following: K12 acetylated, K20 di-methylated and N-terminus, K5, K16 and K31 were other possible acetylation sites. As summarized in Fig. 5, c8+42, c9+42, c10+42 and c11+42 were observed, suggesting one acetylation occurring at fragment 1-8 (SGRGKGGK). Because two peaks representing c5+42 (fragment 1-5 SGRGK) and c6+42 (fragment 1-6 SGRGKG) were also observed, this acetylation should be assigned to either N-terminal serine or K5. Similarly, c series ions such as c12+42, c12+84, c13+42, c13+84, c14+42, c15+42, c15+84 were observed, indicating an additional acetylation present on fragment 12-15 (KGGA). Therefore, this mass shift was assigned as the acetylation in K12. Similarly, mass shifts of 42, 84 and 126 Da representing mono-, di- and tri-acetylation were observed for c17 (fragment 1-17 SRGKGGKGLGKGGAKR), demonstrating K16 as another acetylation site.

The mass shifts of 70 and 112 Da were the primary modifications observed for c22 (fragment 1-22 SGRGKGGKGLGKGGAKRHRKVL) and larger c ions. Compared with the previous mass shifts observed from c12 (fragment 1-12 SGRGKGGKGLGK) through c18 (fragment 1-18 SGRGKGGKGLGKGGAKRH), a mass increase of 28 Da was gained, respectively, suggesting the di-methylation of the protein. Since K20 was the only possible modification site in fragment 18-22 (HRKVL), this di-methylation was assigned to K20 in agreement with the data presented above.

In addition to the N-terminus of the protein, the globular domain was found modified. A mass shift of 154 Da was observed for c37 and c40, which indicated acetylation occurred in the region 22–40 (LRDNIQGITKPAIRRLARR). The most likely location of acetylation is K31.

Most of the z series ions were observed without mass shifts (Fig. 4B) with the exceptions of z12, 42, 44, 49, 51, 75, 77 and those after z84. This data further confirmed that modifications occurred primarily at the N-terminus of this protein. However, the exceptions observed in the c-terminal or core domain of the protein suggested the acetylation of K91 and K31 as well as the methylation of K59. All observations are summarized in Fig. 5.

The unmodified form of products ions z8, z9, z10, z11, z12 and z20 was observed as the C-terminal tail of H4. A doubly charged peak with low abundance was also observed at 683.4254^{2+} m/z, representing z12+42 (fragment 91-102 KRQGRTLYGFGG). In addition, a mass shift of 42 Da was observed for z22 (fragment 81-102 VTAMDVVYALKRQGRTLYGFGG). These observations indicated the acetylation of K91 since it was the only possible acetylation site in this region.

The methylation of K59 was observed in the z ion series. In addition to the unmodified z ions observed from z47 through z68, mono-methylated ions such as z44+14 (fragment 59-102), z49+14 (fragment 54-102) and z51+14 (fragment 52-102) were observed, indicating the mono-methylation the globular domain of H4 (fragment 52-59 EETRGVLK). Since K59 was the only possible methylation site in this region, the methylation was assigned to K59. In addition to the unmodified z60-68 and z75-79

ions, species representing mono-acetylated isoforms of these ions were observed (z75+42, z77+42 corresponding to fragment 24-43; DNIQGITKPAIRRLARRGGV). The mass shift of 42 Da indicated a mono-acetylation that was assigned to the only possible acetylation site in this region: K31.

Starting from z84, a series of positive mass shifts were observed. For example, z84+28, z85+28, z86+28 and z87+28 were observed. As previously mentioned, the z ion series z75-79 were unmodified. These data suggest the presence of two methylations in the region of z87-75 (or fragment 16-28 RHRKVLRDNIQ) that are likely localized on K20. This observation correlates well with the c ion series. Similarly, a mass shift of 42 Da was observed for the product ions of z88, 89 and 91 (fragments 14-102, 13-102 and 12-102). A mass shift of 70 Da was observed for z88-90. These observations further suggest the presence of an isoform that is di-methylated and acetylated. Assuming that K20 is di-methylated the additional acetylation is localized to K16.

Although evidence observed from the c product ions suggested the acetylation of K12, no further acetylations were observed for z ion series fragments containing K12. Mass shifts of 42 Da were observed for z91 (fragment 12-102), 70 Da for z92 (fragment 11-102) and z94 (fragment 9-102). However, these ions possess multiple modification sites and are likely a mixture of isoforms dominated by the dimethyl-K20, mono-acetyl-K16. A mass shift of 112 Da was observed for z98 (fragment 5-102), suggesting the presence of an additional acetylation occurred in the region z98-92 (or fragment 5-10 KGGKGL). However, since the mass shift observed at product ions z95, 96 and 97 (fragment 6-102, 7-102 and 8-102) was 70 Da, this acetylation was assigned to K5 instead of K8. In addition, mass shifts of 70 and 112 Da were observed for z101, which further supports the acetylation of K5. Since no observation was made for z102, no experimental data support the presence of N-terminal acetylation.

3.4. Identification of H4 fragments

After top down sequencing of H4 by ECD, we performed ECD on H4 tryptic fragments. However, among the tryptic digestion peptide fragments, the fragments containing modified lysine residues are usually not abundant enough for a direct ECD. Therefore, we separated the tryptic digest fragments and concentrated them in order to obtain modified fragments at higher concentrations. We successfully increased the relative abundance of several modified fragments such as the acetylated fragment 18-23. However, they were present primarily as the singly charged ions because of the acetylation of the basic lysine side chain and thus cannot be fragmented using ECD. After several trials, we obtained ECD spectra for several unmodified peptide fragments.

For example, a doubly charged peak was observed at 733.9112^{2+} *m/z*, which can be identified either as the unmodified form of fragment 80-92 (TVTAMDVVYALKR, theoretical: 733.9060^{2+} *m/z*, mass accuracy 7.09 ppm), or the di-methylated form of fragment 79-91 (KTVTAMDVVYALK, theoretical: 733.9185^{2+} *m/z*, mass accuracy −9.95 ppm). To identify this peptide's sequence, the precursor ion at 733.9112^{2+} *m/z* was isolated by SWIFT excitation and fragmented by ECD. The singly charged radical cation of the precursor was observed at 1466.8718^{+} *m/z*. Another singly charged peak at 1349.7311^{+} *m/z* can be interpreted as the loss of the N-terminal threonine (T) residue according to the measured mass difference of 118.123^{+} *m/z*, as shown in Fig. 6. In addition, the following peaks were observed at 1250.6647^{+} *m/z*, 1149.6211^{+} *m/z*, 1078.5902^{+}

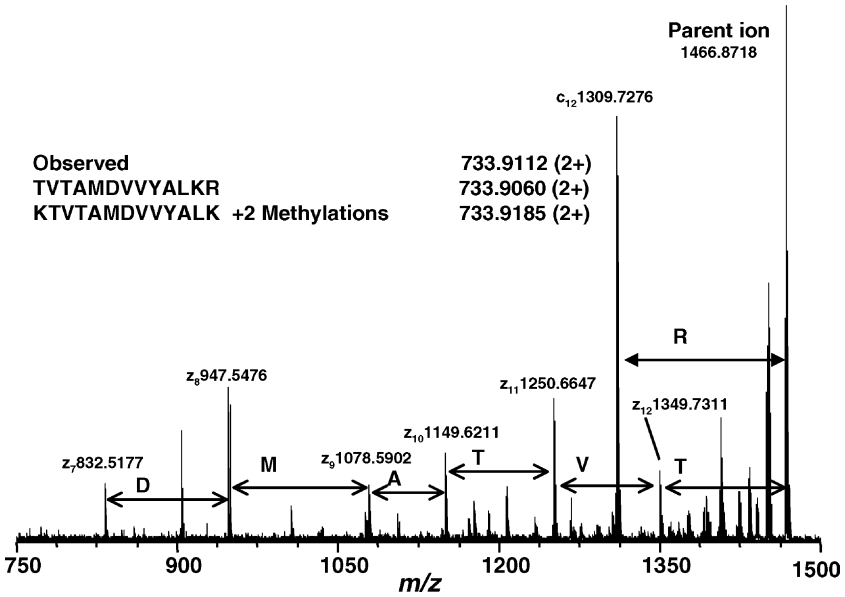

Fig. 6. ECD of the doubly charged tryptic peptide observed at *m/z* 733.9112^{2+}. The peptide was identified as the unmodified form of fragment 80-92 (TVTAMDVVYALKR, theoretical: 733.9060^{2+} *m/z*, mass accuracy 7.09 ppm).

m/*z*, 947.5476^{+} *m*/*z*, 832.5177^{+} *m*/*z*, 634.3832^{+} *m*/*z* and 471.3182^{+} *m*/*z* as z11 to z7, z5 and z4, respectively. According to the mass differences between two neighboring peaks, the missing residue was determined to be valine (V), threonine (T), alanine (A), methionine (M), aspartic acid (D), valine–valine (VV) and tyrosine (Y). Therefore, this fragment was identified as TVTAMDVVYALKR. Several other peptide fragments were identified by use of ECD as the unmodified form of tryptic fragment 24-35 (DNIQGITKPAIR), 60-67 (VFLENVIR) and Glu-C digested fragments 64-102 (NVIRDAVTYTEHAKRKTVTAMDVVYALKRQGRTLYGFGG).

3.5. Comparison of ECD and peptide mapping

Peptide mapping is used as a routine approach for the identification of protein or localizations of posttranslational modification sites. The ultimate success of peptide mapping relies on the highest mass accuracy one can obtain and sequencing by tandem mass spectrometry. When used for the investigation of posttranslational modifications, the complexity of the digestion mixture greatly limits the application of direct peptide mapping if ultra-high mass accuracy cannot be provided. For example, peptides with very similar *m*/*z* may contain different modifications, which require further identification using tandem mass spectrometry. For example, to resolve the mono-acetylated or tri-methylated fragments at 2000 *m*/*z* requires a resolving power of ∼55,000. However, neutral loss MS/MS techniques have been successfully employed to distinguish between these two modifications [48]. Unfortunately, not all of these fragments are abundant enough for the MS/MS experiment and may require substantial fractionation. The coupling of HPLC with a lower resolution MS system may allow isobaric peptides to be separated, identified by mass and selected for confirmation by MS/MS if the peptide proves to be clinical relevant. Once sequences are confirmed, peptides can then be identified in a LC–MS assay using nominal mass and retention times for the modified peptides. Thus, the need for high mass accuracy is reduced. Furthermore, a low-resolution LC–MS system will cost less and is a good platform for screening modifications in both preclinical and clinical settings.

Trypsin is the most commonly used enzyme for the peptide mapping of proteins because of the suitable fragment size it generates and additionally the basic amino acids on the C-terminus of tryptic fragments. However, as we observed in our study, trypsin only gave about 75% sequence coverage after digestion. As a result, some modification information may be missing. Therefore, additional enzymatic digestion will be necessary to ensure complete information will be obtained for the interested residues.

The observed modifications in this work correlate well our prior work and the work of others. Zhang et al. studied the posttranslational modification of histones using MS and LC–MS [41,44]. The posttranslational modifications were determined using MALDI-TOF MS analysis of tryptic digests of histones combined with nano-ESI and MS/MS. Several modifications of H3 and H4 were observed. Our current work corroborates the modifications observed on H4 despite the difference in organisms and demonstrates the highly conserved nature of histone modifications. In a previous study we performed peptide mapping on all four core histones by use of a high resolution FT-ICR MS [27]. We observed modifications of the N-terminal residues, K5, 8, 12 and 16, and several modification sites that included, methylation of K31, 44 and 59 along with acetylation of K20, 31, 77, 79 and 91. In this study, LC–MS was used as an alternative approach for the investigation of posttranslational modifications for

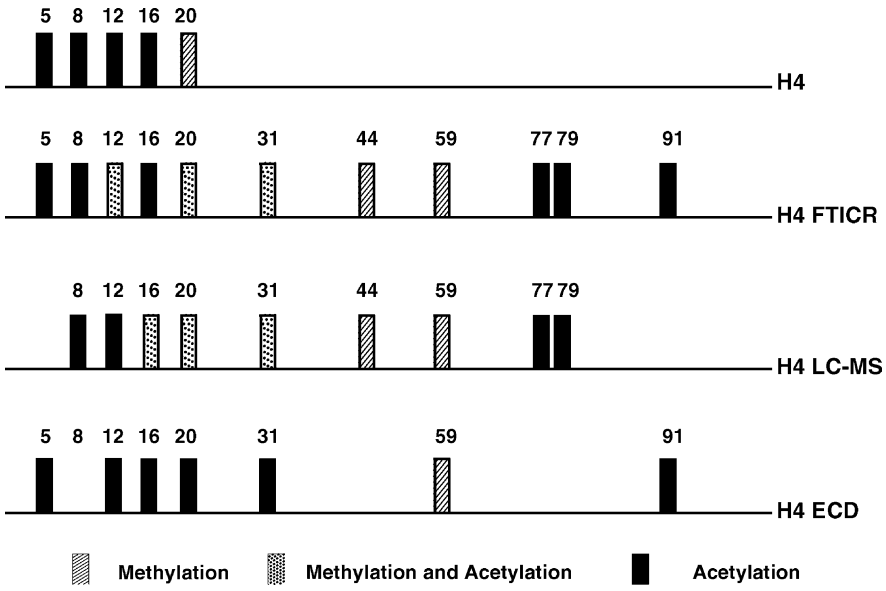

Fig. 7. A comparison of histone modification found using direct FT-ICR MS peptide mapping, LC–MS peptide mapping and ECD sequencing. Good agreement was obtain for the methods evaluated.

histone H4. The results showed that the known modification sites: acetylation on K5, 8, 12 and 16 as well as the methylation of K20, were observed using LC–MS peptide mapping. In addition, several modification sites were observed, such as the acetylation of K20, 77, 79 and the methylation of K16, 31 and 59. A comparison map between the results is given in Fig. 7. All the modifications observed using direct peptide mapping were also observed in LC–MS peptide mapping with the exception of acetylation of K31 and 91.

As an alternate assay, ECD was performed on H4 and its enzymatic digest fragments to determine posttranslational modification sites. ECD of H4 indicated K5, 8, 12, 16, 31, 91 were acetylated and the K20 and 59 were methylated. Both direct peptide mapping and LC–MS peptide mapping showed that K5, 8, 12, 16, 20, 31, 77, 79 and 91 were acetylated while K12, 20, 31, 44, 59 and 79 were methylated. A comparison between peptide mapping and ECD suggests that the peptide mapping technique provided more information with regards to modification sites. One explanation for this observation is the improved dynamic range as a result of the separation of the peptide by HPLC. Another contributing factor may be the relatively low dissociation efficiency in ECD. Thus, only fragments of the highest abundant isoforms were observed. For the purposes of screening modification changes, however, these fragments may provide sufficient information to relate changes in modifications. Such information could be used as a diagnostic to ascertain alterations in acetylation in samples treated with HDAC inhibitors. Furthermore, the ECD method could be coupled with the LC–MS for online separation, analysis of global modification profiles and localization of modifications. For clinical samples, we typically inject 30–300 μg of histone. The chromatographic peak widths (∼2 min) would allow for averaging 60–120 spectra depending on the detection bandwidth. Under these conditions it should be possible to obtain sufficient fragmentation to determine modification patterns of the most abundant species.

4. Conclusion

We have evaluated two techniques as potential clinical assays for assessing posttranslational modifications of histone H4. LC–MS peptide mass mapping was performed on the H4 peptic and tryptic digestion mixture using a QqTOF MS. The results showed that the known modification sites of H4 (acetylation of K8, 12, 16 and methylation of K20) were observed. In addition, modification sites such as the methylation of K31, 44 and 59, acetylation of K20, 77 and 79 were also observed. Both direct peptide mapping by FT-ICR and LC–MS peptide mapping gave similar results. However, due to the isobaric mass difference for both tri-methylation and mono-acetylation (42.0470 Da versus 42.0106 Da) it was not possible to resolve these two modifications on the lower resolving power QqTOF MS. ECD was also performed on both the protein and enzymatic digestion fragments of H4 for the investigation of histone posttranslational modification. ECD of histone H4 indicated the acetylations of K5, 8, 12, 16, 31, 91 and the methylation of K20 and 59. The results from ECD showed good agreement with the results from peptide mapping although fewer sites of modification were observed with ECD. The ECD approach has an important advantage over peptide mass mapping in that enzymatic digestion of the protein and LC separation are not required.

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References

[1] A.T. Annunziato, J.C. Hansen, Gene Expr. 9 (2000) 37.
[2] S.L. Berger, Curr. Opin. Genet. Dev. 12 (2002) 142.
[3] A.P. Jonsson, Cell. Mol. Life Sci. 58 (2001) 868.
[4] M.R. Stallcup, Oncogene 20 (2001) 3014.
[5] F. van Leeuwen, P.R. Gafken, D.E. Gottschling, Cell 109 (2002) 745.
[6] H.H. Ng, Q. Feng, H. Wang, H. Erdjument-Bromage, P. Tempst, Y. Zhang, K. Struhl, Genes Dev. 16 (2002) 1518.
[7] C. Crane-Robinson, T.R. Hebbes, A.L. Clayton, A.W. Thorne, Methods 12 (1997) 48.
[8] N. Suka, Y. Suka, A.A. Carmen, J. Wu, M. Grunstein, Mol. Cell 8 (2001) 473.
[9] W.-S. Lo, R.C. Trievel, J.R. Rojas, L. Duggan, J.-Y. Hsu, C.D. Allis, R. Marmorstein, S.L. Berger, Mol. Cell 5 (2000) 917.
[10] D.G. Edmondson, J.K. Davie, J. Zhou, B. Mirnikjoo, K. Tatchell, S.Y. Dent, J. Biol. Chem. 277 (2002) 29496.
[11] S.D. Shi, M.E. Hemling, S.A. Carr, D.M. Horn, I. Lindh, F.W. McLafferty, Anal. Chem. 73 (2001) 19.
[12] J. Bergquist, M. Palmblad, M. Wetterhall, P. Hakansson, K.E. Markides, Mass Spectrom. Rev. 21 (2002) 2.
[13] K. Bartlett, M. Pourfarzam, J. Inherit. Metab. Dis. 22 (1999) 568.
[14] W.J. Griffiths, A.P. Jonsson, S. Liu, D.K. Rai, Y. Wang, Biochem. J. 355 (2001) 545.
[15] D.R. Goodlett, E.C. Yi, Funct. Integr. Genomics 2 (2002) 138.
[16] G.E. Reid, J.L. Stephenson Jr., S.A. McLuckey, Anal. Chem. 74 (2002) 577.
[17] D.M. Horn, Y. Ge, F.W. McLafferty, Anal. Chem. 72 (2000) 4778.
[18] D.M. Horn, K. Breuker, A.J. Frank, F.W. McLafferty, J. Am. Chem. Soc. 123 (2001) 9792.
[19] D.M. Vigushin, R.C. Coombes, Anticancer Drugs 13 (2002) 1.
[20] A. Melnick, J.D. Licht, Curr. Opin. Hematol. 9 (2002) 322.
[21] G. Kouraklis, S. Theocharis, Curr. Med. Chem. 2 (2002) 477.
[22] W.K. Kelly, O.A. O'Connor, P.A. Marks, Expert Opin. Investig. Drugs 11 (2002) 1695.
[23] R.W. Johnstone, Nat. Rev. 1 (2002) 287.

[24] M. Yoshida, R. Furumai, M. Nishiyama, Y. Komatsu, N. Nishino, S. Horinouchi, Cancer Chemother. Pharmacol. 48 (2001) S20.
[25] P. Marks, R.A. Rifkind, V.M. Richon, R. Breslow, T. Miller, W.K. Kelly, Nat. Rev. 1 (2001) 194.
[26] M. Jung, Curr. Med. Chem. 8 (2001) 1505.
[27] L. Zhang, E.E. Eugeni, M.R. Parthun, M.A. Freitas, Chromosoma 112 (2003) 77.
[28] Y.-P. Ho, P.-H. Hsu, J. Chromatogr. A 976 (2002) 103.
[29] R. Bischoff, Chromatogr. Sci. Ser. 87 (2002) 689.
[30] L.E. Ostrowski, K. Blackburn, K.M. Radde, M.B. Moyer, D.M. Schlatzer, A. Moseley, R.C. Boucher, Mol. Cell. Proteomics 1 (2002) 451.
[31] L. Zhang, M.A. Freitas, J. Wickman, M.R. Parthun, M.I. Klisovic, G. Marcucci, J.C. Byrd, J. Am. Soc. Mass Spectrom. 15 (2004) 77.
[32] K. Zhang, H. Tang, J. Chromatogr. B 783 (2003) 173.
[33] S.C. Galasinski, K.A. Resing, N.G. Ahn, Methods 31 (2003) 3.
[34] I. Sures, D. Gallwitz, Biochemistry 19 (1980) 943.
[35] M.W. Senko, C.L. Hendrickson, L. Pasa-Tolic, J.A. Marto, F.M. White, S. Guan, A.G. Marshall, Rapid Commun. Mass Spectrom. 10 (1996) 1824.
[36] M.W. Senko, C.L. Hendrickson, M.R. Emmett, S.D.-H. Shi, A.G. Marshall, J. Am. Soc. Mass Spectrom. 8 (1997) 970.
[37] M.A. Freitas, E. King, S.D.H. Shi, Rapid Commun. Mass Spectrom. 17 (2003) 363.
[38] M.W. Senko, J.D. Canterbury, S. Guan, A.G. Marshall, Rapid Commun. Mass Spectrom. 10 (1996) 1839.
[39] D.M. Horn, R.A. Zubarev, F.W. McLafferty, J. Am. Soc. Mass Spectrom. 11 (2000) 320.
[40] J.H. Waterborg, Biochem. Cell Biol. 80 (2002) 384.
[41] K. Zhang, K.E. Williams, L. Huang, P. Yau, J.S. Siino, E.M. Bradbury, P.R. Jones, M.J. Minch, A.L. Burlingame, Mol. Cell. Proteomics 1 (2002) 500.
[42] J. Sommerville, J. Baird, B.M. Turner, J. Cell. Biol. 120 (1993) 277.
[43] D. Suckau, M. Przybylski, Proc. Natl. Acad. Sci. U.S.A. 89 (1992) 5630.
[44] K. Zhang, H. Tang, L. Huang, J.W. Blankenship, P.R. Jones, F. Xiang, P.M. Yau, A.L. Burlingame, Anal. Biochem. 306 (2002) 259.
[45] B. Sarg, E. Koutzamani, W. Helliger, I. Rundquist, H.H. Lindner, J. Biol. Chem. 277 (2002) 39195.
[46] D.M. Horn, R.A. Zubarev, F.W. McLafferty, Proc. Natl. Acad. Sci. U.S.A. 97 (2000) 10313.
[47] R.A. Zubarev, Mass Spectrom. Rev. 22 (2003) 57.
[48] K. Zhang, P.M. Yau, B. Chandrasekhar, R. New, R. Kondrat, B.S. Imai, M.E. Bradbury, Proteomics 4 (2004) 1.