Available online at www.sciencedirect.com
ScienceDirect
International Journal of Mass Spectrometry 268 (2007) 168–180
International Journal of Mass Spectrometry
www.elsevier.com/locate/ijms

Identification of *N*,*Nε*-dimethyl-lysine in the murine dioxin receptor using MALDI-TOF/TOF- and ESI-LTQ-Orbitrap-FT-MS

Keyur A. Dave [a,b], Brett R. Hamilton [a], Tristan P. Wallis [a], Sebastian G.B. Furness [c], Murray L. Whitelaw [d], Jeffrey J. Gorman [a,*]

[a] *Protein Discovery Centre, Queensland Institute of Medical Research, PO Royal Brisbane Hospital, Herston, Queensland 4029, Australia*
[b] *Institute for Molecular Bioscience, The University of Queensland, St. Lucia, Queensland 4072, Australia*
[c] *Department of Pharmacology, Monash University, Clayton, Victoria 3800, Australia*
[d] *School of Molecular and Biomedical Science, The University of Adelaide, Adelaide, SA 5005, Australia*

Received 20 February 2007; received in revised form 25 May 2007; accepted 1 June 2007
Available online 6 June 2007

Abstract

Analysis of a tryptic digest of a recombinant D83A mutant of the murine dioxin receptor (D83A-DR) by CapHPLC-MALDI-TOF/TOF-MS/MS revealed a peptide corresponding to cleavage at a lysine that had either been mutated to an arginine or modified by dimethylation or formylation (Ser-Phe-Phe-Ala-Val-Ala-Leu-Me2K/FormK/Arg). High mass accuracy data were obtained for the pseudomolecular ions of synthetic peptides corresponding to the potential alternate structures of the variant D83A tryptic peptide and their CID products using an LTQ-Orbitrap-FTMS instrument. The high mass accuracies obtained by both FT-MS and FT-MS/MS were more than sufficient to enable differentiation between the synthetic peptides. The only potential fragmentation characteristic produced by CID was the formation of ions at −17 u relative to *y* ions of the Arg peptide. The variant D83A-DR tryptic peptide was also detected by CapHPLC-ESI-LTQ-Orbitrap-FT-MS and MS/MS with masses detected for the pseudomolecular and CID product ions being consistent with dimethylation of a C-terminal lysine.

Fragmentation of the synthetic peptides by MALDI-TOF/TOF-MS/MS produced a range of fragmentation characteristics which provided a basis for distinguishing between peptides with C-terminal Me2K, FormK and Arg. These characteristics included specific immonium ions, apparent side chain fragmentation from the precursors of 28 or 30 u and satellite ions at −17 u relative to the *y* ions of the Arg peptide. Comparison of the fragmentation properties of the D83A-DR derived tryptic peptide produced by MALDI-TOF/TOF-MS/MS with those of the synthetic peptides corroborated the mass accuracy-based assignment of a dimethylated lysine at the C-terminus of the D83A-DR tryptic peptide. This represents the first documentation of any post-translational modification of DR, other than two previously described sites of phosphorylation.

Keywords: Dimethyl-lysine; Dioxin receptor; Orbitrap-FT-MS; MALDI-TOF/TOF-MS/MS

1. Introduction

The dioxin receptor (DR) or aryl hydrocarbon receptor (AhR) is a ligand activated transcription factor and is a member of the basic helix-loop-helix (bHLH)/Per-ARNT-Sim (PAS) family of developmental regulators and chemosensors [1]. Several studies over the past decade have suggested important regulatory roles for phosphorylation in the signaling cascade mediated by DR [2–8]. For example, phosphorylation of Ser-36 and Ser-68 in DR was demonstrated to play an important role in nucleocytoplasmic shuttling of the receptor [9–11]. However, little is known about the global post-translational modification profile of DR in terms of multi-site phosphorylation or the presence of other post-translational modifications.

Protein methylation, like phosphorylation, is an important covalent modification resulting in side-chain modifications of nine of the 20 common amino acids [12]. A well characterized paradigm is the site-specific lysine and arginine methylation of histones, which play a pivotal role in regulating chromatin activity and specific genomic functions [13–15]. Transcription factors, such as p53, STAT1, and retinoic acid receptor-α, are also known to be post-translationally methylated in a fashion

* Corresponding author. Tel.: +61 7 3845 3669; fax: +61 7 3845 3504.
E-mail address: jeff.gorman@qimr.edu.au (J.J. Gorman).

1387-3806/$ – see front matter © 2007 Elsevier B.V. All rights reserved.
doi:10.1016/j.ijms.2007.06.001

that regulates their functions [16–20]. Histones have also been shown to be formylated [21].

Mass spectrometry has been used for many years as a specialist technique to define post-translational modifications of proteins [22,23]. In recent years, this application of mass spectrometry (MS) has become a leading analytical technology for characterization of protein post-translational modifications at the proteome level [24–26]. The sensitivity, and specificity of MS are ideally suited to elucidating exactly which residues are modified, especially when measurements are performed with high mass accuracy [27,28]. However, post-translational modifications with the same isobaric masses such as phosphorylation (+nominal 80 u; +79.9663 u exact mass) versus sulfation (+nominal 80 u; +79.9568 u exact mass), dimethylation (+nominal 28 u; +28.031300 u exact mass) versus formylation (+nominal 28 u; +27.994915 u exact mass) and acetylation (+nominal 42 u; +42.0105 u exact mass) versus trimethylation (+nominal 42; +42.0468 u exact mass) are exceedingly difficult to distinguish [29,30]. A recent investigation revealed a significant number of inaccurately assigned *N*,*Nε*,-dimethyllysine residues using routine automatic database search engine parameters (100 ppm error tolerance), which were actually formylated lysine residues resulting from formaldehyde used in the silver staining procedure [31]. This emphasizes the need for mass spectrometry technologies which can distinguish between isobaric modifications. Mass measurements with accuracies of better than 5 ppm may enable conclusive identification of isobaric modifications. The recently described hybrid linear ion trap-Orbitrap-FTMS (LTQ-Orbitrap-FTMS) is routinely capable of sub-ppm accuracy [32–34], especially when enabled with real time calibration on the 'lock mass' [35]. Specific fragmentation properties of post-translationally modified amino acid side chains may also be valuable tools for distinguishing between isobaric modifications. For example, differentiation of acetylated versus trimethylated lysine residues and sulfation versus phosphorylation of serine and threonine has been previously reported based on characteristic immonium ions and modification specific neutral losses using ESI-Quadrupole-quadrupole-TOF technology [29,30].

During investigation of a recombinant D83A mutant DR (D83A-DR) by matrix-assisted laser desorption/ionization-time of flight-time of flight tandem mass spectrometry (MALDI-TOF/TOF-MS/MS), a peptide with a nominal mass increment of +28 u was observed. However, dimethyl-lysine (+28.031300 u), formyl-lysine (+27.994915 u), and mutation of lysine to arginine (+28.00615 u) [21] could explain such a nominal mass increment and cannot be distinguished using the mass accuracy routinely achieved with MALDI-TOF instruments.

This study describes precise assignment of a novel *N*,*Nε*-dimethyllysine modification in D83A-DR by using sub-ppm mass accuracy with LTQ-Orbitrap-FT-MS for parent as well as production mass spectra. The formation of unique product ions during MALDI-TOF/TOF-MS/MS mass spectrometry of synthetic variants of the modified DR peptide, which can assist in distinguishing between these three possibilities, were observed and used to validate the presence of the dimethylated peptide in D83A-DR.

2. Experimental procedures

2.1. Chemicals and reagents

In general, reagents used were obtained from Sigma–Aldrich. Acids and organic solvents were HPLC grade or better. Trypsin (modified sequencing grade) was purchased from Roche Diagnostics. Rapigest SF™ was purchased from Waters Inc., Australia. α-Cyano-4-hydroxy cinnamic acid (α-CHCA) was from Bruker Daltonics (Bremen, Germany). Water was purified by Milli-Q-synthesis A10 system (Millipore, Billerica, USA).

2.2. Synthetic peptides

Three synthetic peptides with the common sequence of 'SFFAVAL', but variously modified to contain formyl-lysine (FormK), dimethyl-lysine (Me2K) or arginine (Arg) at their C-terminii were prepared by Mimotopes Pty. Ltd. (Clayton, Victoria, Australia). Synthetic peptide solutions were prepared at 10 pmol/μl in 0.1% (v/v) formic acid/50% (v/v) acetonitrile for infusion, 1 pmol/μl in 0.1% (v/v) formic acid for LC-MS/MS analysis or 0.05% (v/v) trifluoroacetic acid/2% (v/v) acetonitrile for MALDI-TOF/TOF-MS/MS of 10 pmol per spot.

2.3. Recombinant expression of the D83A mutant form of murine DR (D83A-DR)

Murine D83A-DR was produced in HEK 293T cells stably transfected with a plasmid encoding cMyc and HexaHis affinity tagged, mutant murine DR. Cells were disrupted and tagged D83A-DR isolated using immobilised metal ion affinity adsorption using similar protocols as described previously [36].

2.4. 1D-SDS-PAGE and in-solution digestion

Eluates from the immobilized metal affinity resin were subjected to SDS-PAGE, stained with Coomassie brilliant blue and recovered from gel slices by electroelution [37]. This purification strategy was adopted as the preparations contained some contaminating proteins and D83A-DR was insoluble in non-denaturing solvents, which prevented chromatographic fractionation. The electroeluted protein was co-precipitated with trypsin at −20 °C using methanol (95%, v/v) and washed twice with −20 °C methanol after harvesting by centrifugation. Tryptic digestion was performed by resuspending the precipitated protein in 100 mM NH_4HCO_3 containing 0.1% (w/v) Rapigest SF. Before CapHPLC fractionation and downstream mass spectrometry analysis, tryptic digests of D83A-DR were incubated at 37 °C for 45 min with equal amounts of 0.5% (v/v) TFA to hydrolyse the Rapigest SF.

2.5. LC-MS/MS analysis

LC-MS/MS analysis was carried out using non-metallic Dionex 3000 Ultimate one-dimensional nanoHPLC systems

(Dionex) and C18 reverse phase, 300 Å pore size, 150 mm × 150 μm (i.d.) columns (Vydac). D83A-DR digests were fractionated at 900 nl/min flow rates with 100 min gradients from 5.0 to 80% acetonitrile in 0.1% (v/v) formic acid for LC-ESI-MS or MS/MS analysis or at 1 μl/min using 0.05% (v/v) trifluoroacetic acid (TFA) for LC-MALDI-TOF/TOF-MS or MS/MS analysis. For LC-MALDI-TOF/TOF analyses, fractions were co-spotted with α-cyano-hydroxy-cinnamic acid (α-CHCA) matrix (see below for composition) at 0.5 min intervals using an on-line micro-capillary robot (Dionex Ultimate Probot) onto a stainless steel Anchorchip MALDI target plate (Bruker Daltonics).

2.6. MALDI-TOF/TOF analysis

All mass spectra were acquired in positive ion mode on an Ultraflex II MALDI-TOF/TOF instrument (Bruker Daltonics). The spectra were measured in reflectron mode with typical resolution within the range of 15,000 and 20,000, and accuracies of within 50 ppm for MS measurements and between 60 and 250 ppm for MS/MS measurements. Saturated α-CHCA matrix was prepared in 97% (v/v) acetone containing 0.3 mM ammonium dihydrogen phosphate and 0.1% (v/v) TFA, subsequently diluted 15 times in 6:3:1 ethanol:acetone:10 mM ammonium dihydrogen phosphate and 0.1% (v/v) TFA and used as matrix for all spot and LC-MS or MS/MS analyses at a sample to matrix ratio of 1:2. MS/MS analysis was performed either manually or automatically with 40% higher laser intensity than that used for MS analysis. Spectra were calibrated using a peptide calibration standard mixture (Bruker Daltonics. Kit number 208241) of nine peptides in the mass range of $m/z = 1046$ and $m/z = 3147$, with 62.5 fmol of each peptide applied to calibration spots. High precision MS/MS calibration was achieved using fragment ions derived from all the nine peptides in the MS calibration kit and the associated calibration coefficients were applied to the method file used to acquire MS/MS data. Instrument settings for MS were: ion source 1 potential, 25.00 kV; ion source 2 potential, 21.70 kV; reflectron 1 potential, 26.30 kV; reflectron 2 potential, 13.85 kV and for MS/MS were: ion source 1 potential, 8.00 kV; ion source 2 potential, 7.20 kV; reflectron 1 potential, 29.50 kV; reflectron 2 potential, 13.75 kV; LIFT 1 voltage, 19.00 kV; LIFT 2 voltage, 3.00 kV. Ion selector resolution was set at 0.5% of the mass of the precursor ion.

Assignment of data to specific portions of the D83A-DR sequence was performed by database searches against custom made DR-D83A database using Biotools 3.0 (Bruker Daltonics) and an in-house MASCOT search engine. For more global queries, data obtained from MS and MS/MS analysis were searched against all taxa entries of the NCBI (NCBInr) (http://www.ncbi.nlm.nih.gov) which contained 4.38 million protein sequences using Biotools 3.0 in association with an in-house MASCOT search engine. Search parameters were: enzymatic cleavage, tryptic and semitryptic; fixed modifications, carbamidomethylation of cysteine residues and methionine oxidation; missed cleavages, 2; MS tolerance, 100 ppm; and, MS/MS tolerance, 0.7 u.

2.7. LTQ-Orbitrap analysis

Solutions of individual synthetic peptides at 10 pmol/μl were infused using a syringe pump at 1 μl/min into the LTQ-Orbitrap equipped with a dynamic nanoelectrospray source and distal coated silica emitters (50 μm i.d., 20 μm Tip i.d., New Objective). The LTQ-Orbitrap was controlled using LTQ-Orbitrap Tune plus (Thermo Electron, Bremen, Germany) for infusion experiments. Peptide mixtures were fractionated by CapHPLC in-line with an LTQ-Orbitrap mass spectrometer equipped with a dynamic nanoelectrospray ion source and distal coated silica emitters (50 μm i.d., 20 μm Tip i.d.) and controlled using Xcalibur 2.0 SR1 (Thermo Electron, Bremen, Germany) with DCMS link (Dionex). D83A-DR digests were injected onto the column with a flow rate of 900 nl/min using the gradient described earlier. Analyses were carried out using data dependent acquisitions, whereby the survey scan was acquired with the Orbitrap FT analyzer at a resolution of $R = 100{,}000$ and the MS^2 spectra were acquired sequentially in the Orbitrap at $R = 100{,}000$ after fragmentation of selected precursors in the LTQ mass analyzer. The analyses were further augmented by the use of an inclusion list that contained the peptides of interest, and dynamic exclusion with repeat count 2, repeat duration 8 s, exclusion size 250, and exclusion duration 60 s to maximize the number of different MS^2 spectra collected during the analysis. Polydimethylcyclosiloxane (PCM) ions generated in the electrospray process from ambient air were used for lock-mass real time calibration [35]. Other instrument conditions for MS were: ionspray voltage 1.5 kV, capillary temperature 200 °C, capillary voltage 9 V, and tube lens 100 V, and for MS^2 were: normalized collision energy, 35%; activation q, 0.25; activation time, 50 ms; minimum ion selection threshold, 500 counts.

3. Results

3.1. Detection of structural variation at lysine 87 of D83A-DR by LC-MALDI TOF-TOF-MS/MS

Analysis of a tryptic digest of D83A-DR by CapHPLC-MALDI-TOF/TOF-MS revealed a pseudomolecular ion at retention time of 66.5 min with an m/z of 910.58 (Fig. 1A) that did not correspond to any predicted tryptic peptide or non-tryptic segment of the parent protein when the data were used to search a custom sequence database consisting of the D83A-DR sequence only.

MALDI-TOF/TOF-MS/MS fragmentation of the unassigned ion at $m/z = 910.58$ produced data (Fig. 1B) consistent with this ion being the pseudomolecular ion for a modified form of the D83A-DR peptide, SFFAVALK, which spans residues 80–87. This modification represented a nominal mass increment of +28 u compared to that of the unmodified sequence that was detected in a fraction at retention time of 48.5 min with an m/z of 882.53 (Figs. 1A and B; inverted spectra). For instance, fragment ions were generated from both the modified and unmodified ions consistent with N-terminal fragment ions up to b_5 (b_2– b_5) of the unmodified peptide SFFAVALK (Fig. 1B; Table 1). However, an additional 28 u was required to assign

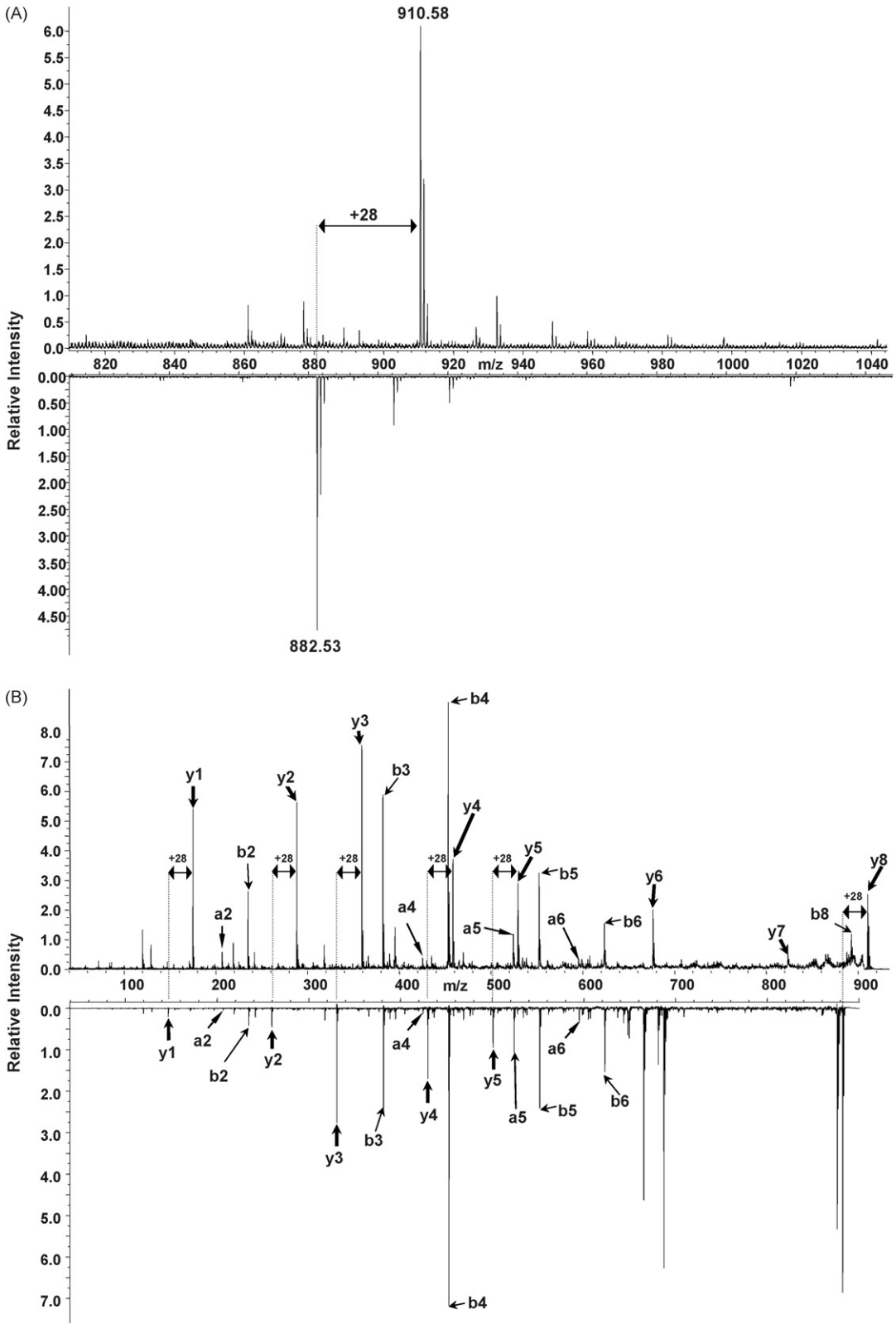

Fig. 1. MALDI TOF/TOF-MS spectra of CapHPLC fractions of a tryptic digest of D83A-DR (A) and MS/MS spectra of selected peptide ions (B). The upper spectrum in panel A shows a precursor ion of interest at *m*/*z* of 910.58 observed at retention time 66.5 min that was subjected to MS/MS to produce the upper spectrum in panel B. The lower (inverted) spectrum in panel A shows an ion of interest at *m*/*z* of 882.53 observed at retention time of 48.5 min that was subjected to MS/MS to produce the lower (inverted) spectrum in panel B. Groups of 25 MS shots were collected and summed to a total of 200 shots and 100 MS/MS shots were acquired in groups and summed to a total of 800 shots. Ions corresponding to *y* and *b* fragments of unmodified SFFAVALK (Table 1) are labeled on the inverted spectrum in panel B plus additional ions that conform with the sequence. Ion in the upper spectrum of panel B also correspond to SFFAVALK if allowance is made for additional 28 u on the C-terminal lysine as referenced by dotted lines and listed in Table 1.

Table 1
Fragment ions corresponding to unmodified and modified residues 80–87 of D83A-DR (SFFAVALK) arising from MALDI-TOF/TOF-MS/MS of precursor ions of $m/z = 882.53^{1+}$ and 910.58^{1+}, respectively

Residue[a]	Fragment ion		SFFAVALK (unmodified)		SFFAVALK (modified)	
	y	*b*	*y*	*b*	*y*	*b*
S	8	1	882.5	ND	910.6	ND
F	7	2	795.4	234.8	823.6	235.0
F	6	3	ND[b]	382	676.5	382.0
A	5	4	501	452.9	529.3	453.1
V	4	5	430	552	458.2	552.2
A	3	6	330.9	623	359.1	623.2
L	2	7	259.9	ND	288.1	ND
K*/R	1	8	146.9	ND	175.0	892.8

[a] K*/R denotes the possibility that the modified sequence is increased in mass by 28 u due to dimethylation, formylation or mutation to arginine.
[b] ND indicates that no corresponding fragment ion was detected.

fragment ions as C-terminal sequence ions up to y_5 (y_1– y_5) for the modified peptide (Fig. 1B; Table 1). The unmodified sequence produced *y* ions consistent with the theoretical C-terminal fragments (Fig. 1B, inverted spectrum; Table 1). The finding that the C-terminal sequence ions, including y_1, displayed a +28 u mass shift suggested that lysine at position 87 was post-translationally modified. The data did not correspond to any tryptic peptide sequence belonging to any other protein in the NCBI database when a more global search was performed with the MS/MS data.

The observed mass increment of +28 u on lysine in the sequence SFFAVALK based on MS and MS/MS fragmentation data could be attributed either to dimethylation (+28.031300 u) or formylation (+27.994915 u) of lysine or mutation of lysine to arginine (+28.00615 u). The mass accuracies required to distinguish between these possibilities is between 11 and 36 ppm (Table 2). However, the mass accuracy of the MALDI-TOF/TOF-MS measurement of the modified peptide was within approximately 50 ppm of the nominal masses of the alternate structures and MS/MS data would be expected to be less accurate using this technique. Thus, it was not possible to distinguish between the potential alternate modifications based on pseudomolecular or fragment ion mass accuracies of MALDI-TOF/TOF measurements.

3.2. Differentiation between the potential alternate structures of the modified tryptic peptide spanning residues 80–87 of D83A-DR using high mass accuracy measurements

The potentially different structures that could represent the modified tryptic peptide spanning residues 80–87 of D83A-DR, as observed above by MALDI-TOF/TOF-MS/MS, are presented in Table 2. These peptides all have a nominal mass of 28 u greater than that of the C-terminal lysyl peptide encoded by the D83A-DR gene. The calculated exact masses of these peptides are also very similar with a range of mass differences between the potential structures of between 11 and 36 ppm (Table 2). In order to evaluate the efficacy of accurate mass measurements with an LTQ-Orbitrap-FTMS to differentiate between the alternate structures they were synthesised and analysed in MS and MS/MS modes with high resolution. As summarized in Table 3, LTQ-Orbitrap-FTMS measurement of the masses of these synthetic peptides was well within the 11 sub-ppm mass accuracy range required to differentiate between these sequences. In fact, masses within 0.5 ppm and 1.4 ppm and 0 and 2.6 ppm were observed for the singly and doubly protonated pseudomolecular ions, respectively, when the peptides were infused for electrospray ionization and measurements made in FT-MS mode with a resolution of 100,000.

The predominant pseudomolecular ion charge species of each peptide was selected for MS/MS measurements in the high resolution FT-MS/MS mode ($R = 100{,}000$). In the cases of the Me2K and Arg peptides, the doubly protonated ions at $m/z = 455.7732$ (Fig. 2A) and $m/z = 455.7606$ (Fig. 2C), respectively, were analysed. In the case of the FormK peptide, the singly charged ion at $m/z = 910.5028$ (Fig. 2B) was used for MS/MS and MS/MS/MS was conducted on the MS/MS fragment at $m/z = 359.2289$ (Fig. 2B; inset) to obtain sequence information on low m/z MS/MS ions. Mass accuracies in the range of +1.4 ppm to −5.7 ppm (RMS error range = 2.0–1.3) and +1.3 ppm and −5.5 ppm (RMS error range 2.8–1.2) were observed for *y*- and *b*-type fragment ions, respectively (Table 4).

Table 2
Elemental compositions of synthetic variants of SFFAVALK with C-terminal Me2K, FormK and Arg and calculated mass differences in ppm between Me2K vs. FormK, Me2K vs. Arg and Arg vs. FormK C-terminal variants

Synthetic peptide	Elemental composition	$(M+H)^+$; m/z	Mass difference (ppm)	
			SFFAVAL(FormK)	SFFAVALR
SFFAVAL(Me2K)	$C_{46}H_{71}N_9O_{10}+H$	910.5397	36	25
SFFAVAL(FormK)	$C_{45}H_{67}N_9O_{11}+H$	910.5033		
SFFAVALR	$C_{44}H_{67}N_{11}O_{10}+H$	910.5145	11	

Table 3
Mass accuracies obtained by direct infusion-ESI-LTQ-Orbitrap FT-MS of synthetic SFFAVAL(Me2K), SFFAVAL(FormK) and SFFAVALR

Synthetic peptide	*m/z*					
	$(M+H)^+$			$(M+H)^{2+}$		
	Calculated	Observed	Error (ppm)	Calculated	Observed	Error (ppm)
SFFAVAL(Me2K)	910.5397	910.5384	1.4	455.7735	455.7728	1.5
SFFAVAL(FormK)	910.5033	910.5028	0.5	455.7553	455.7553	0
SFFAVALR	910.5145	910.5158	1.4	455.7609	455.7597	2.6

The arginyl peptide exhibited a series of satellite ions accompanying *y* ion fragments at *m/z* values of 17 u less than *y* ions. Neither of the other two peptides exhibited any potentially characteristic satellite ions accompanying their *y* ion fragments and no other differential fragmentation characteristics were observed for any of the peptides.

The accuracies of both the pseudomolecular ion and fragment ion mass measurements would appear to be more than adequate to differentiate between the different potential structures that would explain the variant tryptic peptide spanning residues 80–86 of D83A-DR plus a modification at residue 87. The only caveat to this conclusion would be that this peptide could be detected in HPLC-ESI-LTQ-Orbitrap experiments with the tryptic digest of D83A-DR.

3.3. Accurate mass based identification of dimethylation of the side chain of lysine 87 of D83A-DR by CapHPLC-ESI-LTQ-Orbitrap-FT-MS and MS/MS measurements

The tryptic digest of D83A-DR and synthetic peptides were analyzed by CapHPLC-ESI-LTQ-Orbitrap using the same conditions as above. A single peptide was observed in the digest at *m/z* = 455.7731 (Fig. 3) with a retention time of 64.65 min that potentially corresponded to a doubly protonated pseudomolecular ion of the variant residue 80–87 tryptic peptide identified above by MALDI-TOF/TOF-MS/MS experiments (Fig. 1B). The *m/z* value of this ion was approximately 0.87 ppm less than the calculated *m/z* for the doubly protonated form of residues

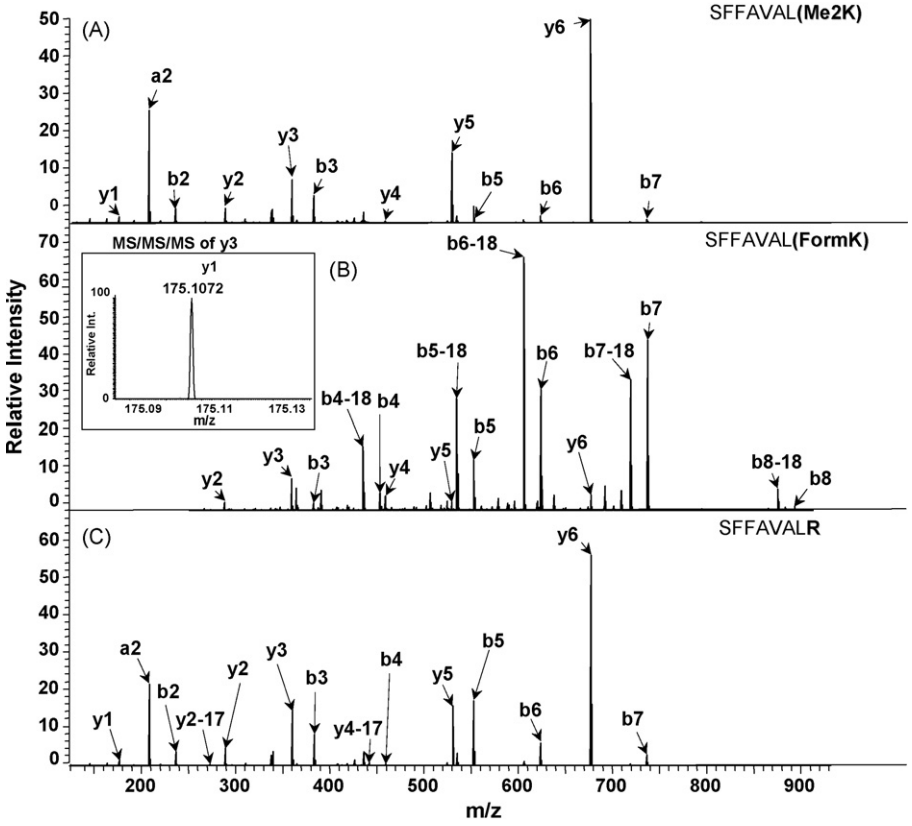

Fig. 2. Direct infusion-ESI-LTQ-Orbitrap FT-MS/MS spectra of synthetic variants of SFFAVALK (A) SFFAVAL(Me2K), (B) SFFAVAL(FormK) and (C) SFFAVALR. The inset in panel B shows the spectrum obtained by MS/MS/MS of the MS/MS fragment at *m/z* 359.2289^{1+}.

Table 4
Mass accuracies obtained by direct infusion-ESI-LTQ-Orbitrap-FT-MS/MS of synthetic SFFAVAL(Me2K), SFFAVAL(FormK) and SFFAVALR and calculated RMS errors in data obtained

Fragment ion	SFFAVAL(Me2K)			SFFAVAL(FormK)			SFFAVALR		
	Calculated	Observed[a]	Δmass (ppm)	Calculated	Observed	Δmass (ppm)	Calculated	Observed	Δmass (ppm)
y_8	910.5397	910.5386	−1.2	910.5033	ND	–	910.5145	910.5158	1.4
y_7	823.5073	823.506	−1.5	823.4709	823.4717	1	823.482	ND	–
y_6	676.4393	676.4371	−3.2	676.4029	676.4026	−0.4	676.414	676.4138	−0.3
y_5	529.3719	529.3689	−5.7	529.3349	529.3339	−1.9	529.346	529.3451	1.7
y_4	458.3333	458.3325	−1.7	458.2969	458.2968	−0.2	458.309	458.3084	−1.3
y_3	359.2653	359.2646	−1.9	359.2289	359.2289	0	359.2401	359.2401	0
y_2	288.2283	288.2278	−1.7	288.1919	288.1919	0	288.2031	288.2033	0.7
y_1	175.1443	175.1438	−2.8	175.1079	175.1072	−4	175.1191	175.119	−0.6
RMS error			2			1.5			1.3
b_1	88.0393	ND	–	88.0393	ND	–	88.0393	ND	–
b_2	235.1077	235.1074	−5.5	235.1077	ND	–	235.1077	235.108	1.3
b_3	382.1761	382.1754	−4.7	382.1761	382.1761	0	382.1761	382.1764	0.8
b_4	453.2132	ND	–	453.2132	453.2128	−0.9	453.2132	453.2133	0.2
b_5	552.2816	552.2801	−4.9	552.2816	552.281	−1.1	552.2816	552.2815	−0.2
b_6	623.3187	623.3175	−3	623.3187	623.3179	−1.3	623.3187	623.319	0.5
b_7	736.4028	736.4016	−2.2	736.4028	736.4021	−0.9	736.4028	736.4034	0.8
b_8	892.5291	ND	–	892.4927	892.4928	0.1	892.5039	ND	–
RMS error			2.8			1.2			1.1

[a] ND indicates that no corresponding fragment ion was detected.

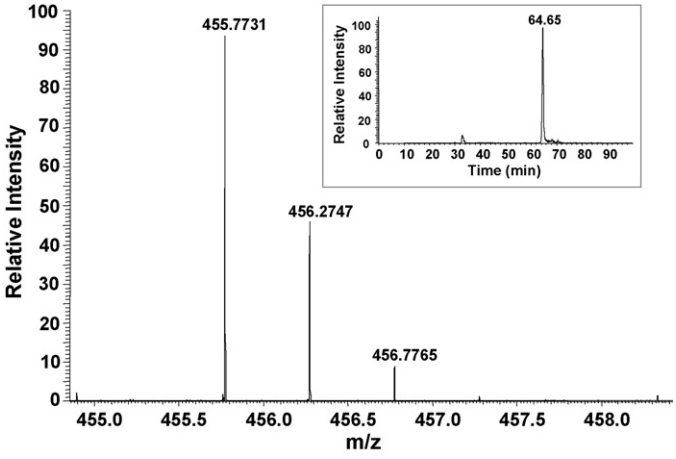

Fig. 3. Extracted ion chromatogram of $m/z = 455.7731$ observed as a doubly charged ion during CapHPLC-ESI-Orbitrap-FT-MS of the tryptic digest of D83A-DR. The figure shows the isotope envelope for the monoisotopic ion of $m/z = 455.7731$ observed at 64.6 min (inset).

80–87 of D83A-DR modified by dimethylation (Tables 3 and 5). By comparison the m/z value of this ion differed by approximately 39 ppm and 27 ppm from the calculated values of the FormK and Arg versions of the sequence, respectively (Table 5), and by approximately 0.22 ppm and 39.3 ppm and 27.4 ppm for the doubly protonated pseudomolecular ion values of the Me2K, FormK and Arg synthetic peptides observed by CapHPLC-ESI-Orbitrap-FTMS, respectively. Elution times for the Me2K and Arg synthetic peptides were 64.04 min and 63.77 min, respectively, which were essentially the same as the variant residue 80–87 tryptic peptide. The synthetic FormK peptide eluted at 57.96 min, which was later then the unmodified residue 80–87 peptide. The later retention times of the modified peptides would be explained largely by their increased hydrophobicities. The Arg peptide possibly eluted later due to increased polar interactions with the column matrix. The retention characteristics of these peptides would not provide any definitive clues to the identification of the variant residue 80–87 peptide in the tryptic digest of D83A-DR. The slightly earlier elution of this peptide compared with CapHPLC-MALDI experiments probably reflects the differences in plumbing of the two HPLC configurations as exactly the same column was used for both experiments.

Table 5
Comparisons of differences (Δ) in m/z of the doubly protonated form of the modified D83A-DR tryptic peptide observed at m/z 455.7731 by CapHPLC-Orbitrap-FT-MS with the calculated and observed m/z values of synthetic SFFAVAL(Me2K), SFFAVAL(FormK) and SFFAVALR

SFFAVAL(Me2K)				SFFAVAL(FormK)				SFFAVALR			
Calculated m/z	Δmass (ppm)	Observed m/z	Δmass (ppm)	Calculated m/z	Δmass (ppm)	Observed m/z	Δmass (ppm)	Calculated m/z	Δmass (ppm)	Observed m/z	Δmass (ppm)
455.7735	0.87	455.7732	0.22	455.7553	39	455.7552	39.3	455.7609	26.7	455.7606	27.4

Experimentally determined *y* series fragment ion masses measured by LTQ-Orbitrap FT-MS/MS of the tryptic digest peptide at *m/z* = 455.7731 were compared with theoretical *y* ion values of the potential alternate structures for this peptide and experimentally determined values obtained with the synthetic alternates (Table 6). Comparison of the D83A-DR digest peptide with the experimentally determined values obtained with Me2K modified sequence revealed an MS/MS RMS error of 1.4 ppm (Table 6). Comparisons with FormK and Arg versions of the sequence revealed MS/MS RMS differences of 13.1 ppm and 12.0 ppm, relative to the experimentally determined *y* fragment ion masses for the digest peptide *y* ion fragments (Table 6). This is in contrast to the MS/MS RMS differences of 1.2 ppm and 1.1 ppm for these two sequences when experimentally determined *y* fragment ion masses obtained with the authentic synthetic structures were compared to theoretical values for the authentic sequences themselves (Table 4). As expected for a Me2K residue at the C-terminus of the D83A-DR digest peptide, the *y* ion *m/z* values compared very well with the calculated and experimental values for the authentic Me2K peptide (Table 6). The degree of correspondence between the experimentally determined fragmentation data for the D83A-DR tryptic peptide and the authentic Me2K peptide is illustrated in Fig. 4.

These experimental mass accuracy measurements strongly favour the assignment of dimethylation of lysine 87 as the modification of residues 80–87 of D83A-DR.

3.4. Comparison of MALDI-TOF/TOF-MS/MS fragmentation characteristics of synthetic variants of SFFAVALK with C-terminal Me2K, FormK and arginine

It was evident that immonium ions formed during MALDI-TOF/TOF-MS/MS of the synthetic variants of SFFAVALK produced a level of differentiation between C-terminal Me2K, FormK and Arg (Fig. 5A). MALDI-TOF/TOF-MS/MS of SFFAVAL(Me2K) and SFFAVAL(FormK) produced a distinct immonium ion at *m/z* 84. SFFAVAL(FormK) also produced an immonium ion at *m/z* 112 (Fig. 5A) which was the only characteristic immonium ion for SFFAVALR (Fig. 5A).

Potentially diagnostic fragmentations were also apparent in the pseudomolecular ion regions of the MALDI-TOF/TOF-MS/MS spectra of these synthetic peptides. The FormK peptide exhibited a major loss of 28 u, probably due to fragmentation of the formyl group (Fig. 5B). The Me2K and Arg peptides showed a loss of 30 u but the relative loss appeared to be higher for the Arg peptide (Fig. 5B).

Diagnostic fragmentations that produced satellites associated with C-terminal *y* ion fragments were evident for the FormK and Arg peptides but not the Me2K peptide (Fig. 5C). The Arg peptide exhibited very substantial satellites with masses of 17 u less than *y* ions and the FormK peptide exhibited much less intense satellites at 18 u lower masses than *y* ions. This satellite effect, as illustrated with the y_3 ion in Fig. 5C, and was consistent for the vast majority for the *y* ions but not presented comprehensively for reasons of brevity. A more detailed presentation of the satellite effect appears in Table 7. It is noteworthy that none of the Me2K or FormK peptide *y* ions exhibited a

Table 6
Comparison of the experimentally determined *y* series fragment ion mass values of the D83A-DR tryptic digest peptide at *m/z* 455.7731 with calculated and experimental *y* ion mass values of synthetic SFFAVAL(Me2K), SFFAVAL(FormK) and SFFAVALR obtained by CapHPLC-ESI-LTQ-Orbitrap-FT-MS/MS and corresponding mass differences in ppm

Residue[a]	Fragment ion	SFFAVALK*/R (D83A-DR)	SFFAVAL(Me2K)[c]			SFFAVAL(FormK)			SFFAVALR		
		Observed[b]	Calculated	Observed		Calculated	Observed		Calculated	Observed	
S	y_8	ND	910.5386	ND	ND	910.5033	ND	ND	910.5145	ND	ND
F	y_7	ND	822.506	ND	ND	822.4709	ND	ND	822.482	ND	ND
F	y_6	676.4386	676.4393	(−1)	676.4394	(−1.2)	676.4029	52.7	676.4031	52.5	676.414	36.4	676.4102	42
A	y_5	529.3699	529.3719	(−3.7)	529.3705	(−1.1)	529.3349	66.1	529.3338	68.2	529.346	45.1	529.3441	49
V	y_4	458.3339	458.3333	(−1)	ND	ND	458.2969	80.7	458.297	80.5	458.309	54.3	458.3087	55
A	y_3	359.2652	359.2653	(−0.3)	359.2657	(−1.4)	359.2289	101	359.2292	100.2	359.2401	70	359.2412	67
L	y_2	288.2285	288.2283	−0.7	288.2286	(−0.3)	288.1919	127	288.192	126.6	288.2031	88.1	288.2047	82.6
K*/R	y_1	175.1441	175.1443	(−1.1)	175.1439	(−1.1)	175.1079	206.6	ND	ND	175.1191	142.7	175.1203	136
RMS error				1.6		1.4		14.5		13.1		12.1		12

[a] K*/R denotes the possibility that the modified sequence is increased in mass by 28 u due to dimethylation, formylation or mutation to arginine.
[b] ND indicates that no corresponding fragment ion was detected.
[c] Values in parentheses are ppm differences in *m/z* values observed for the D83A-DR tryptic peptide and the relevant synthetic peptide.

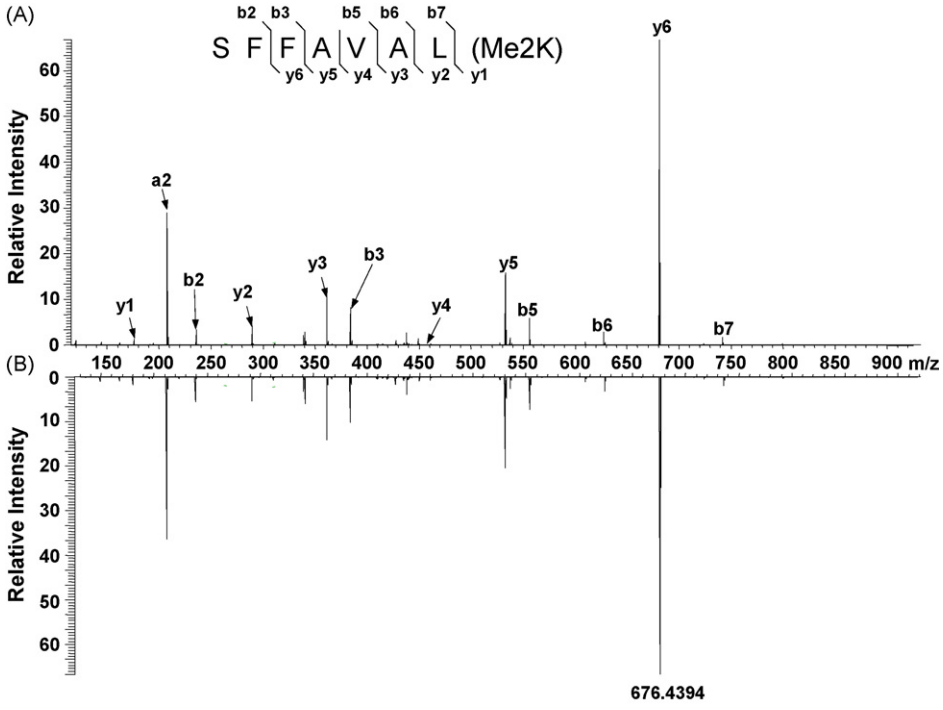

Fig. 4. Comparison of CapHPLC-ESI-Orbitrap-FT-MS/MS spectra data for (A) the D83A-DR tryptic peptide observed at 64.6 min and (B) synthetic SFFAVAL(Me2K).

−17 u satellite and that the −17 u satellites were also uniquely observed for the Arg synthetic peptide during MS/MS using the LTQ-Orbitrap-FT-MS (Fig. 2C).

The MALDI-TOF/TOF-MS/MS data obtained for the variant tryptic peptide of interest at $m/z = 910.58$ in the digest of D83A-DR (Fig. 1; Table 8) was reviewed taking into account the MALDI-TOF/TOF-MS/MS fragmentation characteristics of the synthetic peptides (Table 7). The D83A-DR tryptic peptide exhibited an immonium ion at $m/z = 84$ but not $m/z = 112$ and no evidence of −17 u *y* ion satellites or loss of either 28 u or 30 u from the precursor ion (Fig. 6; Table 8). These observations support the contention that the D83A-DR tryptic peptide at $m/z = 910.58$ was modified by dimethylation of the C-terminal lysine 87 when compared to the fragmentation characteristics of the authentic potential alternate structures (Table 7). A formylated peptide would have been expected to have exhibited a pronounced 28 u loss from the pseudomolecular ion. Furthermore, the 30 u loss from the pseudomolecular ion of the synthetic Me2K peptide was much less pronounced than from the synthetic Arg peptide. An Arg at position 87 in D83A-DR would have been expected to have led to pronounced −17 u satellites.

Table 7
MALDI-TOF/TOF-MS/MS signature ions of synthetic SFFAVAL(Me2K), SFFAVAL(FormK) and SFFAVALR

Characteristic fragment ions	SFFAVAL(Me2K)	SFFAVAL(FormK)	SFFAVALR
Immonium ions	84	84	–
	–	112	112
Parent ion losses	−30	–	−30
	–	−28	–
y ion satellite	–	−18	−17

4. Discussion

There is growing appreciation that post-translational modifications overlay the primary amino acid sequences of proteins to provide chemical signals as codes to modulate protein interactions and function [27]. The classic example of this is the histone code that regulates chromatin activity [14,15,38]. Transcription factor stability [39–42], interactions [20,43] and function [44] also appear to be controlled by similar chemical coding systems [45]. The findings presented herein arose as part of a study of the post-translational regulation of signal activated basic-helix-loop-helix transcription factor, DR. Prior to this report only two sites of post-translational modification have been described that regulate any facet of the biology of the DR. Phosphorylation of serines 36 and 68 were detected by peptide specific phosphoserine antibodies as regulators of nuclear-cytoplasmic shuttling of DR [9–11]. While the present report does not address a biological role for the modification described, it does highlight the need for analytical rigor prior to embarking on downstream biological investigations of the role of post-translational modifications. The finding, by MALDI-TOF/TOF-MS/MS, of a peptide in a tryptic digest with a nominal mass increment of 28 u on its C-terminus, relative to the peptide predicted by the gene sequence would be most easily explained by mutation of the predicted

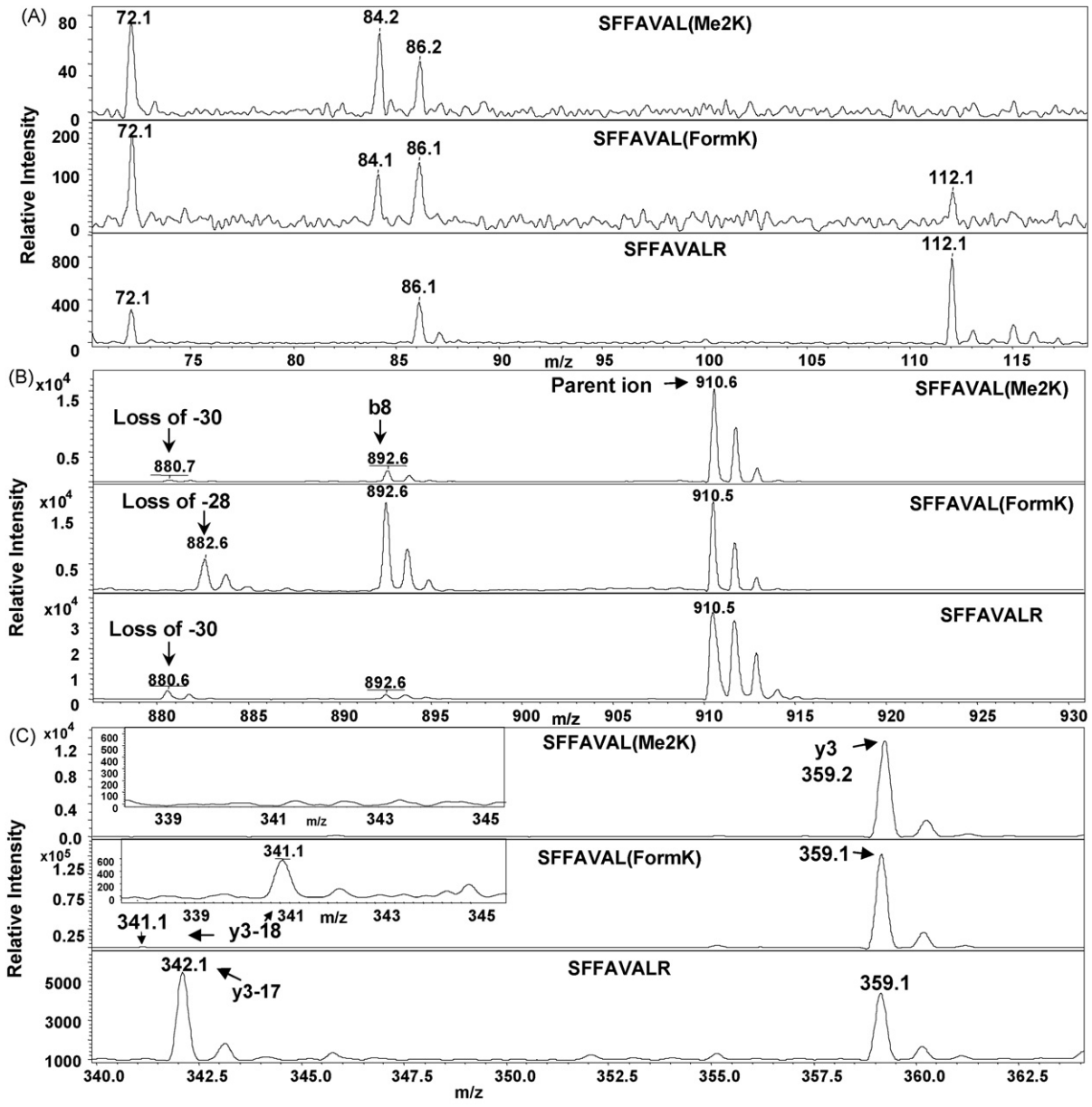

Fig. 5. MALDI-TOF/TOF-MS/MS spectra of synthetic variants of SFFAVALK with C-terminal Me2K, FormK and Arg. Equal quantities of each peptide were spotted onto a MALDI target for analysis. Data shown represent 800 laser shots summed in groups of 100 shots. Segments of the MS/MS spectra are shown corresponding to (A) unique immonium ion region, (B) characteristic fragmentations of the parent ions (B) and (C) ions characteristically associated with C-terminal *y* ion fragments. Insets in C represent 20 times expansions of the spectra around −18 u from y_3 fragments of the corresponding sequences.

C-terminal Lys to Arg. This would accommodate both the magnitude of the mass increment and tryptic cleavage specificity. The other modifications that could cause a mass increment of 28 u would be dimethylation or formylation. Established dogma on the lack of susceptibility of Me2K to tryptic cleavage should have ruled this modification out at the C-terminus of the peptide spanning residues 80–87 of D83A-DR [46]. Formylation appears to be a rarer natural modification but has been reported with a histone [21] and a bee venom peptide [47,48]. In the latter case it was reported that trypsin cleaved peptide bonds C-terminal to FormK [47,48]. The analytical approaches used in this study have demonstrated that the commercially available trypsin used was able to cleave at the C-terminus of a Me2K residue of D83A-DR. We have also demonstrated such cleavage with the commercial trypsin using synthetic peptides containing internal Me2K and at Me2K residues in methylated trypsin preparations (Promega sequencing grade trypsin modified by TPCK and reductive methylation) that had been further modified by reduction and alkylation (data not shown). Tryptic cleavage on the C-terminal side of Me2K has also been observed with histone H1 variants [21] and histone H3 (data not shown). LysC protease was assessed for its cleavage capacity at Me2K residues using the reduced and alkylated modified trypsin that also contained unmodified lysine residues. LysC protease cleaved only

Table 8
Comparison of characteristic MALDI-TOF/TOF-MS/MS 'y', 'y-17', 'y-18' and '*b*' series ions of synthetic SFFAVAL(Me2K), SFFAVAL(FormK) and SFFAVALR and the tryptic peptide derived from D83A-DR with *m/z* 910.58[1+]

Residue[a]	Fragment ion		SFFAVAL(Me2K)[b]				SFFAVAL(FormK)				SFFAVALR				SFFAVAL(K*/R) (D83A-DR)			
	y	*b*	*y*	*y*-17	*y*-18	*b*	*y*	*y*-17	*y*-18	*b*	*y*	*y*-17	*y*-18	*b*	*y*	*y*-17	*y*-18	*b*
S	8	1	910.6	ND	ND	ND	910.5	ND	ND	ND	910.5	892.6	ND	ND	910.6	ND	ND	ND
F	7	2	823.8	ND	ND	235.0	823.6	ND	ND	235.0	823.6	806.6	ND	235.0	823.6	ND	ND	234.0
F	6	3	676.6	ND	ND	382.2	676.5	ND	658.4	382.1	676.5	659.4	ND	382.1	676.5	ND	ND	382.0
A	5	4	529.4	ND	ND	453.2	529.3	ND	511.3	453.2	529.3	512.3	ND	453.1	529.3	ND	ND	453.1
V	4	5	458.3	ND	ND	552.3	458.3	ND	440.2	552.3	458.2	441.2	ND	552.2	458.2	ND	ND	552.2
A	3	6	359.2	ND	ND	623.4	359.2	ND	341.1	623.3	359.1	342.1	ND	623.3	359.1	ND	ND	623.2
L	2	7	288.2	ND	ND	736.5	288.1	ND	270.1	736.5	288.1	271.0	ND	736.5	288.1	ND	ND	ND
K*/R	1	8	175.1	ND	157.1	892.6	175.0	ND	157.1	892.6	175.0	158.0	ND	892.6	175.0	ND	ND	892.8

[a] K*/R denotes the possibility that the modified sequence is increased in mass by 28 u due to dimethylation, formylation or mutation to arginine.
[b] ND indicates that no corresponding fragment ion was detected.

Fig. 6. Expanded segments of the MALDI-TOF/TOF-MS/MS spectrum shown in Fig. 1B (upper) of the ion at *m/z* of 910.58 in the spectrum of the nanoHPLC fraction of the tryptic digest of D83A-DR that eluted at min 66.5 min. The immonium ion (A) and parent ion (B) regions are depicted.

at the unmodified lysines (data not shown), thus, this protease would not be useful for distinguishing between Me2K modification and arginine mutation. We have not assessed LysC protease for cleavage at FormK substrates, however, if such sites were susceptible this protease may be useful for distinguishing formylation from the other isobaric possibilities.

The present work highlights the need to adopt an "eyes-wide-open" approach to mapping post-translational modifications and the value of high mass accuracy measurements for differentiating between isobaric modifications. MALDI-TOF/TOF-MS and -MS/MS proved to be valuable tools in this "eyes-wide-open" approach, both in the present study and in previous studies [49], wherein unanticipated post-translational modifications were encountered. Coupling capillary HPLC with the MALDI ionization method was particularly effective for screening tryptic digests for post-translationally modified peptides in a way that allowed components at particular elution times to be addressed in a more detailed way at a later time. In turn, the high mass accuracy of the FT-MS and -MS/MS measurements enabled definitive characterization of the modification spotted in the MALDI-TOF/TOF-MS and -MS/MS screens. The present study also indicated that specific MALDI-TOF/TOF-MS/MS fragmentation characteristics may be useful in differentiating between the occurrence of Me2K, FormK and Arg at the C-termini of peptides. This will need to be confirmed by examination of a larger number of instances of these modifications but the finding of three windows of characteristics indicates a high likelihood that the MALDI-TOF/TOF-MS/MS fragmentation characteristics will be useful. In particular, comparison of the immonium ions formed from SFFAVAL (Me2K), SFFAVAL(FormK) and SFFAVALR showed diagnostic differences. SFFAVALR formed an immonium ion at *m/z* 112 while an immonium ion at *m/z* 84 was observed with the

SFFAVAL(Me2K) peptide. These findings are consistent with previously described mechanisms for immonium ion formation from Me2K and Arg. An unmodified lysine in a peptide generally produces immonium ions at $m/z = 129$ (α-amino-ε-caprolactam), arginine also gives rise to an immonium ion at $m/z = 129$ [50–52]. The immonium ion of lysine at $m/z = 129$ fragments further to produce an immonium ion $[C_5H_{10}N]^+$ at $m/z = 84$ while arginine undergoes cyclization of the immonium ion by elimination of ammonia resulting in an immonium ion at $m/z = 112$ [50,52]. Formation of an immonium ion at m/z 112 is impossible for lysine modified by dimethylation as the immonium ion of this derivative at $m/z = 129$ can only undergo further rearrangement by elimination of $(CH_3)_2NH$ to produce the same immonium ion at $m/z = 84$ [30] as produced by lysine. Formylated lysine in the sequence SFFAVAL(FormK) on the other hand showed the presence of both the immonium ions at m/z 84 as well as m/z 112. The reason for presence of immonium ion at m/z 112 is that formylated lysine may undergo ammonia or amine elimination from either ε-amine or α-amine to produce the $[C_5H_{10}N]^+$ ion at $m/z = 84$ and the $[C_6H_{10}NO]^+$ ion at $m/z = 112$. The characteristic immonium ions observed in this study for the modified form of residues 80–87 of D83A-DR, which showed the presence of an immonium ion at m/z 84 and not $m/z = 112$, reinforced the conclusion from high mass accuracy measurements that K87 was modified by dimethylation.

Development of the analytical approach described herein stands us in good stead to detect and characterize any further post-translational modifications that may be present on the DR protein. This process is currently underway with one of the major objectives to discover if K87 is also dimethylated in the wild-type protein sequence. DR is activated by stimuli that reflect both its developmental and xenobiotic response roles [53]. These stimuli result in rearranging protein-protein interactions in the cytoplasm, nuclear-cytoplasmic shuttling and protein-protein interactions in the nucleus [54]. It will be of interest to see if there is a post-translational coding system that controls these events. Clearly, this will involve a dynamic interplay of experimentation using the analytical approach described herein with complementary molecular and cellular biology approaches. Methylation of K87 is the first description of a methylation of DR and only second type of any post-translational modification identified on this transcription factor. Mutagenesis of this residue will provide an excellent starting point for investigation of the role of post-translational modifications in regulation of the activity of DR.

Acknowledgements

This paper is dedicated to the important contributions to the field of biomolecular mass spectrometry made by Professor Peter Roepstorff. We wish to thank Fiona Whelan for providing dioxin receptor preparations and the National Health and Medical Research Council of Australia for financial support.

References

[1] Y.Z. Gu, J.B. Hogenesch, C.A. Bradfield, Annu. Rev. Pharmacol. Toxicol. 40 (2000) 519.
[2] I. Pongratz, P.E. Stromstedt, G.G. Mason, L. Poellinger, J. Biol. Chem. 266 (1991) 16813.
[3] S.S. Singh, G.H. Perdew, Arch. Biochem. Biophys. 305 (1993) 170.
[4] F. Matsumura, Biochem. Pharmacol. 48 (1994) 215.
[5] M.J. Mahon, T.A. Gasiewicz, Arch. Biochem. Biophys. 318 (1995) 166.
[6] S. Park, E.C. Henry, T.A. Gasiewicz, Arch. Biochem. Biophys. 381 (2000) 302.
[7] G.D. Minsavage, S.K. Park, T.A. Gasiewicz, J. Biol. Chem. 279 (2004) 20582.
[8] M. Backlund, M. Ingelman-Sundberg, Cell Signal. 17 (2005) 39.
[9] T. Ikuta, Y. Kobayashi, K. Kawajiri, Biochem. Biophys. Res. Commun. 317 (2004) 545.
[10] K. Kawajiri, T. Ikuta, J. Health Sci. 50 (2004) 215.
[11] T. Ikuta, Y. Kobayashi, K. Kawajiri, J. Biol. Chem. 279 (2004) 19209.
[12] S. Clarke, Curr. Opin. Cell Biol. 5 (1993) 977.
[13] D. Chen, H. Ma, H. Hong, S.S. Koh, S.M. Huang, B.T. Schurter, D.W. Aswad, M.R. Stallcup, Science 284 (1999) 2174.
[14] S. Rea, F. Eisenhaber, D. O'Carroll, B.D. Strahl, Z.W. Sun, M. Schmid, S. Opravil, K. Mechtler, C.P. Ponting, C.D. Allis, T. Jenuwein, Nature 406 (2000) 593.
[15] Y. Zhang, D. Reinberg, Genes Dev. 15 (2001) 2343.
[16] S. Chuikov, J.K. Kurash, J.R. Wilson, B. Xiao, N. Justin, G.S. Ivanov, K. McKinney, P. Tempst, C. Prives, S.J. Gamblin, N.A. Barlev, D. Reinberg, Nature 432 (2004) 353.
[17] M.M. Huq, N.P. Tsai, S.A. Khan, L.N. Wei, Mol. Cell Proteom. (2007).
[18] J.M. Aletta, T.R. Cimato, M.J. Ettinger, Trends Biochem. Sci. 23 (1998) 89.
[19] W. Zhu, T. Mustelin, M. David, J. Biol. Chem. 277 (2002) 35787.
[20] C.D. Knights, J. Catania, S. Di Giovanni, S. Muratoglu, R. Perez, A. Swartzbeck, A.A. Quong, X. Zhang, T. Beerman, R.G. Pestell, M.L. Avantaggiati, J. Cell Biol. 173 (2006) 533.
[21] J.R. Wisniewski, A. Zougman, S. Krueger, M. Mann, Mol. Cell Proteom. (2006).
[22] J. Stenflo, P. Fernlund, W. Egan, P. Roepstorff, Proc. Natl. Acad. Sci. U. S. A. 71 (1974) 2730.
[23] S.A. Carr, K. Biemann, Methods Enzymol. 106 (1984) 29.
[24] J.J. Gorman, G.L. Corino, B.J. Shiell, Biomed. Environ. Mass Spectrom. 19 (1990) 646.
[25] M.R. Larsen, P. Roepstorff, Fresenius, J. Anal. Chem. 366 (2000) 677.
[26] R. Aebersold, M. Mann, Nature 422 (2003) 198.
[27] M. Mann, O.N. Jensen, Nat. Biotechnol. 21 (2003) 255.
[28] O.N. Jensen, Curr. Opin. Chem. Biol. 8 (2004) 33.
[29] K.F. Medzihradszky, Z. Darula, E. Perlson, M. Fainzilber, R.J. Chalkley, H. Ball, D. Greenbaum, M. Bogyo, D.R. Tyson, R.A. Bradshaw, A.L. Burlingame, Mol. Cell Proteom. 3 (2004) 429.
[30] K. Zhang, P.M. Yau, B. Chandrasekhar, R. New, R. Kondrat, B.S. Imai, M.E. Bradbury, Proteomics 4 (2004) 1.
[31] J.A. Oses-Prieto, X. Zhang, A.L. Burlingame, Mol. Cell Proteom. (2006).
[32] Q. Hu, R.J. Noll, H. Li, A. Makarov, M. Hardman, R. Graham Cooks, J. Mass Spectrom. 40 (2005) 430.
[33] A. Makarov, E. Denisov, O. Lange, S. Horning, J. Am. Soc. Mass Spectrom. 17 (2006) 977.
[34] M. Scigelova, A. Makarov, Proteomics 6 (Suppl. 2) (2006) 16.
[35] J.V. Olsen, L.M. de Godoy, G. Li, B. Macek, P. Mortensen, R. Pesch, A. Makarov, O. Lange, S. Horning, M. Mann, Mol. Cell Proteom. 4 (2005) 2010.
[36] D.J. Peet, D. Lando, D.A. Whelan, M.L. Whitelaw, J.J. Gorman, Methods Enzymol. 381 (2004) 467.
[37] J.J. Gorman, Anal. Biochem. 160 (1987) 376.
[38] H. He, N. Lehming, Brief Funct. Genom. Proteom. 2 (2003) 234.
[39] E. Appella, C.W. Anderson, Pathol. Biol. (Paris) 48 (2000) 227.
[40] L. Feng, T. Lin, H. Uranishi, W. Gu, Y. Xu, Mol. Cell Biol. 25 (2005) 5389.
[41] M. Ivan, K. Kondo, H. Yang, W. Kim, J. Valiando, M. Ohh, A. Salic, J.M. Asara, W.S. Lane, W.G. Kaelin Jr., Science 292 (2001) 464.
[42] P. Jaakkola, D.R. Mole, Y.M. Tian, M.I. Wilson, J. Gielbert, S.J. Gaskell, A. Kriegsheim, H.F. Hebestreit, M. Mukherji, C.J. Schofield, P.H. Maxwell, C.W. Pugh, P.J. Ratcliffe, Science 292 (2001) 468.

[43] J. Vervoorts, J. Luscher-Firzlaff, B. Luscher, J. Biol. Chem. 281 (2006) 34725.
[44] M.J. Barrero, S. Malik, Mol. Cell. 24 (2006) 233.
[45] T.L. Tootle, I. Rebay, Bioessays 27 (2005) 285.
[46] L. Poncz, D.G. Dearborn, J. Biol. Chem. 258 (1983) 1844.
[47] F.P. Barry, S. Doonan, C.A. Ross, Biochem. J. 193 (1981) 737.
[48] S. Doonan, A.J. Garman, J.M. Hanson, A.G. Loudon, C.A. Vernon, J. Chem. Soc. Perkin Trans. I. (1978) 1157.
[49] D. Lando, D.J. Peet, D.A. Whelan, J.J. Gorman, M.L. Whitelaw, Science 295 (2002) 858.
[50] T. Yalcin, A.G. Harrison, J. Mass Spectrom. 31 (1996) 1237.
[51] F. Fenaille, J.C. Tabet, P.A. Guy, Rapid Commun. Mass Spectrom. 18 (2004) 67.
[52] P.M. Gehrig, P.E. Hunziker, S. Zahariev, S. Pongor, J. Am. Soc. Mass Spectrom. 15 (2004) 142.
[53] K.W. Bock, C. Kohle, Biochem. Pharmacol. 72 (2006) 393.
[54] R.J. Kewley, M.L. Whitelaw, A. Chapman-Smith, Int. J. Biochem. Cell Biol. 36 (2004) 189.