Sulfonation and Phosphorylation of Regions of the Dioxin Receptor Susceptible to Methionine Modifications*§

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Tagged murine dioxin receptor was purified from mammalian cells, digested with trypsin, and analyzed by capillary HPLC-MALDI-TOF/TOF-MS and -MS/MS. Several chromatographically distinct semitryptic peptides matching two regions spanning residues Glu409–Arg424 and Ser547–Arg565 of the dioxin receptor were revealed by de novo sequencing. Methionine residues at 418 and 548 were detected in these peptides as either unmodified or modified by moieties of 16 (oxidation) or 57 amu (S-carboxamidomethylation) or in a form corresponding to degradative removal of 105 amu from the S-carboxamidomethylated methionine. MS/MS spectra revealed that the peptides containing modified methionine residues also existed in forms with a modification of +80 amu on serine residues 411, 415, and 547. The MS/MS spectra of these peptide ions also revealed diagnostic neutral loss fragment ions of 64, 98, and/or 80 amu, and in some instances combinations of these neutral losses were apparent. Taken together, these data indicated that serines 411 and 547 of the dioxin receptor were sulfonated and serine 415 was phosphorylated. Separate digests of the dioxin receptor were prepared in H216O and H218O, and enzymatic dephosphorylation was subsequently performed on the H218O digest only. The digests were mixed in equal proportions and analyzed by capillary HPLC-MALDI-TOF/TOF-MS and -MS/MS. This strategy confirmed assignment of sulfonation as the cause of the +80-amu modifications on serines 411 and 547 and phosphorylation as the predominant cause of the +80-amu modification of serine 415. The relative quantitation of phosphorylation and sulfonation enabled by this differential phosphatase strategy also suggested the presence of sulfonation on a serine other than residue 411 within the sequence spanning Glu409–Arg424. This represents the first description of post-translational sulfonation sites and identification of a new phosphorylation site of the latent dioxin receptor.

Furthermore this is only the second report of serine sulfonation of eukaryotic proteins. Mutagenesis studies are underway to assess the functional consequences of these modifications. Molecular & Cellular Proteomics 8: 706–719, 2009.

Dioxin receptor (DR)†, also called aryl hydrocarbon receptor, is a ligand-activated transcription factor involved in mediating toxic and carcinogenic effects of a wide variety of environmental pollutants such as dioxin (2,3,7,8-tetrachlorodibenzo-p-dioxin) and benzo(a)pyrene (1, 2). DR belongs to a family of chemosensors and developmental regulators that are grouped as bHLH/PAS (basic helix-loop-helix/PER, ARNT, SIM) proteins characterized by shared structural motifs (3). Post-translational modifications (PTMs), particularly phosphorylation, have been suggested to play an important role in regulating DR activation pathways (4–10). However, apart from phosphorylation of serines at position 36 and 68 identified using anti-phosphoserine antibodies and shown to be essential for nucleocytoplasmic shuttling of DR (11–13), information on other phosphorylation events of the DR is lacking (3). In a recent study we identified N,N’-dimethyllysine at position 87 in a recombinant D83A mutant DR (D83A-DR) using advanced mass spectrometry technologies (14). This indicated the possibility of other modifications of DR besides phosphorylation that may regulate its function.

Post-translational modifications underpin signaling cascades and determine the biological outcomes of environmental signals received by cells. Almost 300 PTMs of proteins are known to occur physiologically (15). An emerging theme in the field of mass spectrometry-based characterization of PTMs is accurate identification and quantitation of protein phosphorylation (16–19) to be able to relate phosphosite identification to protein regulation (15, 20). Although protein phosphorylation is an important and probably the best characterized PTM,
protein sulfonation remains a potentially underrated modification that may be of widespread occurrence (21). Protein O-sulfonation on tyrosine is a common enzymatic modification that has been described previously (22–25). However, O-sulfonation of serine and threonine residues of eukaryotic proteins is a more recent discovery with proposed functional involvement in protein assembly and signal transduction (21). The reason for this may be that both phosphorylated and sulfonated forms of the same peptide could exist as a mixture in protease digests with isobaric masses (21), and PTMs contributing +80 amu have been assumed to represent phosphorylation. At the moment the only general means of discrimination between sulfonation and phosphorylation of serine and threonine residues is through inspection of MS/MS spectra for specific neutral loss characteristics. Elimination of the sulfonate moiety from the parent ion during MS/MS resulting in a neutral loss of 80 amu is a strong determinant for the presence of O-sulfonation of serines and threonine residues in peptides (21, 26) as opposed to the neutral loss of 98 amu that typifies serine and threonine phosphorylation. However, the reliability of this approach has not been extensively tested. Consequently there is a need for more specific means of detection of sulfonation that also permits distinction of this modification from the possibility of the isobaric modification of peptides by phosphorylation.

Advances in MS technologies, especially mass spectrometers that can achieve high resolution and high accuracy in the MS and MS/MS modes, have been extremely useful in discriminating isobaric modifications (14, 27–29). Mass spectrometers capable of high mass accuracy measurements generally use ESI of sample molecules (27, 28). However, MALDI-TOF/TOF-MS/MS is a valuable preliminary screening tool for most sample analysis because of its relative tolerance toward interfering compounds, sensitivity, and the ability to archive sample plates to allow more judicious and rational selection of precursor ions at a later time (30, 31). However, as the mechanisms of sample ionization in MALDI and ESI are different not every peptide ion observed in the MALDI spectrum will show a corresponding ion by ESI analysis of the same sample, and only a fraction of peptides produced from a given protein have been detected in common after both MALDI and ESI (30–35). Consequently methods not entirely dependent on high mass accuracy measurement or one particular mass spectrometry technology are required for the differentiation of isobaric modifications such as phosphorylation and sulfonation (36).

Detection of phosphopeptides using specific enzymatic dephosphorylation has been described previously (36, 37), and incorporation of isotopically distinct functional groups prior to enzymatic dephosphorylation has recently been used as a strategy for quantitative phosphoproteomics studies (16–18, 20, 38). Many different chemistries are available for isotopic labeling of peptides (16, 18, 39, 40). However, isotopic labeling with 18O (41–44) is a relatively simple and convenient methodology for protein quantification (45, 46) including for application to quantitative phosphoproteomics (20, 38). Incorporation of 18O into C-terminal carboxylic acids can be enzymatically catalyzed during standard proteolytic digestion procedures (41–44, 46). Trypsin-catalyzed 16O/18O isotope-coding technology is free of unwanted side reactions such as chemical derivatization of nonspecific amino acid residues and does not alter the ionization and the chromatographic behavior of differentially labeled peptides (45).

During investigation of DR by CapHPLC-MALDI-TOF/TOF-MS and -MS/MS numerous peptide ions were observed that did not match with true tryptic peptides of DR, initiating a detailed investigation of these peptides. De novo sequence analysis indicated that these non-tryptic peptides were derived from two different regions of DR sequence, Glu409–Arg424 and Ser547–Arg555, with each region exhibiting different modifications. Several serine residues were modified by a moiety of +80 amu along with chemically modified methionine residues. A trypsin-catalyzed 16O/18O isotope-coding technology in combination with enzymatic dephosphorylation was used to distinguish between phosphorylation and sulfonation on these serines and for the relative quantitation of phosphorylation of one serine. As a consequence of this study we describe for the first time a post-translational phosphorylation event at Ser415 and two sulfonation sites at Ser411 and Ser547 in the latent DR. This is only the second report of sulfonation of serine residues in eukaryotic proteins.

**Experimental Procedures**

**Chemicals and Reagents**—Acids and organic solvents were HPLC grade or better. Trypsin (modified sequencing grade) was purchased from Roche Diagnostics. Triethylammonium bicarbonate buffer (TEAB; 1 M) and benzamidine hydrochloride (99%) were purchased from Sigma–Aldrich. ortho-Phosphoric acid (85%) was purchased from Fluka Biochemica (Sigma–Aldrich). α-Cyano-4-hydroxycinnamic acid was from Bruker Daltonics (Bremen, Germany). Antarctic phosphatase, λ-phosphatase, and associated buffers were obtained from New England Biolabs, Inc. (Ipswich, MA). Water containing ≥97 atom % excess H18O was purchased from Marshall Isotopes Ltd. (Tel-Aviv, Israel). Routinely used water was purified by a Milli-Q synthesis A10 system (Millipore, Billerica, MA).

**Recombinant Expression and Isolation of the D83A Mutant Form of Murine DR**—Murine D83A-DR was produced using protocols described previously (14, 47). Briefly a mutant D83A-DR construct was stably transfected into the human embryonic kidney cell line 293T with a plasmid encoding c-Myc and His6, affinity tags contiguous with the DR coding sequence (His-myc-mDR). Cells were disrupted, and the tagged D83A-DR was isolated using IMAC.

**Purification and Tryptic Digestion of DR**—Recombinant D83A-DR was further purified to remove contaminating proteins by a process of SDS-PAGE (48) followed by electroelution (49). This protocol was mandated because of the insolubility of recombinant DR in non-denaturing buffers. The eluate from the IMAC step above was concentrated using Amicon® Ultra-4 centrifugal filter devices (Millipore) and subsequently subjected to SDS-PAGE on 7.5% gels that had been cured at 4 °C for 24 h after polymerization to allow decomposition of the ammonium persulfate. Gels were stained with Coomassie Brilliant Blue R-250 (Sigma–Aldrich) to visualize the DR band, which was subsequently excised, and the stained DR was electroeluted as described previously (14). The electroeluted protein was harvested.
and co-precipitated with trypsin (1 μg) using 9 volumes of HPLC grade methanol at −20 °C. The precipitate was harvested after 16 h at −20 °C by centrifugation at 4 °C and washed twice with −20 °C methanol. Tryptic digestion was performed by resuspending the methanol-precipitated protein in 100 mM TEAB buffer with the aid of sonication and incubating the resuspended protein at 37 °C for 2 h prior to adding a further 1 μg of trypsin and continuing the incubation for a further 3 h. Prior to HPLC fractionation, tryptic digests containing TEAB buffer were acidified with 1 μl of 50% TFA.

**Differential Stable Isotope Labeling of Peptides** — For differential stable isotope labeling of peptides, electroeluates were divided into exactly two equal portions, co-precipitated separately with identical quantities of trypsin, and processed as described above. The two samples of harvested protein were resuspended separately in H216O water or ≥97.6% H218O, and a 0.1-volume aliquot of 1 mM TEAB in H216O was added to each sample. Tryptic digestion was subsequently allowed to proceed for 18 h at 37 °C. The 16O-labeled peptide pool was immediately frozen at −20 °C, and the 16O-labeled peptide pool was subjected to enzymatic dephosphorylation.

**Enzymatic Dephosphorylation of the Peptide Mixture** — The 16O-labeled peptide sample from above was divided into exactly two halves. One half was treated with phosphatases, and the second half was subjected to a mock incubation procedure without phosphatases. Prior to enzymatic dephosphorylation, a 0.1-volume aliquot of 1 mM benzamidine in H216O was added to inhibit residual trypsin activity. Dephosphorylation was effected using two different phosphatases, Antarctic phosphatase and λ-phosphatase. Freshly prepared ZnCl2 (2.5-μl volumes of 100 mM) and MgCl2 (2.5 volumes of 100 mM) were added to both the treated and untreated samples. After the addition of Antarctic phosphatase (1 μl) to one sample only (treated sample), the samples were incubated for 18 h at 37 °C. Freshly prepared MnCl2 (final concentration of 1 mM) and λ-phosphatase (1 μl) were added to the Antarctic phosphatase-containing sample, whereas an equivalent volume of Milli-Q water was added to the mock sample. Dephosphorylation was allowed to continue for a further 18 h at 37 °C.

The 16O-labeled samples were mixed separately in 0.2 volumes of 50% (v/v) phosphoric acid in H216O, and equivalent aliquots of the 18O-labeled digest were added to each sample after making allowance for volume changes to the 18O-labeled samples associated with addition of buffers, salts, and/or enzymes to achieve dephosphorylation or mock treatments.

**HPLC Fractionation and Tandem Mass Spectrometry** — Non-metallic Dionex 3000 Ultimate one-dimensional nano-HPLC systems (Dionex) and C18 reverse phase, 300-Å-pore size, 150-mm × 150-μm (inner diameter) monomeric columns (Vydac Everest) were used for all HPLC fractionations. For LC-MALDI-TOF/TOF analyses, fractions were co-spotted at 0.5-min intervals onto a polished stainless steel MALDI target plate (Bruker Daltonics) with α-cyano-4-hydroxycinnamic acid matrix using an on-line micropipetillary robot (Dionex Ultimate Probot) as described previously (14). All mass spectra were acquired in positive ion mode on a Ultraflex III MALDI-TOF/TOF instrument (Bruker Daltonics). Instrument settings and the calibration procedure for MS and MS/MS have been described in detail previously (14). MALDI-MS measurements were executed in stable isotope labeling experiment (SILE) work flow mode in which differentially labeled peptides occurring in all mixtures were identified as pairs (SILE pairs) in the MS spectra. In this study the standard automatic Bruker SILE work flow was reconfigured to pause after MS acquisition and peak picking. This strategy allowed targeted selection of precursor ions of interest for subsequent manual acquisition of MS/MS data.

Assignment of data to specific portions of the D83A-DR sequence was performed by database searches against custom-made DR-D83A database using Biologs 3.0 (Bruker Daltonics) and an in-house MASCOT search engine. Search parameters were as follows: enzymatic cleavage, tryptic and semitryptic; fixed modifications, S-carboxamidomethylation of cysteine residues; variable modifications, 13C at one C-terminal oxygen and 15O at both C-terminal oxygen, methionine oxidation, S-carboxamidomethylated methionine, deamidation of Glu and Asp, dimethylamino and dinitrophenylation, and phosphorylation (Ser, Thr, and Tyr); missed cleavages, 2; MS tolerance, 100 ppm; and, MS/MS tolerance, 0.7 amu.

**Data Analysis** — To estimate the degrees of phosphorylation of specific peptides the ratio of 16O:18O components of the isotope clusters of the peptides of interest in mixed mock and dephosphorylated samples was quantitated. This was accomplished by calculating the theoretical isotope peak distribution of peptides of interest using the MS isotope pattern calculator (20). All calculations were performed as described by Bonenfant el al. (20). The equation to obtain the ratio R of mixed mock and dephosphorylated samples is as follows: 

$$R = I_0/(I_0 - V_1 \times I_0) + (I_2 - V_2 \times I_0)$$

where I0, I1, and I2 are the experimental peak intensities for 16O, 18O, and 16O2, respectively, and V1 and V2 are the calculated fractional intensities for 2 × 13C and 4 × 15C isopes of corresponding monoisotopes (20).

**RESULTS**

A full-length form of the murine DR was expressed with attached His6 and c-Myc affinity tags by stable transfection of mammalian cells. The cells were lysed in buffers containing protease and phosphatase inhibitors, and the product was subsequently isolated using metal ion affinity fractionation followed by electrophoresis of Coomassie-stained protein from SDS-PAGE bands of the appropriate molecular weight. The cells were grown without addition of xenobiotic ligands and with care to avoid detachment before disruption to avoid DR activation. Thus the isolated protein represented unstimulated or latent DR. Analysis of tryptic digests of the protein by CapHPLC-MALDI-TOF/TOF-MS and -MS/MS revealed two peptides that were not true tryptic peptides but could be identified as semitryptic peptides Glu409–Arg424 and Ser547–Arg555 in part by matching their masses with masses of the relevant regions of the D83A-DR sequence. In addition, MS/MS of these peptides revealed sequence tag matches for Glu409–Arg424 (PLPIR; supplemental Fig. 1SA) and Ser547–Arg555 (QNEEFFR; supplemental Fig. 1SB) when the experimental data were used to perform a database search against a database containing only the D83A-DR sequence. It was noteworthy that several other components of the digest with masses that did not match tryptic or semitryptic peptides of D83A-DR revealed the same sequence tags (Table I). These findings were suggestive of the existence of the peptides spanning Glu409–Arg424 and Ser547–Arg555 as multiple chemically modified forms in the latent murine DR.

**Characterization of Modifications Detected on the Peptide Spanning Residues Glu409–Arg424 of the Latent Dioxin Receptor** — Precursor ions that were detected at m/z = 1750.9, 1798.9, 1814.9, 1830.9, 1855.9, and 1894.9 exhibited distinct chromatographic retention times during CapHPLC-MALDI-TOF/TOF-MS, and CapHPLC-MALDI-TOF/TOF-MS/MS revealed the common sequence tag PIPRI.
The MS/MS spectrum of the peptide at \( m/z \) 1798.91\(^{+}\) displayed partial C- (\( y_1 \text{--} y_{15} \); \( y_8 \) was not observed) and N (\( b_1 \text{--} b_9 \)) sulfenic acid from methionine sulfoxide (50). This was indicative of neutral loss of methane-peptide (Fig. 1A). Mass differences of \(-48, +16, +32, +57, \text{and} +96 \text{ amu, respectively, were evident for the peptides observed at } m/z 1750.91^{+}, m/z 1814.91^{+}, m/z 1830.91^{+}, m/z 1855.91^{+}, \text{and} m/z 1894.91^{+} \text{ relative to the mass of the unmodified peptide observed at } m/z 1798.91^{+} \) (Table I).

A comparison of the MS/MS spectra of the precursor ions at \( m/z \) 1750.91\(^{+}\) and \( m/z \) 1798.91\(^{+}\) \((\text{Fig. 1, A and B})\) showed that the C-terminal ions \( y_1 \text{--} y_6 \) and the N-terminal ions \( b_2 \text{--} b_9 \) to \( b_9 \) \((b_1 \text{ was not observed in either spectrum, and} b_2 \text{ was not observed in Fig. 1A})\) were essentially identical for both precursors. However, the successively larger ions after \( y_6 \) and \( b_9 \) of the modified peptide \((\text{Fig. 1B})\) could only be assigned if allowance was made for a mass shift of \(-48 \text{ amu relative to the masses of } y_7 \text{ to } y_{15} \text{ and } b_{10} \text{ to } b_{13} \text{ and } b_{15} \) of the unmodified peptide \((\text{Fig. 1A})\). Presence of the mass shift of \(-48 \text{ amu on both the } y_7 \text{ and } b_{10} \text{ but not the } y_6 \text{ and } b_9 \text{ fragment ions of the modified peptide (Fig. 1B) indicated the degradation of Met}^{418}\).

The peptide observed at \( m/z \) 1814.91\(^{+}\) also produced \((\text{Fig. 1C})\) essentially identical C- and N-terminal ions up to \( y_6 \) and \( b_9 \) \((b_1 \text{ and } b_2 \text{ ions were not observed})\) as compared with the unmodified peptide at \( m/z \) 1798.91\(^{+}\) \((\text{Fig. 1A})\). However, successively larger fragment ions from \( y_9 \) and \( b_{11} \) \((y_7, y_9, \text{and} b_{10} \text{ were not observed})\) in the MS/MS spectrum of the modified peptide exhibited a mass shift of \(+16 \text{ amu (Fig. 1C) relative to the fragment ion series of the unmodified peptide (Fig. 1A). Unmodified fragment ions detected up to } y_6 \text{ and } b_9 \text{ in the MS/MS spectrum of the modified peptide (Fig. 1C) indicated that the mass shift of } +16 \text{ amu was located at Met}^{418}\). Moreover, a neutral loss of 64 amu from the parent ion at \( m/z \) 1814.91\(^{+}\) observed in the MS/MS spectrum of the modified peptide \((\text{Fig. 1C})\) was indicative of neutral loss of methanesulfenic acid from methionine sulfoxide (50).

The MS/MS spectrum of the peptide at \( m/z \) 1855.91\(^{+}\) \((\text{Fig. 1D})\) displayed essentially identical C- and N-terminal ions \( y_1 \text{ to } y_6 \) \((y_4 \text{ was not observed})\) and \( b_1 \text{ to } b_9 \) \((b_2 \text{ and } b_4 \text{ were not observed})\) as the unmodified peptide \((\text{Fig. 1A})\). However, ion series starting from \( y_7 \) of the variant peptide exhibited a mass shift of 57 amu as compared with the corresponding fragment ions of the unmodified peptide \((\text{Fig. 1D})\). The MS/MS spectrum of the modified peptide also displayed a neutral loss of 105 amu from the parent ion at \( m/z \) 1855.91\(^{+}\) \((\text{Fig. 1D})\). A mass shift of 57 amu for \( y_7 \) but not \( y_6 \) indicated that a modification was located at Met\(^{418}\) in the modified peptide. No modifications were evident to b ion series with either peptide, but none of the observed ions contained Met\(^{418}\). The magnitude of the mass increase associated with the modification on Met\(^{418}\) of \(+57 \text{ amu was consistent with alkylation with a carboxamidomethyl group.}

The MS/MS spectrum of the variant peptide at \( m/z \) 1830.91\(^{+}\) \((\text{Fig. 2B})\) displayed a continuous C-terminal fragment ion series up to \( y_9 \) \((y_1 \text{--} y_9 \text{ essentially identical to that } y_1 \text{--} y_9 \text{ of the unmodified peptide at } m/z 1798.91^{+} \) \((\text{Fig. 2A})\). However, the requirement to allow for a mass shift of \(-48 \text{ amu to enable alignment between } y_7 \text{ through } y_{13} \text{ fragments of the modified (Fig. 2B) and unmodified peptides (Fig. 2A) suggested a loss of 48 amu from Met}^{418} \text{ in the modified peptide. A mass shift of } +80 \text{ amu was observed for } b_9 \text{ through } b_9 \text{ fragment ions of the modified peptide (Fig. 2B) compared with the unmodified peptide, whereas essentially identical } m/z \text{ values were observed corresponding to } b_1 \text{ and } b_2 \text{ fragment ions of the modified and unmodified peptides. A dominant neutral loss of 80 amu was observed from the parent ion of the modified peptide (Fig. 2B) but not the unmodified peptide (Fig. 2A). Taken together, this information is indicative that the modified peptide contained a decomposed Met}\(^{418}\) and modification of Ser\(^{411}\) with sulfate or phosphate. The dominant neutral loss of 80 amu and the absence of a 98-amu neutral loss from the modified peptide favors the existence of a sulfate \((21)\).

The variant peptide at \( m/z \) 1894.91\(^{+}\) exhibited C- and N-terminal fragment ions from \( y_1 \text{ to } y_6 \) and \( b_1 \text{ to } b_4 \) \((\text{Fig. 2C})\) with essentially identical masses as those of the fragment ions of the unmodified peptide at \( m/z 1798.91^{+} \) \((\text{Fig. 2A})\). However, the \( y_7 \) and \( y_9 \) fragment ions of the modified peptide displayed a mass shift of \(+16 \text{ amu (Fig. 2C) relative to the } y_7 \text{ and } y_9 \text{ fragment ions of the unmodified peptide (Fig. 2A). Fragment ions } y_{11} \text{ and } y_{12} \text{ of the modified peptide (Fig. 2C) displayed a mass shift of } +96 \text{ amu compared with the corresponding fragment ions of the unmodified peptide (Fig. 2A). In addition,}

### Table I

| Characteristic | PLPIR | m/z* | QNEEFFR |
|---------------|-------|------|---------|
| Retention time (min) | 68.5 | 76 | 52.5 |
| ∆ mass (amu) | -48 | 16 | 16 |

a Values in parentheses are the theoretical \( m/z \) values for the proposed structures of the variant peptides.

b Calculated mass difference (\( \Delta \)) between the \( m/z \) values of the variant peptides and \( m/z \) values of the unmodified forms of D83A-DR peptides observed at \( m/z \) 1798.91\(^{+}\) and 1187.51\(^{+}\).
the N-terminal b7 but not b1 to b4 fragment ions of the modified peptide displayed a mass shift of +80 amu relative to the corresponding fragment ions of the unmodified peptide (Fig. 2A). Although the mass shifts of +16 amu of the y7 and y9 fragment ions of the modified peptide may have represented the presence of methionine sulfoxide at position 418, the mass shifts of +80 amu at b7, y11, and y12 fragment ions may have represented modification of Ser415 with a moiety of 80 amu. Neutral losses of 64, 80, 98, 144, and 162 amu were observed from the parent ion of the modified peptide at m/z 1894.91 (Fig. 2C) but not the unmodified peptide (Fig. 2A). The neutral loss of 64 amu is consistent with the loss of methanesulfenic acid from the presumptive methionine sulfoxide at position 418. Although the loss of 98 amu was consistent with loss of phosphoric acid from a phosphoserine, presumably at position 415, loss of 80 amu may represent loss of phosphate or sulfate (21, 36) from the same or another position. The larger neutral losses appear to represent combinations of the neutral losses discussed above as indicated by the labels on Fig. 2C.

Characterization of Modifications Detected on the Peptide Spanning Residues Ser547–Arg555 in the Latent Dioxin Receptor—Precursor ions that were detected at m/z 1139.5, 1187.5, 1203.5, 1219.5, and 1244.5 exhibited distinct chromatographic retention times during CapHPLC-MALDI-TOF/TOF-MS and -MS/MS but revealed the sequence tag QNEEFFR in common (Table I).

The MS/MS spectrum of the precursor ion at m/z 1187.51 displayed continuous C- and N-terminal fragment ion series that corresponded to y1 through y16 and b1 through b7 fragment ions of the unmodified sequence EISSPFSPIMDPLPIR (Fig. 3A). The modified peptides observed at m/z 1139.51, m/z
1203.51, m/z 1219.51, and m/z 1244.51 represented mass differences of −48, +16, +32, and +57 amu, respectively, relative to the unmodified peptide observed at m/z 1187.51 (Table I).

The MS/MS spectrum of the variant peptide at m/z 1139.51 (Fig. 3B), which represented a mass difference of −48 amu relative to the unmodified peptide (Table I), revealed C-terminal fragment ions y1 through y7 and the N-terminal b1 fragment ion that corresponded to the C- and N-terminal fragment ions (y1−y7 and b1) of the unmodified peptide (Fig. 3A). However, a mass shift of −48 amu was required to align y8 and b2 to b9 (b8 was not observed) fragment ions of the modified peptide (Fig. 3B) with those of the unmodified peptide (Fig. 3A). Based on these observations it was apparent that Met548 was present in a decomposed form in the variant peptide (Fig. 3B).

The modified peptide observed at m/z 1203.51 (Table I) produced a partial C-terminal fragment ion series, y1 to y7, and the N-terminal b1 fragment ion corresponding to y and b fragments of the sequence of EISSPFSPIMDPLIR are labeled on the spectra and denoted on the corresponding sequence insets. Mass differences between fragment ions of modified and unmodified sequences are indicated by dotted lines on the spectra of the modified ions. The profile of the precursor ion observed in MS-only mode for the unmodified peptide is shown as an inset (A). In all cases, 100 MS/MS shots were acquired in groups and summed to a total of 800 shots. The spectra presented in A and D was magnified 2× to clearly demonstrate low abundance fragment ions. u, amu. Asterisks indicate modified residues.

The modified peptide observed at m/z 1244.51 (Table I) produced essentially identical C-terminal y1 to y7 fragment ions and the
N-terminal b₁ fragment ion (Fig. 3D) as the unmodified peptide (Fig. 3A). Successively larger y series fragment ions were not observed for the modified peptide, and b₂ through b₉ (b₇ was not observed) fragment ions of the modified peptide (Fig. 3D) could only be aligned with those of the unmodified peptide (Fig. 3A) if an allowance was made for a mass shift of 57 amu on the fragment ions of the modified peptide (Fig. 3D). These findings indicated that Met548 of the modified peptide was alkylated with a carboxamidomethyl moiety.

Both the modified peptide of m/z 1219.51 (Fig. 4A) and the unmodified peptide of m/z 1187.51 (Fig. 4B) displayed essentially identical continuous C-terminal fragment ion series (y₁–y₇). Fragment ions (b₂–b₆ and b₉) of the modified peptide (Fig. 4A) showed a mass shift of +32 amu relative to the unmodified peptide (Fig. 4B). However, the fragment ion at y₉ in the MS/MS spectrum of the modified peptide (Fig. 4A) appeared with a mass shift of −48 amu relative to the unmodified peptide (Fig. 4B). The predominant neutral loss of 80 amu exhibited by the modified peptide (Fig. 4A) but not the unmodified peptide (Fig. 4B) was a strong indication of the presence of a sulfate moiety on the N-terminal serine of the modified peptide (21, 26). Consequently the mass shift of +32 amu as exhibited by the N-terminal sequence ions of the modified peptide (Fig. 4A) may have been the result of an increase in the mass value of +80 amu due to the presence of sulfate on the N-terminal serine residue and a mass deduction of −48 amu due to decomposition of methionine at position 548.

Differentiation between the Presence of Sulfate and Phosphate on Serines of Modified Peptides Spanning Residues Glu⁴₀⁹–Arg⁴₂⁴ and Ser⁵₄⁷–Arg⁵₅₅ of DR—CapHPLC-MALDI-TOF/TOF-MS and -MS/MS were performed on a sample com-
praising a tryptic digest of D83A-DR that was prepared in H$_2^{16}$O and further processed by a mock enzymatic dephosphorylation procedure without any addition of phosphatase enzymes subsequently mixed in a ratio of 1:1 with a tryptic digest of D83A-DR prepared in H$_2^{18}$O and not subjected to enzymatic dephosphorylation or the mock incubation. Another sample was also analyzed that differed only by way of treatment of the H$_2^{16}$O tryptic digest by enzymatic dephosphorylation prior to mixing 1:1 with the H$_2^{18}$O tryptic digest. The H$_2^{16}$O and H$_2^{18}$O digests were also separately subjected to the mock enzymatic dephosphorylation procedure and analyzed by CapHPLC–MALDI-TOF/TOF-MS and -MS/MS without any mixing.

The peptide at m/z 1814.7$^{+}$ corresponding to oxidized Met$^{118}$ was detected in all samples. The MALDI-TOF/TOF-MS spectrum of the ion at m/z 1814.7$^{+}$ in the mixed H$_2^{16}$O–H$_2^{18}$O sample prepared without phosphatase treatment of either digest component indicated an incorporation ratio of 1:1.04 $^{16}$O/$^{18}$O (Fig. 5A and Table II). A very similar pattern of incorporation of oxygen isotopes was observed by merging data obtained by running the mock-treated H$_2^{16}$O and H$_2^{18}$O digests separately (data not shown). However, the peptide ion at m/z 1814.9$^{+}$ was observed in the mixed sample that contained the phosphatase-treated H$_2^{16}$O digest with an isotopic cluster similar to the isotopic pattern obtained from the H$_2^{16}$O-only sample without phosphatase treatment. In this sample the $^{16}$O isotope-only peak abundance at m/z 1814.9$^{+}$ was substantially increased relative to $^{18}$O, and $^{18}$O$_2$ isotope abundances as apparent in the isotope peaks at m/z 1816.9$^{+}$ and m/z 1818.9$^{+}$ (Fig. 5B). The incorporation of $^{16}$O relative to $^{18}$O was apparent at a ratio of 1:0.38 (Table II). Calculation of the ratio of incorporation of $^{16}$O/$^{18}$O isotope peaks in the mixed samples with and without phosphatase treatment of the H$_2^{16}$O digest component revealed a 2.73-fold increase in the abundance of the $^{16}$O isotope peak upon phosphatase treatment. These data strongly suggested that this peptide existed in a form with both methionine sulfoxide and a phosphoserine residue prior to phosphatase treatment.

A similar analysis was performed for the ion at m/z 1894.0 corresponding to residues Glu$^{409}$–Arg$^{424}$ of D83A-DR presumed to have had methionine sulfoxide and phosphoserine at positions 418 and 415, respectively. This ion was observed in the mixed sample that was prepared without phosphatase treatment of either H$_2^{16}$O or H$_2^{18}$O digests together with ions corresponding to $^{15}$O incorporation (Fig. 5C). The isotope cluster of this ion indicated a ratio of 1:0.76 for $^{16}$O/$^{18}$O incorporation (Fig. 5C and Table II). The $^{16}$O component of this cluster was markedly depleted in the mixed sample that was prepared using the H$_2^{16}$O digest subjected to phosphatase treatment prior to mixing with the H$_2^{18}$O digest (Fig. 5D) resulting in an apparent ratio of $^{16}$O/$^{18}$O of 1:1.91 (Table II). This represented a decrease in the $^{16}$O component to 0.4 upon phosphatase treatment. These data indicated that the peptide represented by this ion contained a substantial phosphate content prior to phosphatase treatment and the possibility of a trace of sulfate that was resistant to phosphatase treatment. Removal of sulfate from the H$_2^{16}$O digest component probably accounted for the increase in the $^{16}$O component of the ion at m/z 1814.7$^{+}$ upon phosphatase treatment as described above (Fig. 5B). A component of sulfonation would account for the neutral loss of 80 amu from this peptide as described above (Fig. 2C).

By comparison, similar analyses of the variant peptide ion previously detected at m/z 1830.9$^{+}$ and interpreted to contain a modified Met$^{118}$, resulting in a mass decrease of 48 amu for certain fragment ions relative to the unmodified sequence,
and the presumptive presence of sulfate on Ser$^{411}$ failed to exhibit phosphatase sensitivity (Fig. 5F). The $^{16}$O:$^{18}$O ratio for the isotope cluster relating to this peptide was 1:1.3 for the sample prepared without phosphatase treatment of the H$_2^{16}$O digest component of the mixture (Fig. 5E and Table II). The $^{16}$O:$^{18}$O ratio for the corresponding isotope cluster of the sample in which the H$_2^{16}$O digest component was treated with phosphatase prior to mixing was also 1:1.3 (Fig. 5F and Table II). These observations strongly support the contention that this ion represents a modified form of residues Glu$^{409}$–Arg$^{424}$ with modified Met$^{418}$ and sulfonated Ser$^{411}$.

**Table II**

*Ratio of incorporation of $^{16}$O:$^{18}$O isotopes*

| Parent ion | $^{16}$O:$^{18}$O | -Fold change |
|-----------|-----------------|-------------|
|           | -ppase | +ppase | $R_{-ppase}/R_{+ppase}$ | $R_{+ppase}/R_{-ppase}$ |
| 1814.9$^{1+}$ | 1:1.04 | 1:0.38 | 2.73 | 0.36 |
| 1894.0$^{1+}$ | 1:0.76 | 1:1.91 | 0.4 | 2.5 |
| 1830.9$^{1+}$ | 1:1.3 | 1:1.3 | 1 | 1 |
| 1139.5$^{1+}$ | 1:1.4 | 1:1.38 | 1.01 | 0.98 |
| 1219.5$^{1+}$ | 1:1.3 | 1:1.3 | 1 | 1 |
The ion at \( m/z \) 1139.6\(^{11}\) was observed in both samples that were prepared by mixing \( \mathrm{H}_2\mathrm{O}^{16}\)O and \( \mathrm{H}_2\mathrm{O}^{18}\)O digests regardless of whether the \( \mathrm{H}_2\mathrm{O}^{16}\)O digest was treated with phosphatase (Fig. 6, A and B). The MS/MS spectra of both peptide ions suggested that they corresponded to the sequence spanning residues of D83A-DR with Met548 degraded by loss of 48 amu (Fig. 3B). The isotope clusters of this ion were not influenced by phosphatase treatment. A ratio of 1:1.4 was evident for the \( ^{16}\mathrm{O}^{18}\)O isotope cluster for the sample containing the \( \mathrm{H}_2\mathrm{O}^{16}\)O digest not pretreated with phosphatases (Fig. 6A and Table II), and a ratio of 1:1.38 was evident for the \( ^{16}\mathrm{O}^{18}\)O isotope cluster for the sample containing the \( \mathrm{H}_2\mathrm{O}^{16}\)O digest that was treated with phosphatase prior to mixing (Fig. 6B and Table II).

The variant form of the peptide spanning residues Ser547–Arg555 at \( m/z \) 1219.5\(^{11}\), which was previously observed in the unlabeled D83A-DR sample and interpreted as containing mass shifts of \( +80 \) amu at Ser547 and \( -48 \) amu at Met548 (Fig. 4A), was also observed at \( m/z \) 1219.5\(^{11}\) regardless of whether the \( \mathrm{H}_2\mathrm{O}^{16}\)O digest was treated with phosphatases prior to mixing with \( \mathrm{H}_2\mathrm{O}^{18}\)O digests (Fig. 6, C and D). The \( ^{16}\mathrm{O}^{18}\)O ratios of the isotope clusters observed in samples prepared without or with phosphatase treatment of the \( \mathrm{H}_2\mathrm{O}^{16}\)O digests were both 1:1.3 (Fig. 6, C and D, and Table II). These observations support the interpretation above that the ion at \( m/z \) 1219.5 rep-
represented a modified form of the sequence spanning residues of D83A-DR with a decomposed Met\(^{418}\), resulting in the loss of 48 amu relative to unmodified Met\(^{418}\), and sulfonated Ser\(^{547}\), resulting in a compensating gain of 80 amu to produce an ion of 32 amu higher in mass than the unmodified peptide.

**DISCUSSION**

It is believed that regulation of the transcriptional activity of the signal-activated bHLH/PAS transcription factor DR involves a variety of post-translational events (4, 7–13) For instance, previous studies indicated that phosphorylation of Ser\(^{38}\) and Ser\(^{48}\) is involved in regulating nucleocyttoplasmic shuttling of DR (12). We have previously reported PTM of D83A-DR at lysine position 87 by dimethylation (14). However, there is no other definitive information regarding PTM sites of DR. Discovery of the PTMs presented herein arose from our studies aimed at comprehensively defining the post-translational regulation of DR.

Detection of two groups of peptides from DR that displayed common de novo sequence tags during CapHPLC–MALDI-TOF/TOF-MS and -MS/MS of a tryptic digest of D83A-DR prompted a detailed investigation of these peptides. All of the peptides were observed at distinct chromatographic retention times (Table I) and appeared to reflect “hot spots” of modifications between residues Glu409 and Arg424 (EISSPFSPIMDPLPIR) and between residues Ser547 and Arg555 (SMQNEEFFFR) of the DR sequence. Several peptides displayed modifications on methionine residues Met\(^{418}\) and Met\(^{448}\). Mass shifts of −48, +16, and 57 amu were observed on both methionine residues (Figs. 1, B–D, and 3, B–D). In some instances, additional modifications by moieties of +80 amu on serine residues were also observed (Figs. 2, B and C, and 4A) on the peptides.

The peptides at m/z 1814.91\(^{+}\) and m/z 1203.51\(^{+}\), which showed a mass increment of +16 amu on Met\(^{418}\) and Met\(^{448}\) respectively, as compared with their corresponding unmodified forms (Figs. 1C and 3C), indicated conversion of methionine to methionine sulfoxide, which is a commonly observed phenomenon during storage and sample handling (50). A characteristic neutral loss of 64 amu was observed from both of these peptides due to the elimination of methanesulfenic acid (CH\(_3\)SOH, −64 amu) as an indicator for the presence of oxidized methionine (50).

MALDI-TOF/TOF-MS/MS spectra also revealed that methionines 418 and 548 in the peptides at m/z 1855.91\(^{+}\) and m/z 1244.51\(^{+}\) were modified by +57 amu (Figs. 1D and 3D), which suggested the presence of S-carboxamidomethylated methionine (mass difference, +57 amu) resulting from use of iodoacetamide when modifying cysteinyl residues to prevent the formation of disulfide bonds (51, 52). This was supported by the observation that both peptides modified by +57 amu displayed neutral losses of 105 amu from their respective parent ions (Figs. 1D and 3D) that may be attributed to loss of a carboxamido group, a sulfur atom, a methyl group, and one hydrogen atom from the modified methionine (52). In addition, MS/MS spectra of distinct peptides at m/z 1750.91\(^{+}\) and m/z 1139.51\(^{+}\) (Figs. 1B and 3B) had a difference in mass of −48 amu compared with the corresponding unmodified sequences that could possibly have arisen as a consequence of chemical decomposition of S-carboxamidomethylated methionine. This is consistent with observations that peptides containing S-carboxamidomethylated methionine may display chemical degradation during HPLC and mass spectrometric analysis due to exposure to acid resulting in elimination of S-methyl, S-carboxymethylsulfonium side chain and concomitant dehydration leading to a mass difference of −48 amu relative to the unmodified methionine (51). The methionine decomposition observed in this study apparently occurred prior to HPLC and/or MS analyses as the relevant peptides eluted at chromatographically distinct times relative to their S-carboxamidomethylated counterparts.

The mass shift of 32 amu observed for the peptides at m/z 1830.91\(^{+}\) (unmodified m/z 1798.91\(^{+}\)) (Fig. 2B) and m/z 1219.51\(^{+}\) (unmodified m/z 1187.51\(^{+}\)) (Fig. 4A) could potentially be explained by a double oxidation event at the methionine residues present in the sequences. However, the MS/MS spectra of these variant peptides produced y series fragment ions at −48 amu compared with y series ions corresponding to the unmodified sequences along with a prominent neutral loss of 80 amu from the parent ion (Figs. 2B and 4A). These observations indicated that these peptides contained a modification consistent with the chemical degradation product of S-carboxamidomethyl methionine and another modification representing an actual post-translational event of +80 amu. The MS/MS spectra also revealed mass shifts of +80 amu on serine residues 411 and 547 (Figs. 2B and 4A). Thus, the mass shifts of 32 amu on the modified peptides resulted from post-translationally modified serine residues present in the peptides with accompanying chemically degraded methionines (51, 52) rather than the presence of methionine sulfone. Various previous studies have reported that phosphorylated peptides preferentially display a neutral loss of 98 amu, whereas sulfonated serine residues exhibit a loss of a sulfono moiety (−80 amu) as a favored dissociation process (21, 26, 53–55). The dominant neutral losses of 80 amu associated with these peptides indicated that serine residues 411 and 547 were sulfonated.

A peptide ion at m/z 1894.91\(^{+}\) was also detected with a modification of +80 amu on serine at position 415 in a peptide that also contained methionine sulfoxide at position 418 (Fig. 2C). Apart from the neutral loss of methanesulfenic acid (−64 amu), predominant neutral losses of 98 and 162 amu (98 + 64 amu) were seen, and neutral losses of 80 and 144 amu (80 + 64 amu) were also evident in the MS/MS spectrum to a lesser extent (Fig. 2C). The neutral loss of 98 amu from this peptide indicated that serine 415 was certainly phosphorylated. However, the indication of a neutral
loss of 80 amu from this peptide ion may indicate that serine 415 or serines 411 or 412 were also sulfonated.

The observation of sulfonated serine residues on proteins isolated from a biological context has only been reported once previously (21). The evidence for discrete sulfonation of serines 411 and 547 and the possibility that another serine in the peptide spanning Glu^409–Arg^424 was also sulfonated in DR was based largely on the observation of the neutral loss of 80 amu from relevant peptide ions. Thus, we deemed it highly desirable to confirm the presence of sulfate on serines 411 and 547 and to establish the likelihood of additional serine sulfonation in the peptide spanning residues Glu^409–Arg^424 using an orthogonal analytical technique. Two possible approaches were assessed as sources of the confirmatory data. These were high mass accuracy MS and phosphatase sensitivity.

The mass accuracy required to differentiate between phosphorylation and sulfonation (phosphate addition = 79.9663; sulfate addition = 79.9568) of the peptides of interest in this study was between 4.96 and 7.71 ppm. However, mass accuracy achieved by MALDI-TOF/TOF-MS (10–47 ppm) was not sufficient to reliably distinguish between these possibilities. Unfortunately attempts at high mass accuracy measurements of the peptides of interest at m/z 1894.91+, m/z 1830.91+, and m/z 1219.51+ using CapHPLC-ESI-LTQ-Orbi-trap-FT-MS and -MS/MS were not successful due to failure to detect the relevant ions presumably because of inefficient ionization of these peptides under ESI conditions.

Phosphatase sensitivity measurements in combination with differential stable isotope labeling by proteolysis in H_2^{18}O, however, did provide data supporting the presence of sulfonation of serines 411 and 547 and phosphorylation as the substantive modification of serine 415 as the sources of +80 amu observed on peptides in this study. This strategy emanated from a separate larger study in which the efficacies of different stable isotope labeling reagents were assessed for their suitability for use in conjunction with phosphatase treatment (56).

Essentially the increase in the relative abundance of the ^16O isotope peak at m/z 1814.91+, Glu^409–Arg^424 (+16 amu) (Fig. 5B), after enzymatic dephosphorylation of the H_2^{16}O digest component prior to mixing with the H_2^{18}O digest for CapHPLC MALDI-TOF/TOF-MS and -MS/MS as compared with the corresponding isotopic peak in a sample in which neither digest component of the mixture was phosphatase-treated (Fig. 5A) strongly supported the contention that Ser^415 was phosphorylated prior to phosphatase treatment. This increase could only have occurred if a phosphate had been removed from the Glu^409–Arg^424 (+16 and +80 amu) component of the H_2^{16}O digest. The ^16O isotope peak of the peptide at m/z 1894.91+, Glu^409–Arg^424 (+16 and +80 amu), showed a concomitant depletion of the ^16O component upon phosphatase treatment of ~2.51-fold (Table II, R_{ppase} / R_{ppase}) relative to the ^16O component present in the tryptic digest (Fig. 5D).

Although these data substantiated post-translational phosphorylation of Ser^415, the presence of a subpopulation of peptides containing sulfonated serine cannot be ruled out based on the detection of a ^16O component resistant to enzymatic dephosphorylation in the phosphatase-treated H_2^{16}O digest (Fig. 5D). This is consistent with observation of a neutral loss of 80 amu described above for the ion at m/z 1894.9, representing Glu^409–Arg^424 (+16 and +80 amu), and the report that such a neutral loss is most likely to be a consequence of the presence of a sulfonated serine or threonine (21). The purity of the ^18O water used in this study was specified by the supplier to be ≥97%, which may be another factor that introduced a response into the ^16O channel from the heavy sample. Consequently the ^16O component present in the phosphatase-treated sample of the peptide at m/z 1894.91+ may be a combination of both factors described above.

The ^16O components of the peptides at m/z 1830.91+ and m/z 1219.51+, interpreted as containing a modification of 80 amu on Ser^411 and Ser^547, respectively, with accompanying decomposition of methionine side chains, were impervious to phosphatase treatment (Figs. 5, E and F, and 6, C and D), and the ^16O: ^18O ratios of the isotope profiles were identical in the data obtained on samples with and without phosphatase treatment (Table II). Isotopic profiles of the unmodified variant of m/z 1219.51+ at m/z 1139.61+ (Fig. 6, A and B) present in the mock and dephosphorylated samples also displayed identical ^16O: ^18O ratios of ~1:1.4 (Table II). Taken together, the data strongly indicated that serum residues at positions 411 and 547 are post-translationally modified by sulfonation.

In this study we have demonstrated the likely presence of phosphorylation and sulfonation of DR using tandem mass spectrometry. These observations were strongly supported using an ^16O/^18O labeling approach in combination with selective phosphatase treatment. This approach was used previously with phosphopeptide-enriched samples (20, 38) for relative quantitation of phosphorylation. However, the use of phosphopeptide enrichment prior to phosphatase treatment precludes differentiation between phosphorylation and sulfonation. In contrast, the technique used in the present study has a potential to provide a comprehensive survey of all the phosphopeptides that may be present in a complex sample without any need for prior enrichment of phosphopeptides (38). Furthermore it offers the distinct advantage of using enzymatic specificity to distinguish between phosphorylation and sulfonation. We demonstrated this by differentiating between these modifications as the causes of the mass increment of +80 amu at Ser^411, Ser^415, and Ser^547 of DR (Figs. 2, 4, 5, and 6). A potential limitation that may have affected the outcome of application of this strategy to DR could have been sequence context-specific resistance of phosphoserine residues to phosphatase treatment. However, the strategy used a combination of Antarctic and λ-phosphatases to minimize this possibility. Furthermore such a limitation has not been evident.
from extensive studies with phosphopeptides from ovalbumin and β-, α1- and α2-caseins.2

We have previously suggested the possibility of a post-translational coding system that may regulate diverse roles of dioxin receptor in both xenobiotic as well as developmental functions (14). Identification of phosphorylation at serine 415 and sulfonation at 411 and 547 raises a unique scenario where proximal serine residues in the same peptide are subjected to two different post-translational events. These post-translational events occur in the vicinity of a domain of DR responsible for transactivation. Moreover hyper-reactivity of methionines in these regions suggests that the protein may be folded in such a way whereby these residues are exposed to the external environment. Numerous analyses conducted by us on D83A-DR samples have detected only one other methionine residue (Met342), detected in only one other analysis, with apparent S-carboxymethylation. However, methionines at position 418 and 548 were consistently found to be modified by an apparent S-carboxamidomethylation in the peptides that contained serines 411, 415, and 547 in all analyses performed on the latent dioxin receptor. The possibility that the post-translationally modified serine residues contribute to the chemical reactivity of the methionines in their vicinity is a feature of the chemistry of DR that warrants further investigation. We have described here for the first time two sulfonated motifs of the dioxin receptor. This study is only a second description of O-sulfonation of serine residues in eukaryotic proteins (21). Interestingly one of these serines was in close sequence juxtaposition to another serine that was post-translationally phosphorylated, and these modifications appeared to occur as alternates as they were not found together on the same peptide. The possibility that they did occur on the same peptide that was not detected cannot be ruled out especially in view of the anticipated lability and potentially poor ionization properties of such a peptide. Mutagenesis of the above mentioned serine and methionine residues is underway to unravel the implications of these post-translational events to the biology of DR. Further studies to assess whether or not occurrence of modifications of these residues of DR is affected by exposing cells to conditions that activate the receptor will shed further light on the relevance of the modifications described herein to the biological function of DR.

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REFERENCES

1. Barouki, R., Coumoul, X., and Fernandez-Salgueiro, P. M. (2007) The aryl hydrocarbon receptor, more than a xenobiotic-interacting protein. FEBS Lett. 581, 3608–3615

2. Gu, Y. Z., Hogenesch, J. B., and Bradfield, C. A. (2000) The PAS superfamily: sensors of environmental and developmental signals. Annu. Rev. Pharmacol. Toxicol. 40, 519–561

3. Furness, S. G., Lees, M. J., and Whitehead, M. L. (2007) The dioxin (aryl hydrocarbon) receptor as a model for adaptive responses of bHLH/PAS transcription factors. FEBS Lett. 581, 3616–3625

4. Backlund, M., and Ingelman-Sundberg, M. (2005) Regulation of aryl hydrocarbon receptor signal transduction by protein tyrosine kinases. Cell Signal. 17, 39–48

5. Mahon, M. J., and Gasiewicz, T. A. (1996) Ah receptor phosphorylation: localization of phosphorylation sites to the C-terminal half of the protein. Arch. Biochem. Biophys. 316, 166–174

6. Matsumura, F. (1994) How important is the protein phosphorylation pathway in the toxic expression of dioxin-type chemicals? Biochem. Pharmacol. 48, 215–224

7. Missavage, G. D., Park, S. K., and Gasiewicz, T. A. (2004) The aryl hydrocarbon receptor (AhR) tyrosine 9, a residue that is essential for AhR DNA binding activity, is not a phosphoserine but augments AhR phosphorylation. J. Biol. Chem. 279, 20582–20593

8. Park, S., Henry, E. C., and Gasiewicz, T. A. (2000) Regulation of DNA binding activity of the ligand-activated aryl hydrocarbon receptor by tyrosine phosphorylation. Arch. Biochem. Biophys. 381, 302–312

9. Pongratz, I., Stromstedt, P. E., Mason, G. G., and Poellinger, L. (1991) Inhibition of the specific DNA binding activity of the dioxin receptor by phosphatase treatment. J. Biol. Chem. 266, 16813–16817

10. Singh, S. S., and Perdew, G. H. (1993) Alterations in the Ah receptor level after staurosporine treatment. Arch. Biochem. Biophys. 305, 170–175

11. Ikuta, T., Eguchi, H., Tachibana, T., Yoneda, Y., and Kawajiri, K. (1998) Nuclear localization and export signals of the human aryl hydrocarbon receptor. J. Biol. Chem. 273, 2985–2990

12. Ikuta, T., Kobayashi, Y., and Kawajiri, K. (2004) Phosphorylation of nuclear localization signal inhibits the ligand-dependent nuclear import of aryl hydrocarbon receptor. Biochem. Biophys. Res. Commun. 317, 545–550

13. Ikuta, T., Kobayashi, Y., and Kawajiri, K. (2004) Cell density regulates intracellular localization of aryl hydrocarbon receptor. J. Biol. Chem. 279, 19209–19216

14. Dave, K. A., Hamilton, B. R., Wallis, T. P., Furness, S. G. B., Whitelaw, M. L., and Gorman, J. J. (2007) Identification of N,N-dimethyl-lysin in the murine dioxin receptor using MALDI-TOF/TOF- and ESI-LTQ-Orbitrap FT-MS. Int. J. Mass Spectrom. 268, 168–180

15. Witze, E. S., Old, W. M., Resing, K. A., and Ahn, N. G. (2007) Mapping protein post-translational modifications with mass spectrometry. Nat. Methods 4, 796–806

16. Gruler, A., Olsen, J. V., Mohammed, S., Mortensen, P., Faergeman, O., Mann, M., and Jensen, O. N. (2005) Quantitative phosphoproteomics applied to the yeast phenome signaling pathway. Mol. Cell Proteomics 4, 310–327

17. Ibarrola, N., Kalume, D. E., Gronborg, M., Iwahori, A., and Pandey, A. (2003) A proteomic approach for quantitation of phosphorylation using stable isotope labeling in cell culture. Anal. Chem. 75, 6043–6049

18. Smith, J. C., and Figeys, D. (2008) Recent developments in mass spectrometry-based quantitative phosphoproteomics. Biochem. Cell Biol. 86, 137–148

19. Steen, H., Jebanathirajah, A. J., Springer, M., and Kirschner, M. W. (2005) Stable isotope-free relative and absolute quantitation of protein phosphorylation stoichiometry by MS. Proc. Natl. Acad. Sci. U.S.A. 102, 3948–3953

20. Bonenfant, D., Schmelze, T., Jacinto, E., Crespo, J. L., Mini, T., Hall, M. N., and Jenoe, P. (2003) Quantitation of changes in protein phosphorylation: a simple method based on stable isotope labeling and mass spectrometry. Proc. Natl. Acad. Sci. U.S.A. 100, 880–885

21. Medzhiradskyz, K. F., Darula, Z., Persson, E., Fainzliber, M., Chalkley, R. J., Ball, H., Greenbaum, D., Bogoy, M., Tyson, D. R., Bradshaw, R. A., and
Burlingame, A. L. (2004) O-Sulfonation of serine and threonine: mass spectrometric detection and characterization of a new posttranslational modification in diverse proteins throughout the eukaryotes. Mol. Cell. Proteomics 3, 429–440

22. Beisserwinder, R., Corbell, D., Vannier, C., Thiele, C., Dohrmann, U., Kellner, R., Ashman, K., Niehrs, C., and Huttner, W. B. (1998) Existence of distinct tyrosylprotein sulfotransferase genes: molecular characterization of tyrosylprotein sulfotransferase-2. Proc. Natl. Acad. Sci. U. S. A. 95, 11134–11139

23. Huttner, W. B. (1984) Determination and occurrence of tyrosine O-sulfate in proteins. Methods Enzymol. 107, 200–223

24. Kehoe, J. W., and Bertozzi, C. R. (2000) Tyrosine sulfation: a modulator of extracellular protein-protein interactions. Chem. Biol. 7, R57–R61

25. Moore, K. L. (2003) The biology and enzymology of protein tyrosine O-sulfation. J. Biol. Chem. 278, 24243–24246

26. Nemeth-Cawley, J. F., Karnik, S., and Rouse, J. C. (2001) Analysis of sulfated peptides using positive electrospray ionization tandem mass spectrometry. J. Mass Spectrom. 36, 1301–1311

27. Hu, G., Noll, R. J., Li, H., Makarov, A., Hardman, M., and Graham Cooks, R. (2005) The Orbitrap: a new mass spectrometer. J. Mass Spectrom. 40, 430–443

28. Olsen, J. V., de Godoy, L. M., Li, G., Macek, B., Mortensen, P., Pesch, R., Makarov, A., Lange, O., Honning, S., and Mann, M. (2005) Parts per million mass accuracy on an Orbitrap mass spectrometer via lock mass injection into a C-trap. Mol. Cell. Proteomics 4, 2010–2021

29. Wisniewski, J. R., Zougman, A., Krueger, S., and Mann, M. (2006) Mass spectrometric mapping of linker histone H1 variants reveals multiple acetylations, methylations and phosphorylation as well as differences between cell culture and tissue. Mol. Cell. Proteomics 6, 72–87

30. Kast, J., Parker, C. E., van der Drift, K., Dial, J. M., Milgram, S. L., Wilm, M., Moore, K. L. (2003) The biology and enzymology of protein tyrosine O-sulfation. J. Biol. Chem. 278, 24243–24246

31. Yang, Y., Zhang, S., Howe, K., Wilson, D. B., Moser, F., Irwin, D., and Regnier, F. (2000) Strategy for qualitative and quantitative analysis in proteomics based on signature peptides. J. Chromatogr. B Biomed. Sci. Appl. 745, 197–210

32. Gorman, J. J., Wallis, T. P., and Pitt, J. J. (2002) Protein disulfide bond determination by mass spectrometry. Mass Spectrom. Rev. 21, 183–216

33. Rose, K., Savoy, L. A., Simona, M. G., Offord, R. E., and Wingfield, P. (1988) C-terminal peptide identification by fast atom bombardment mass spectrometry. Biochim. J. 250, 253–259

34. Wallis, T. P., Huang, C. Y., Nimkar, S. B., Young, P. R., and Gorman, J. J. (2004) Determination of the disulfide bond arrangement of dengue virus NS1 protein. J. Biol. Chem. 279, 20729–20741

35. Wallis, T. P., Pitt, J. J., and Gorman, J. J. (2001) Identification of disulfide-linked peptides by isotope profiles produced by peptic digestion of proteins in 50% 18O water. Protein Sci. 10, 2251–2271

36. Huttner, W. B. (1984) Determination and occurrence of tyrosine O-sulfate in proteins. Methods Enzymol. 107, 200–223

37. Stewart, I. I., Thomson, T., and Figeys, D. (2001) 18O labeling: a tool for proteomics. Rapid Commun. Mass Spectrom. 15, 2456–2465

38. Lees, M. J., and Whitehead, M. L. (1999) Multiple roles of ligand in transforming the dioxin receptor to an active basic helix-loop-helix/PAS transcription factor complex with the nuclear protein Amt. Mol. Cell. Biol. 19, 5811–5822

39. Gorman, J. J. (1987) Fluorescent labeling of cysteine residues to facilitate electrophoretic isolation of proteins suitable for amino-terminal sequence analysis. Anal. Chem. 59, 2157–2213

40. Jensen, O. N. (2002) Phosphopeptide detection and sequencing by MALDI-TOF, ESI-QTOF, and ESI-ion trap mass spectrometers. J. Am. Soc. Mass Spectrom. 14, 704–718

41. Jones, M. D., Merewether, L. A., Clogston, C. L., and Lu, H. S. (1994) Identification of oxidized methionine in peptides. Rapid Commun. Mass Spectrom. 10, 1905–1910

42. Jones, M. D., Merewether, L. A., Clogston, C. L., and Lu, H. S. (1994) Peptide map analysis of recombinant human granulocyte colony stimulating factor: elimination of methionine modification and nonspecific cleavages, Anal. Biochem. 216, 135–146

43. Lapko, V. N., Smith, D. L., and Smith, J. B. (2000) Identification of an artifact comprised mass spectrometry of proteins derivatized with iodoacetamide. J. Mass Spectrom. 35, 572–575

44. Liao, P. C., Leykam, J., Andrews, P. C., Gage, D. A., and Allison, J. (1994) An approach to locate phosphorylation sites in a phosphoprotein: mass mapping by combining specific enzymatic degradation with matrix-assisted laser desorption/ionization mass spectrometry. Anal. Chem. 66, 3413–3421

45. Waller, C., Chu, F., Ball, H., Wolfender, F., Finzilber, M., Baldwin, M. A., and Burlingame, A. L. (1999) Identification of tyrosine sulfation in Conus pennaceus conotoxins α-PnIA and α-PnIB: further investigation of labile sulfo- and phosphopeptides by electrospray, matrix-assisted laser desorption/ionization (MALDI) and atmospheric pressure MALDI mass spectrometry. J. Mass Spectrom. 34, 447–454

46. Bennett, K. L., Stensballe, A., Podtelezhnikov, A. V., Moniatte, M., and Jensen, O. N. (2002) Phosphopeptide detection and sequencing by matrix-assisted laser desorption/ionization quadrupole time-of-flight tandem mass spectrometry. J. Mass Spectrom. 37, 179–190

47. Gorman, J. J., Wallis, T. P., Dave, K. A., Hamilton, B. R., Headlam, M. J., Linke, S., and Peet, D. J. (2007) Quantitative approaches for analysis of regulatory post-translational modifications, in Eighth International Symposium on Mass Spectrometry in the Health & Life Sciences: Molecular and Cellular Proteomics, San Francisco, August 19–23, 2007 (Bradshaw, R. A., ed) University of California, San Francisco