

# Residue-specific Mass Signatures for the Efficient Detection of Protein Modifications by Mass Spectrometry

Haining Zhu,<sup>†,‡</sup> Thomas C. Hunter,<sup>†,‡</sup> Songqin Pan,<sup>†</sup> Peter M. Yau,<sup>§</sup> E. Morton Bradbury,<sup>§,||</sup> and Xian Chen<sup>\*,†</sup>

C-ACS, MS M888, Chemistry Division, and BN-2, Bioscience Division, Los Alamos National Laboratory, Los Alamos, New Mexico 87544, and Department of Biological Chemistry, School of Medicine, University of California at Davis, Davis, California 95616

**Currently available mass spectrometric (MS) techniques lack specificity in identifying protein modifications because molecular mass is the only parameter used to characterize these changes. Consequently, the suspected modified peptides are subjected to tandem MS/MS sequencing that may demand more time and sample. We report the use of stable isotope-enriched amino acids as residue-specific “mass signatures” for the rapid and sensitive detection of protein modifications directly from the peptide mass map (PMM) without enrichment of the modified peptides. These mass signatures are easily recognized through their characteristic spectral patterns and provide fingerprints for peptides containing the same content of specific amino acid residue(s) in a PMM. Without the need for tandem MS/MS sequencing, a peptide and its modified form(s) can readily be identified through their identical fingerprints, regardless of the nature of modifications. In this report, we demonstrate this strategy for the detection of methionine oxidation and protein phosphorylation. More interestingly, the phosphorylation of a histone protein, H2A.X, obtained from human skin fibroblast cells, was effectively identified in response to low-dose radiation. In general, this strategy of residue-specific mass tagging should be applicable to other posttranslational modifications.**

Posttranslational protein modifications such as phosphorylation and methionine oxidation are essential in regulating many cellular functions.<sup>1–6</sup> Phosphorylation is one of the most important protein modifications and plays critical roles in signal transduction

pathways.<sup>1–3</sup> Methionine residues are readily oxidized by biological oxidants, such as hydrogen peroxide and superoxide, and this oxidation is thought to be involved in biological processes such as mutagenesis, carcinogenesis, and aging.<sup>4–6</sup>

The precise identification and quantification of these modifications are major tasks for high-throughput proteomics.<sup>7,8</sup> The covalent modification of an amino acid residue in a protein increases its molecular mass, and this mass change can be determined by mass spectrometry (MS). Peptide mass mapping (PMM), the commonly used MS-based method, measures  $m/z$  values of proteolytic peptides of proteins and can be used to screen for modified peptides according to the predicted mass changes.<sup>8,9</sup> Frequently, protein modifications are misidentified because of peptide mass degeneracy, occurrence of genetic variants, low instrumental mass accuracy, signal artifacts, or contaminations.<sup>10,11</sup>

Tandem MS/MS sequencing of proteolytic peptides is subsequently required for the reliable assignment of modified residues.<sup>12–17</sup> Postsource decay (PSD) experiments in MALDI-TOF MS can determine the partial sequences of certain peptide ions.<sup>15</sup> Alternatively, using electrospray ionization (ESI) MS, peptide ions can be individually selected for collision-induced dissociation (CID) to generate daughter spectra for peptide sequencing.<sup>13–17</sup> However, to obtain high-quality MS/MS spectra for unambiguous de novo peptide sequencing, large amounts of the precursor ions may be required. Because most of the modified proteins are of low abundance, additional steps to enrich modified

\* To whom correspondence should be addressed: (tel) 505-665-3197; (fax) 505-665-3024; (e-mail) chen\_xian@lanl.gov.

<sup>†</sup> Chemistry Division, Los Alamos National Laboratory.

<sup>‡</sup> Authors contributed equally to this work.

<sup>§</sup> University of California at Davis.

<sup>||</sup> Bioscience Division, Los Alamos National Laboratory.

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peptides then become necessary.<sup>13,14</sup> Further, the number of putative peptides is enormous for higher eukaryotes, thus reducing the effectiveness of the MS/MS strategy to determine peptide modifications in a high-throughput manner. Also, ESI mass spectra are complex because each peptide in a mixture is present in multiply charged states. Thus, for large proteins, or complexes, or mixtures, sample-consuming chromatographic separation is essential for the unambiguous assignment of the large numbers of proteolytic peptides from different proteins. All these difficulties make it challenging to perform high-throughput identification of modified peptides by tandem MS/MS-based methods.

Previously we reported the use of residue-specific mass tagging with stable isotopes for high-throughput protein identifications.<sup>18,19</sup> Here, this residue-specific mass-tagging strategy is readily adapted to the rapid and efficient identification of protein modifications, e.g., methionine-specific oxidation, and the phosphorylation of serine (Ser), tyrosine (Tyr), and threonine (Thr) residues. Thus, "heavy" methionine-*S*-methyl-*d*<sub>3</sub> (Met-*d*<sub>3</sub>) and serine-2,3,3-*d*<sub>3</sub> (Ser-*d*<sub>3</sub>) residues can be used as mass tags to identify their corresponding modifications. These mass tags allow for the selective retrieval of Met-*d*<sub>3</sub>, or Ser-*d*<sub>3</sub>-tagged peptides from a peptide mixture through their characteristic mass-split patterns. Because of the specificity of particular mass-tagged signals, naturally occurring protein modifications can be identified directly from a PMM with high confidence without either pre-enrichment of modified peptides or tandem MS/MS sequencing.

In this report, we have unambiguously identified methionine oxidations in a PMM through the recognition of Met-*d*<sub>3</sub> tags in methionine-containing peptides. Ser-*d*<sub>3</sub> tags have been used to label serine residues to follow an *in vitro* phosphorylation of a GST-fusion protein containing a protein kinase A site. Furthermore, this technique has been successfully employed for the sensitive detection of a rapid cellular response to ionizing radiation (IR), i.e., the low-dose radiation-induced phosphorylation. Histone H2A.X, a replacement histone, is found in the chromatin of all tissues and developmental stages so far examined.<sup>20</sup> Similar to other histones, H2A.X contains multiple sites for several post-translational modifications.<sup>21</sup> It has been shown that H2A.X is rapidly phosphorylated following the induction of DNA double-strand breaks (DSBs) by exposure to IR and this response is independent of other DNA repair pathways.<sup>22</sup> There are several hypotheses suggesting that the IR-induced phosphorylation of H2A.X may serve as a signal to recruit protein complexes involved in the repair of DNA DSBs. Another possible role of H2A.X phosphorylation is to restructure chromatin into the conformation required for DNA repair.<sup>23</sup> To precisely identify the IR-induced phosphorylation of histone H2A.X, we have studied the exposure of human skin fibroblast (HSF) to IR using the mass-tagging approach.

## MATERIALS AND METHODS

**Chemicals.** Stable isotope-enriched amino acid precursors, L-methionine-*S*-99.9%-*d*<sub>3</sub> (Met-*d*<sub>3</sub>), and D,L-serine-2,3,3-99.9%-*d*<sub>3</sub> (Ser-*d*<sub>3</sub>) were purchased from Isotec Inc. (Miamisburg, OH). Unlabeled amino acids, protease inhibitors, and  $\alpha$ -cyano-4-hydroxycinnamic acid were obtained from Sigma (St. Louis, MO). Sequencing-grade modified trypsin and micrococcal nuclease were purchased from Roche Diagnostics (Indianapolis, IN). *Saccharomyces cerevisiae* strains and diploid human skin fibroblasts (strain 55) were obtained from the American Type Culture Collection (Manassas, VA). The  $\alpha$ -minimal essential medium ( $\alpha$ -MEM), fetal bovine serum, and streptomycin sulfate for HSF cell culture were purchased from Life Technology (Rockville, MD). Precast SDS polyacrylamide gels and nylon membrane were obtained from Invitrogen (Carlsbad, CA). ECL western blotting kit and protein A Sepharose beads were purchased from Amersham Pharmacia Biotech (Piscataway, NJ).

### Residue-Specific Labeling of Yeast Cells and Sample

**Preparation.** Yeast cells were inoculated into 10 mL of synthetic complete (SC) medium<sup>19</sup> and incubated overnight. The SC medium consists of 0.67% yeast nitrogen base containing all the essential vitamins, salts, trace metal elements, 0.5% ammonium sulfate, 2% dextrose, and a mixture of 20 amino acids. The yeast cells from the overnight culture were diluted to a starting optical density (OD) of 0.1 in 100 mL of the SC medium supplement with the labeled amino acid precursors, such as Met-*d*<sub>3</sub> (200 mg/L) or Ser-*d*<sub>3</sub> (500 mg/L). The labeled and unlabeled amino acid precursors can be mixed in different ratios and added to the SC medium with other unlabeled amino acids to produce 0, 50, and 100% residue-specifically labeled proteins, respectively. Cells were harvested during log phase and washed twice with millipore H<sub>2</sub>O (Millipore Corp. Bedford, MA) to remove excess medium. The cells were then resuspended in 10 mM Tris HCl (pH 8), 10 mM dithiothreitol, 10 mM EDTA, 100 mM NaCl, 0.1% sodium dodecyl sulfate (SDS), and 5 mg/mL protease inhibitors including leupeptin, pepstatin A, and chemostatin A (Sigma). The cell lysate was prepared by vortexing with glass beads for 10 min at 4 °C followed by centrifugation at 14 000 rpm for 10 min at 4 °C to clarify the sample. The denatured cell lysates were then separated using polyacrylamide SDS gels and stained using Coomassie stain G-250.

**Protein Digestion and Peptide Extraction.** Coomassie-stained gel slices were destained with acetonitrile and 50 mM NH<sub>4</sub>HCO<sub>3</sub> at 1:1 ratio and were then dried using a speed-vacuum centrifuge. Trypsin was added in a final concentration of 10  $\mu$ g/mL, and the mixture was incubated overnight at 37 °C. The gel slices were extracted with 50% acetonitrile/5% acetic acid for 45 min, and the supernatant was collected and lyophilized.

**In Vitro Phosphorylation/Dephosphorylation.** The pGEX-2TK vector (Pharmacia), encoding a GST fusion protein containing a protein kinase A (PKA) site, was transformed into *Escherichia coli* BL21 cells. The M9 minimum media with and without Ser-*d*<sub>3</sub> precursors were used for cell growth. Because of the unique metabolic pathways for the synthesis of amino acids in *E. coli* cells, isotopic scrambling can occur to some extent during the labeling of nonessential amino acids including serine residues. Thus, it is necessary for the labeled amino acid precursors to be much more concentrated than the other amino acids in the medium for residue-specific labeling. The protein

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expression was induced by 1 mM IPTG for 1 h at 37 °C. After sonication of the IPTG-induced cell pellets, the GST protein was purified by the glutathione-affinity agarose beads (Pharmacia). The purified GST protein was then treated with various units of PKA before the proteolytic digestion with thrombin or trypsin. The proteolytic digests were desalted using C<sub>18</sub> ZipTips before MS analysis.

**Residue-Specific Labeling of HSF Cells and Radiation Treatment.** *Mycoplasma*-free HSF cells from passage 7 were cultured at 37 °C under 5% CO<sub>2</sub>–95% air in the  $\alpha$ -MEM supplemented with dialyzed fetal bovine serum, 100  $\mu$ g/mL streptomycin sulfate, and 100 units/mL penicillin. During the final period of 12 h, the cells were cultured in a modified  $\alpha$ -MEM supplemented with a mixture of 100 mg/L L-serine and 100 mg/L L-serine-*d*<sub>3</sub>. Subconfluent, proliferating cultures were irradiated with  $\gamma$ -ray using a Mark I model 68A high-dose-rate <sup>137</sup>Cs Source Chamber Irradiator (J. L. Shepherd and Associates). Cells were irradiated with 0.5 Gy of  $\gamma$ -rays at a rate of 0.42 Gy/min. Cells were harvested 20 min after irradiation using trypsin. The cell pellet was washed once with phosphate-buffered saline (PBS) at 4 °C and kept frozen in a methanol–dry ice bath at –70 °C until further use.

**Nucleosome Isolation from HSF Cells.** Nucleosomes were isolated from the control and irradiated HSF cells using the method of Imai et al.<sup>24</sup> with minor modifications. Briefly, cells were lysed in buffer A (10 mM Tris-Cl, pH 8.0, 1 mM CaCl<sub>2</sub>, 1.5 mM MgCl<sub>2</sub>, 0.2 mM PMSF, 0.25 M sucrose) by douching on ice. Crude nuclei were extracted from the cellular lysate by centrifugation through buffer B (buffer A plus 0.8 M sucrose) at 1000*g* at 4 °C. Crude nuclei were washed with buffer A several times and checked by microscopy until all cytoplasmic contaminants were removed. The washed nuclei were then resuspended in micrococcal nuclease (MNase) digestion buffer (10 mM Tris-Cl, pH 8.0, 1.5 mM MgCl<sub>2</sub>, 1.0 mM CaCl<sub>2</sub>, 0.25 M sucrose) to an OD<sub>260</sub> of 1.0 and warmed to 37 °C. Digestion was carried out with 25 units of MNase for 30 min at 37 °C and then stopped by the addition of 5 mM EGTA. The digest was incubated on ice for 20 min. Mononucleosomes were purified by ultracentrifugation through a linear sucrose gradient (10–25% in buffer A) at 100000*g* for 16 h at 4 °C.

**Western Analysis of Mononucleosome.** The purified mononucleosomes (20  $\mu$ g) were resolved on 18% SDS polyacrylamide gels and transferred to a nylon membrane using Semi-phor semidry blot apparatus (Hoefer Scientific Instruments, San Francisco, CA). Ponceau S staining of the membranes ensured that equal amounts of protein were loaded and transferred in all lanes. Membranes were blocked for 2 h at room temperature using 2% BSA, phosphate-buffered saline (BPBS). Proteins were detected using the antibody against either intact H2A.X or phosphorylated H2A.X at a 1:5000 dilution for 12 h at 4 °C. The antibody against phosphorylated H2A.X is specific for the C-terminal phosphorylation of Ser139. Horseradish peroxidase secondary antibody was used at a 1:10 000 dilution and bands were visualized using chemiluminescence reagents.

**Immunoprecipitation of Intact and Phosphorylated H2A.X.** Mononucleosomes (100  $\mu$ g) were resuspended in PBS and precleared by adding protein A Sepharose beads for 1 h at room

temperature. Mononucleosomes purified from control and irradiated cells were incubated with the antibody against H2A.X or phosphorylated H2A.X, respectively, for 4 h at 4 °C. Protein–antibody conjugates were precipitated by protein A Sepharose at 4 °C for 2 h. The precipitate was washed three times with PBS containing 0.1% Tween-20, followed by three times washes with PBS. The washed samples were resuspended in a 2 $\times$  protein loading buffer, boiled for 10 min, and subjected to 18% SDS–PAGE for protein separation. The protein bands were visualized by silver stain, and the band corresponding to H2A.X was cut out of the gel. Trypsin digestion and peptide extraction were carried out as described earlier. The tryptic peptides from intact and phosphorylated H2A.X were then analyzed by MALDI-TOF MS.

**Sample Preparation and Mass Spectrometric Measurements.** All gel extracts were resuspended in a 0.1% TFA solution and further desalted using C<sub>18</sub> ZipTips (Millipore Corp). One microliter of desalted sample was mixed with 1  $\mu$ L of a saturated solution (10 mg/mL) of  $\alpha$ -cyano-4-hydroxycinnamic acid (Sigma) which was prepared by dissolving 10 mg in 1 mL of a 1:1 solution of acetonitrile and 0.1% TFA. All MS experiments were carried out on a PE Voyager DE-STR Biospectrometry workstation equipped with a N<sub>2</sub> laser (337 nm, 3-ns pulse width, 20-Hz repetition rate) in both linear and reflectron modes (PE Biosystems, Framingham, MA). The mass spectra of proteolytic digests were acquired in the reflectron mode with delayed extraction (DE). The *m/z* values of proteolytic peptides were calibrated externally with Calmix 1 (PE Biosystems).

## RESULTS

**Identification of Methionine Oxidation Using Met-*d*<sub>3</sub> Mass Tags in a PMM.** The tryptic PMM of a one-dimensional (1D) gel band excised from the 50% Met-*d*<sub>3</sub>-labeled yeast cells is shown in Figure 1. Mass-split doublets of 3 Da at *m/z* values of 1750.82, 2246.98, and 2263.05 Da show that these peptides contain a single methionine residue.

In the tryptic PMM, two peptide signals at 2246.98 and 2263.05 Da, each with identical 3-Da mass-split patterns, are separated by ~16 Da, which is equivalent to the mass of an oxygen atom. This observation suggests that the peptide at 2263.05 Da is the oxidized form of the Met-*d*<sub>3</sub>-tagged peptide at 2246.98 Da. Further, a signal without a mass split at *m/z* of 2199.12 Da is found in the mass spectra of both unlabeled (Figure 1b) and 50% Met-*d*<sub>3</sub>-labeled samples. This peak at 2198.80 Da is ~67 mass units from the Met-*d*<sub>3</sub>-oxidized peptide (the Met-*d*<sub>3</sub>-labeled M<sup>+</sup> ion at 2266.16 Da, Figure 1a) and 64 Da from the Met-oxidized peptide (the unlabeled M<sup>+</sup> ion at 2262.86 Da, Figure 1b), respectively. Previously, a cleavage pathway showing that an oxidized methionine can lose a methanesulfenic acid group (CH<sub>3</sub>SOH) after the formation of a methionine sulfoxide had been reported for peptides containing methionine sulfoxides from tandem MS/MS-based studies such as fast atom bombardment, MALDI ion trap, triple quadrupole, and Q-TOF instruments.<sup>10,25</sup> Because the methyl group of a Met-*d*<sub>3</sub> precursor carries the 3-Da label, the removal of CH<sub>3</sub>SOH or CD<sub>3</sub>SOH results in the loss of the 3-Da mass-split pattern for the corresponding Met-containing peptides. Thus, the peptide at 2199.12 or 2198.80 Da results from the 64- or 67-Da loss of CH<sub>3</sub>-

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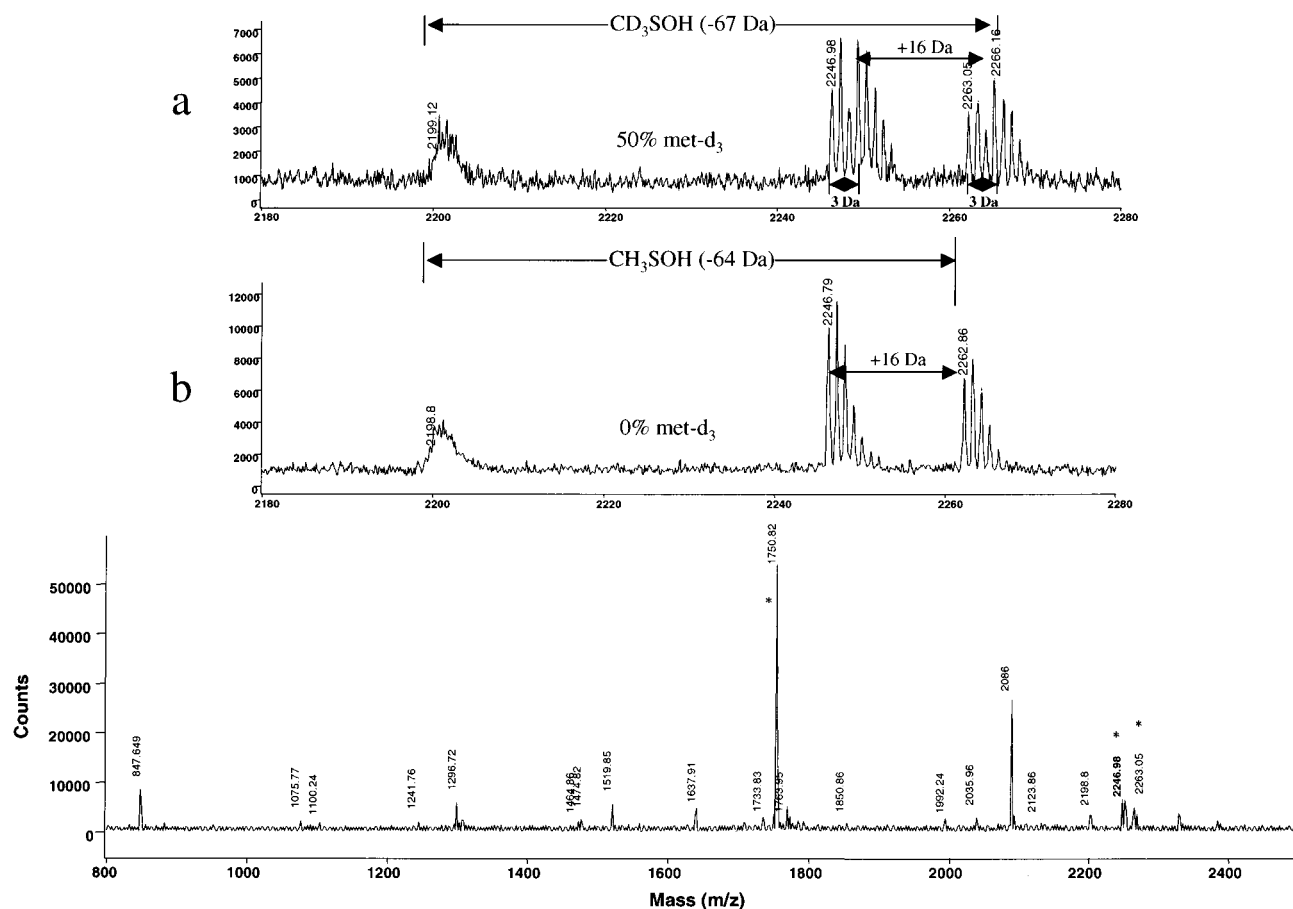


Figure 1. MALDI-TOF MS PMM of an in-gel tryptic digestion of a 1D SDS-PAGE gel band obtained from yeast cells labeled with 50% Met- $d_3$ . Asterisks indicate those peptides containing one methionine as evidenced by the 3-Da mass-split pattern. A Met- $d_3$  generates a 3-Da mass increase for each methionine because  $^2\text{H(D)}$  labels in the methyl group of a methionine residue. The expanded spectra, which show the characteristic pattern for methionine oxidation and the subsequent loss of methanesulfenic acid, were obtained from (a) yeast cells labeled with 50% Met- $d_3$ , (b) the unlabeled cell culture.

SOH or  $\text{CD}_3\text{SOH}$  from either the unlabeled or labeled peptide ions, respectively. This result therefore suggests that the loss of methanesulfenic acid can also occur on the time scale of a MALDI-TOF experiment. The methyl- $d_3$  group of the methionine residue acts as an internal marker and provides direct evidence for this dissociation process. Presumably, low-energy pathways induce metastable fragmentations in the free-drift region during the MALDI ionization process and proton transfers from the acidic matrix to peptides further facilitate the cleavage. Here, Met- $d_3$  labels were used to trace a series of Met-containing signals arising from methionine oxidation (+16 Da) and the subsequent loss of methane sulfenic acid (−64 Da). As a result, this characteristic pattern associated with methionine oxidation can be used as an additional constraint in database searching of MALDI-TOF PMMs. Further, the relative intensity ratio of the unmodified to the oxidized Met-containing peptide signals allows for quantitation of methionine oxidation.

The  $m/z$  values of the peptides were submitted to the MS-FIT website for protein search using the NCBIInR database and 200 ppm error tolerance. The detailed procedure using Met-containing peptides as an extra search constrain can be found in our earlier publication.<sup>19</sup> The Met-tagged tryptic peptide at 1750.82 ( $\text{M}^+$ ) was identified as (K)DFLQLCM\*NIDPNEK(M) in the first-round search, and its theoretical mass was then used as an internal

calibrant for recalibrating the PMM. The major component of the 1D gel band was identified as asparagine synthetase 2 (Asn2) with a 21% sequence coverage of the recalibrated PMM. However, no match was found for the peptide pair at 2246.98 and 2263.05 Da. A second proteolytic digestion was performed on the same gel band using Asp-N protease and the same protein, Asn2, was identified. From the examination of the Asn2 protein sequence, the Met-tagged peptide at 2246.98 Da was matched to the peptide sequence (K)KDRIVAARDPIGVVTLYM\*GR(S) with two tryptic sites remaining uncleaved in the tryptic digestion (asterisk denotes a methionine oxidation site). In this case, the use of a different protease for “orthogonal” digestion of the same protein band increases the sequence coverage of the PMM in the putative identification. This correlation of both the tryptic and Asp-N digests lends high confidence to the identification of Asn2, and this is supported by the characteristic 3-Da mass-split patterns of Met- $d_3$ -containing peptides.

**Residue-Specific Labeling: A Mass Signature for Phosphopeptides.** Here, we describe the use of Ser- $d_5$  mass tags for tracking the formation of phosphoserine peptide(s) in an in vitro phosphorylation/dephosphorylation model system. The pGEX-2TK plasmid encodes a GST protein with a cAMP-dependent protein kinase A (PKA) site fused to its C-terminus. Thrombin protease can cleave the *E. coli* expressed fused protein to yield a

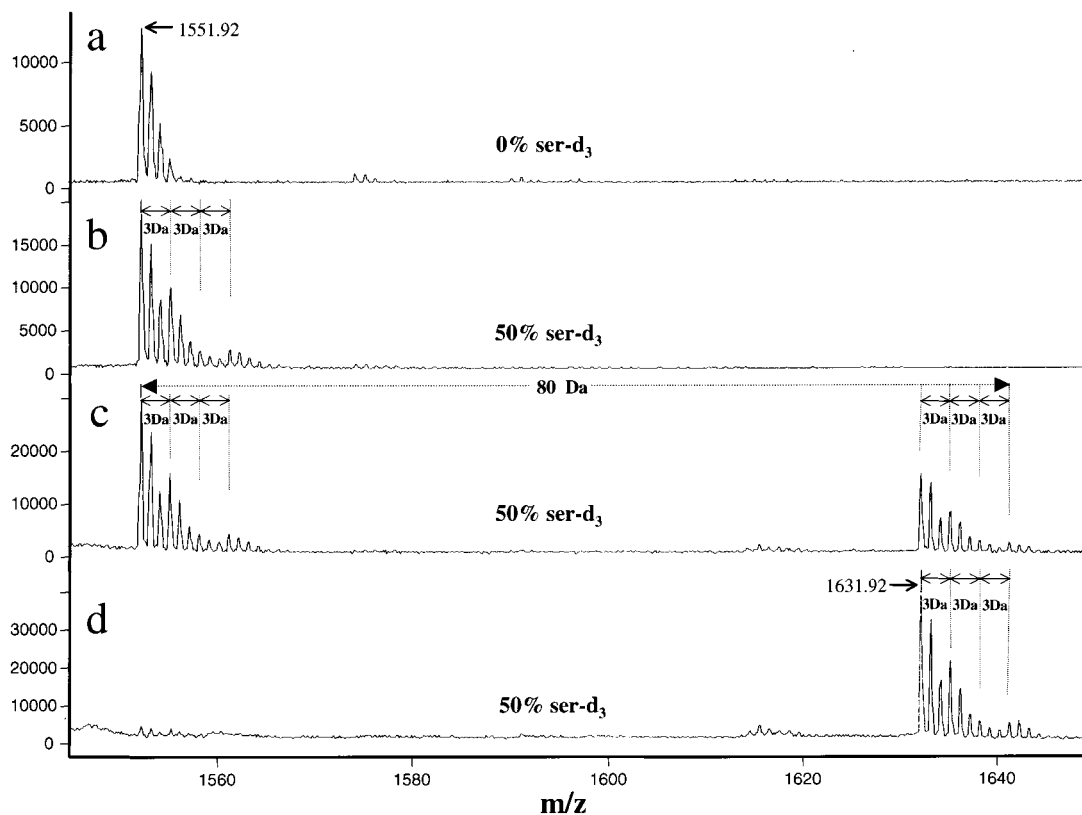


Figure 2. MALDI-TOF MS spectra of the thrombin-cleaved peptide, GSRRAS\*VGSPGIHRD, obtained from (a) the GST protein expressed in the unlabeled M9 medium, (b) the GST protein expressed in the 50% Ser- $d_3$ -labeled M9 medium, (c) the 50% Ser- $d_3$ -labeled-GST protein phosphorylated by PKA (1 unit/ $\mu$ g of protein), and (d) the 50% Ser- $d_3$ -labeled-GST protein phosphorylated by PKA (5 units/ $\mu$ g of protein). Each 3-Da increment for a monoisotopic peak set corresponds to a Ser- $d_3$  residue.

peptide with the sequence GSRRAS\*VGSPGIHRD (asterisk denotes the PKA phosphorylation site). The labeled precursor, Ser-2,3,3- $d_3$ , was used to label all serine residues. Figure 2a shows the monoisotopic distribution of the peptide at  $m/z$  of 1551.92 Da ( $M^+$  ion,  $M$  refers to the mass of the most abundant isotope) with the natural abundance of isotopes,  $M^+:(M+1)^+:(M+2)^+:(M+3)^+$ . In contrast, the mass-split pattern of the 50% Ser- $d_3$ -labeled peptide displays a 9-Da spacing between the unlabeled and the fully Ser- $d_3$ -labeled monoisotopic peak set (Figure 2b). With the known 3-Da mass tag for each Ser- $d_3$  precursor, the 9-Da mass-split pattern reveals a content of three serines in this peptide. As shown in Figure 2c, following treatment of the Ser- $d_3$ -labeled GST with PKA (1 unit of protease/ $\mu$ g of protein), thrombin digestion of the phosphorylated protein released the fused peptide at 1631.92 Da with the same 9-Da mass split but heavier by 80 Da than the peptide at 1551.92 Da. With increasing amounts of PKA, the intensity of the peptide peak at 1631.92 Da increases in parallel with the disappearance of the original peptide signal at 1551.92 (Figure 2d). Furthermore, following alkaline phosphatase treatment of the peptide at 1631.92 Da, the mass spectrum as shown in Figure 2b is recovered. Clearly, this 80-Da mass increase corresponds to the addition of a phosphate group ( $HPO_3$ ) to the thrombin-released peptide to generate the phosphoserine peptide at 1631.92 Da. Importantly, the characteristic mass-split pattern induced by Ser- $d_3$  labels provides a signature to identify those peptides with the same serine content in mass spectra during the course of phosphorylation or dephosphorylation. These mass-split patterns are directly correlated with the serine content of pro-

teolytic peptides and remain the same for those peptides with the identical amino acid composition regardless of peptide modifications.

**Direct Identification of Phosphopeptides Through Ser- $d_3$  Mass Tags in a Tryptic PMM.** To demonstrate the use of Ser- $d_3$  tags for the direct identification of phosphoserine peptide(s) in a PMM, we have analyzed a pool of tryptic peptides from the 50% Ser- $d_3$ -enriched GST protein following PKA phosphorylation (Figure 3a). Among other tryptic peptides displaying normal monoisotopic distribution (expanded views of Figure 3a), both peaks at 1251.72 and 1331.73 Da (Figure 3b) gave identical 6-Da mass-split patterns from two Ser- $d_3$  residues. This pair of peaks, separated by 80 mass units, is assigned to the peptide RAS\*VGSPGIHRD and its phosphorylated form. Besides the match of the mass shift due to the modification, the finding that these peptides contain the same number of serine residues greatly increases confidence in the assignment of the upper mass peak set to a phosphopeptide. Furthermore, alkaline phosphatase treatment of these peptides resulted in the disappearance of the higher mass peak, which unambiguously verified our assignment.

**Rapid Detection of a Phosphoserine Site in a Replacement Histone Protein H2A.X in Response to Low-Dose IR.** We have employed the serine-specific labeling strategy to analyze in situ the very early response of HSF cells to low-dose IR. The results of this study are summarized in Figure 4. Western analysis of HSF nuclear extracts using an anti-phospho-H2A.X antibody shows that the H2A.X protein is phosphorylated within 10 min of

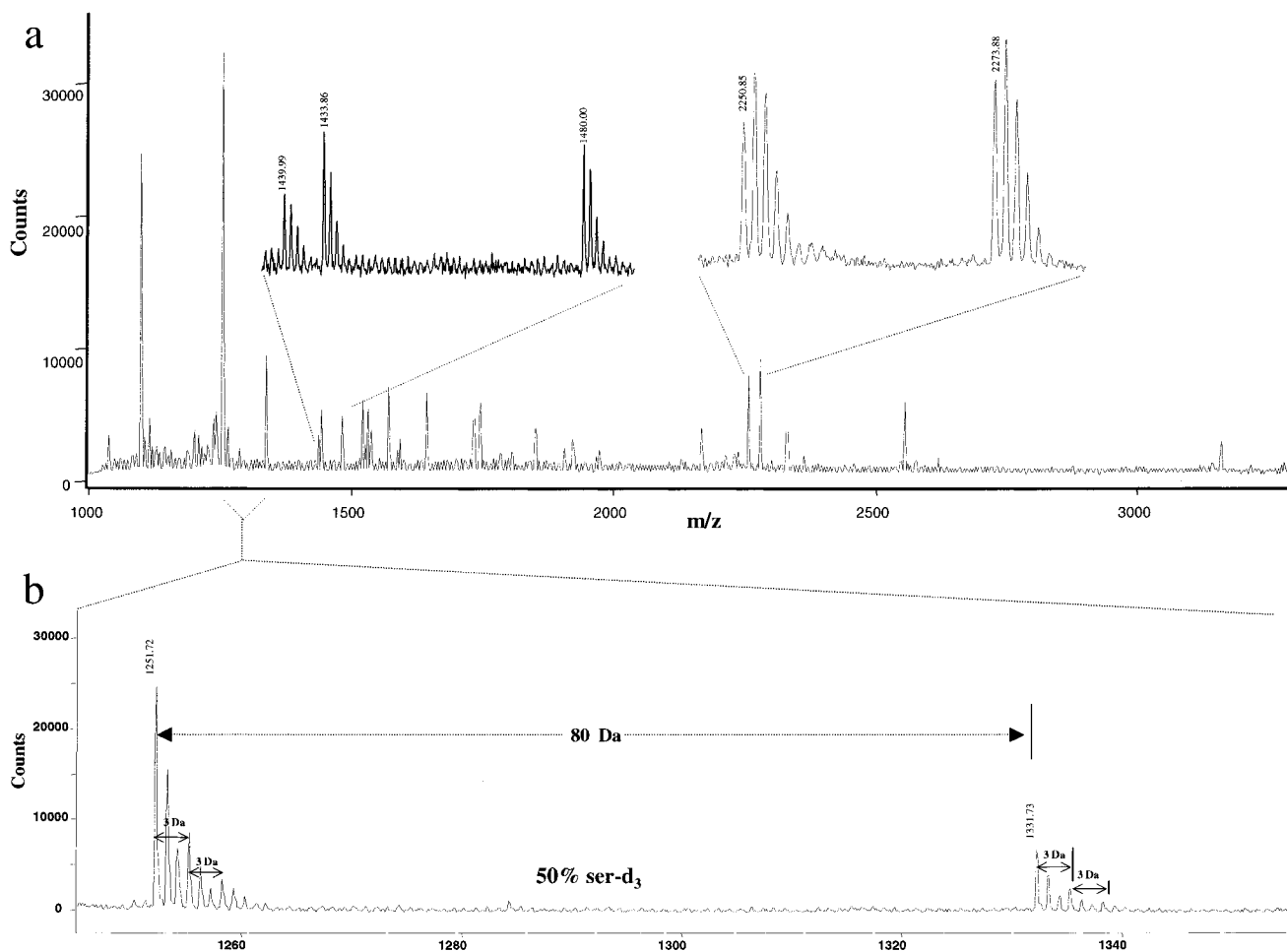


Figure 3. MALDI-TOF MS PMM of (a) the tryptic digestion of the PKA-phosphorylated GST protein labeled with 50% Ser- $d_3$ . The monoisotopic distribution patterns for the tryptic peptides at  $m/z$  values of 1439.99, 1433.86, 1480.00, 2250.85, and 2273.88 Da that contain no serine residues are shown in the expanded spectra. (b) A pair of peptides at  $m/z$  values of 1251.72 and 1331.73 Da having the identical 6-Da mass-split pattern corresponds to the unmodified and phosphorylated peptide RAS\*VGSPGIHRD.

exposure to 0.5-Gy  $\gamma$ -radiation (Figure 4a). The H2A.X protein and its phosphorylated form were immunoprecipitated from control and irradiated HSF cells using antibodies specific for the native or phosphorylated form of H2A.X, respectively. Immunoprecipitated proteins were separated using an 18% SDS-PAGE gel. The H2A.X protein band was then subjected to an in-gel digestion with trypsin, and its MALDI-TOF MS PMM is shown in Figure 4b. Comparison of the PMM of the H2A.X protein obtained from the control (Figure 4c I) and the irradiated (Figure 4c II) HSF cells shows a new peak at 1105.46 Da, an 80-Da mass increase from the signal at 1025.52 Da upon 0.5-Gy radiation. Under the same experimental condition, the H2A.X PMM obtained from the irradiated HSF cells grown in the presence of Ser- $d_3$  labels reveal that both peaks at 1025.52 and 1105.46 Da contain a serine residue through their characteristic 3-Da mass-split pattern while other tryptic fragments display the normal isotopic pattern. The data search for the fragment with an  $m/z$  value of 1025.52 Da corresponds to the H2A.X tryptic peptide sequence of  $^{134}\text{KATQASQEY}^{142}$ . The peak at 1105.46 Da can be assigned to its phosphorylated form as  $^{134}\text{KATQASpQEY}^{142}$ . The sensitive response of H2A.X to radiation through the phosphorylation was rapidly detected by our tagging method.

## DISCUSSION

Our stable isotope labeling strategy is highly specific for detecting mass-tagged peptides with particular types of amino acid residues and characterizes the peptides not only by their  $m/z$  values but also by the content of the particular residues. The distinctive isotope distribution pattern of tagged peptides allows them to be easily distinguished from other peptides in a mixture. This residue-specific labeling approach has been validated for *E. coli*, yeast, and human cells. Without demanding ultrahigh mass accuracy, commercially available mass spectrometers such as the PE Voyager DE-STR MALDI-TOF have sufficient mass resolution to resolve monoisotopic distributions for the accurate determinations of incorporated mass signatures.

This strategy represents a convenient approach to obtain the molecular signatures of proteins, thus allowing modified peptides to be identified directly in a PMM through a single MS experiment. For example, phosphopeptides of interest often have masses that are the same as, or extremely close to, other unrelated, unphosphorylated proteolytic peptides. Such mass degeneracy becomes more pronounced with the increasing molecular mass of a protein or the number of protein species in a complex or mixture. Further, the phosphorylation of a peptide can result in

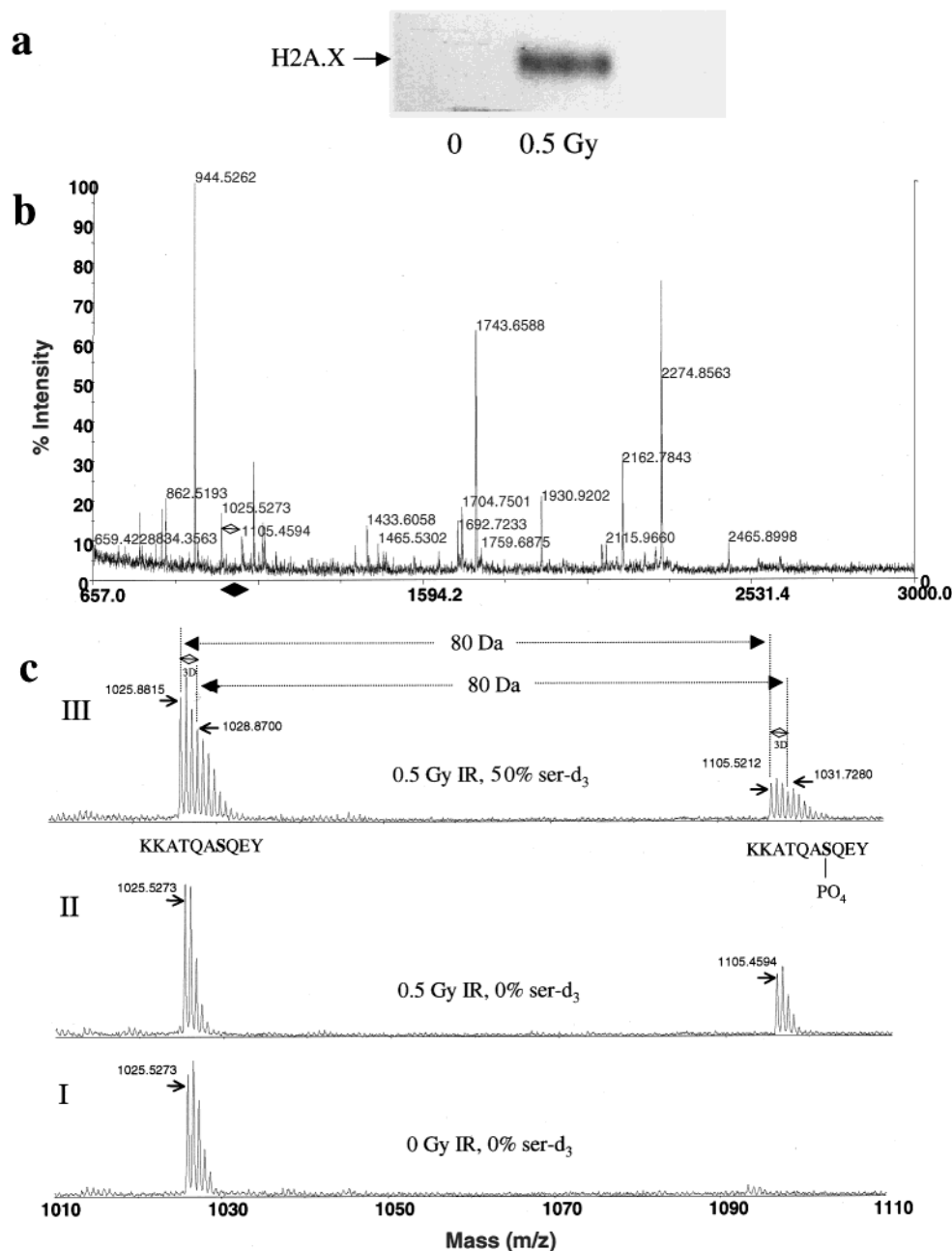


Figure 4. (a) Western analysis of HSF cell nuclear extracts with the anti-phospho-H2A.X antibody. Lane 1 is from control cells while lane 2 is from cells exposed to 0.5-Gy  $\gamma$ -radiation; (b) PMM of immunoprecipitated phospho-H2A.X from irradiated HSF cells. The peak with  $m/z$  value of 1025.5273 corresponds to the tryptic peptide  $^{133}\text{KKATQASQEY}^{142}$  while the peptide with  $m/z$  1105.4594 is tentatively assigned as the phosphorylated form of  $^{133}\text{KKATQASQEY}^{142}$ . The PMM was initially calibrated with the trypsin autocleaved peptide at 2273.52 Da; (c) expanded MALDI-TOF spectra of peptides in the range of  $m/z$  985–1150. The peptides were from (I) unlabeled control cells, (II) unlabeled cells exposed to 0.5-Gy  $\gamma$ -radiation, and (III) 50% Ser- $d_3$ -labeled cells exposed to 0.5-Gy  $\gamma$ -radiation.

mass increases of either 80 (+HPO<sub>3</sub>) or 98 Da (+H<sub>3</sub>PO<sub>4</sub>). The mass tagging of particular amino acid residues involved in possible modifications gives characteristic mass-split signatures that can resolve immediately these ambiguities in a large pool of peptides.

Because the modified peptides can be directly detected in PMM, both LC separation and MS/MS fragmentation steps, which need more samples, can be eliminated. In this regard, our method is relatively less time and sample consuming than an LC/MS/MS setup. In most cases, more intense precursor signals are needed to obtain high-quality MS/MS spectra for unambiguous peptide sequencing whereas our method does not require further

fragmentation of those precursor ions containing modifications. Most importantly, many modified peptides are present in low abundance in mass spectra making it very difficult, if not impossible, to analyze them by tandem MS/MS fragmentation. Using the above strategy, low-abundance peptides carrying amino acid-specific signatures can be recognized and their relationship with the parent peptides can be established in the presence of other abundant peptides. Therefore, the sensitivity and efficiency of the detection of modified peptides can be dramatically improved in our "one-step" PMM analysis of total proteolytic digests without the need for HPLC separation and tandem MS/MS sequencing.

Because this is the first demonstration of our labeling method, alkaline phosphatase digestion was used as precaution to show the accuracy of our detection of phosphopeptides. This demonstrates that the assignment of modifications using our labeling method has high confidence and does not require further verification through alkaline phosphatase treatment. It is a common practice to verify the results from a new approach with additional experiments using existing methods. Moreover, the phosphatase treatment has another important role, i.e., we can demonstrate that the unique isotope distribution pattern has been conserved during both the phosphorylation and dephosphorylation. This is critical because the conservation of isotope distribution pattern underlines the universal application of this labeling technique to any other kinds of protein modifications.

Here, using the serine-tagging technique, we have demonstrated the rapid detection of the phosphorylation of histone H2A.X which may be one of the initial responses to IR-induced DNA double-strand breaks. The phosphorylation of H2A.X is assigned based on two observations in a single PMM: a pair of peaks with

the same mass-split pattern and a mass separation corresponding to the phosphorylation. The combination of both observations greatly increases confidence in the assignment that the higher mass peptide is the phosphorylated form of the lower mass peptide. In addition, mass tagging provides a general and effective approach for studies of other posttranslational modifications.

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