Anal. Chem. **2002,** *74,* 607−616

Phosphoprotein Isotope-Coded Affinity Tags: Application to the Enrichment and Identification of Low-Abundance Phosphoproteins

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The use of a phosphoprotein isotope-coded affinity tag (PhIAT), which employs differential isotopic labeling and biotinylation, has been shown capable of enriching and identifying mixtures of low-abundance phosphopeptides. A denatured solution of *β*-casein was labeled using the PhIAT method, and after proteolytic digestion, the labeled peptides were isolated using immobilized avidin. The recovered peptides were separated by capillary reversed-phase liquid chromatography and identified by tandem mass spectrometry. PhIAT-labeled peptides corresponding to known O-phosphorylated peptides from *β*-casein were identified along with the phosphorylated peptides from α_{S1}-casein and α_{S2}-casein, known low-level (<5%) contaminants of commercially available *β*-casein. All of the casein-phosphorylated residues identified by the present PhIAT approach correspond to previously documented sites of phosphorylation. The results illustrate the efficacy of the PhIAT-labeling strategy to not only enrich mixtures for phosphopeptides but also, more importantly, permit the detection and identification of low-level phosphopeptides. In addition, the differences in the phosphorylation state could be determined between phosphopeptides in comparative samples by stoichiometric conversion using the light and heavy isotopic versions of the PhIAT reagents. Overall, our results exemplify the application of the PhIAT approach and demonstrate its utility for proteome-wide phosphoprotein identification and quantitation.

Protein phosphorylation and dephosphorylation are the predominant mechanisms of signal transduction in mammalian cells, involving nearly one-third of all proteins expressed.[1] This signaling mechanism, operating within the cellular integrated network of regulatory pathways, allows various cellular events and responses to external and internal stimuli to be coordinated. The phosphorylation state of a protein can be regulated via phosphorylation of accessory proteins that control the levels of secondary messengers, phosphorylation of protein kinases and phosphatases themselves, or through the reversible phosphorylation of their substrates. Key regulatory proteins can exist in either a phosphorylated or a dephosphorylated form with the steady-state levels of phosphorylation dictating their relative activity as well as reflecting the activities of the protein kinases and phosphatases that moderate phosphorylation flux.[2] Due to the importance of phosphorylation, it would be valuable to study protein phosphorylation events on a global level, allowing multiple signal transduction events to be mapped as they occur within the cell. Many phosphoproteins, however, are present in the cell at very low concentrations with a low stoichiometry of phosphorylation at any specific site. The dynamic range of present analytical methods makes it extremely difficult to confidently identify phosphopeptides in a complex mixture containing all other types of modified or unmodified peptides.

The most common method for analyzing protein phosphorylation employs metabolic labeling of cellular proteins with radioactive inorganic phosphate ($^{32}P_i$) followed by a two-dimensional polyacrylamide gel electrophoretic (2D-PAGE) separation. The phosphorylated proteins are visualized by autoradiography, excised from the gel, enzymatically digested, and analyzed by mass spectrometry (MS).[3−6] Changes in protein phosphorylation state are determined by comparing spot intensities between autoradiographs obtained from different samples. This method not only suffers from the difficulties associated with working with radioactive samples but is also labor-intensive and time-consuming. Other methods designed to preferentially isolate phosphopeptides from a global pool of peptides have primarily used immunoaffinity or metal affinity columns. Unfortunately, both of these techniques often result in the isolation of many nonphosphorylated peptides through nonspecific interactions that ultimately complicate subsequent analysis. In addition, unless a metabolic or postextraction isotopic labeling step is incorporated, neither method can quantify the relative phosphorylation states of phosphopeptides from different samples.

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10.1021/ac015528g CCC: $22.00
Published on Web 12/08/2001

Three new approaches for the enrichment of phosphopeptides have been recently reported.[7−9] The method of Oda et al. modifies O-linked phosphoproteins with 1,2-ethanedithiol and a thiolate reactive reagent containing a maleimide group and biotin tag, thereby using avidin chromatography to isolate modified phosphopeptides. One of the disadvantages of this method compared to our method is that the maleimide group undergoes partial hydrolysis, resulting in two products for each modified peptide. The other method reported by Zhou et al. chemically blocks reactive amine and carboxylate groups on peptides allowing phosphate groups to be selectively modified to facilitate the covalent attachment of the peptides to an immobilized matrix via the modified phosphate group. This covalent coupling of the phosphopeptides allows stringent washing conditions, resulting in highly enriched mixtures of phosphopeptides. This strategy is able to quantify differences in relative phosphopeptide abundance by using isotopic variants of ethanolamine to block the carboxylate groups. Unlike our method, the isotopic label is not incorporated at the site of phosphorylation.

In this paper, we report the use of phosphoprotein isotope-coded affinity tags (PhIAT)[9] to enrich and identify mixtures of phosphoproteins. The PhIAT method uses stable isotope labeling and biotinylation to prepare highly enriched mixtures of seryl and threonyl phosphopeptides. The use of a biotin tag in conjunction with immobilized avidin chromatography results in fewer nonspecific interactions than obtained with immunoaffinity or metal affinity isolation methods, allowing the detection and identification of lower abundance phosphopeptides. We describe our results showing the use of the PhIAT strategy to identify low-level phosphopeptides from a sample of commercially available β-casein. The utility of PhIAT labeling to detect differences in the relative abundance of phosphopeptides is also presented.

EXPERIMENTAL PROCEDURES

Materials. The materials used in all experiments were obtained from commercially available sources and used without further purification unless otherwise noted. 1,2-Ethanedithiol (EDT-D_0, $HSCH_2CH_2SH$, >98%) and ethane-d_4-1,2-dithiol (EDT-D_4, $HSCD_2CD_2SH$, 99 atom %D) were purchased from Fluka and C/D/N Isotopes (Pointe-Claire, PQ, Canada), respectively. β-Casein (from bovine milk, minimum 90% purity) and trifluoroacetic acid (TFA) (HPLC grade) was purchased from Sigma (St. Louis, MO). (+)-Biotinyliodoacetamidyl-3,6-dioxaoctanediamine (iodoacetyl-PEO-biotin) and tris(2-carboxyethyl) phosphine hydrochloride (TCEP-HCl) were purchased from Pierce (Rockford, IL). Acetonitrile (HPLC grade) and glacial acetic acid (ACS reagent grade) were purchased from Aldrich (Milwaukee, WI). Water used in all experiments was purified using a Barnstead Nanopure Infinity water purification system (Dubuque, IA).

β-Casein Sample Preparation. A 10 mg/mL stock solution of β-casein was prepared by dissolving measured lyophilized protein (by weight) into 0.1 M NH_4HCO_3, pH 8.2, containing 6 M guanidine hydrochloride (GdnHCl). For the experiments to evaluate the quantitative aspects of the PhIAT approach, samples containing various concentrations of β-casein were prepared, transferred into separate Eppendorf tubes, lyophilized, and stored at −20 °C until PhIAT labeling was performed. To maintain the same labeling reaction conditions, the protein samples destined for heavy isotope labeling (PhIAT-D_4) were diluted with 0.1 M NH_4HCO_3, pH 8.2, containing 6 M GdnHCl to match the light isotope labeling (PhIAT-D_0) sample volume prior to lyophilization.

β-Elimination and PhIAT Labeling of Protein Samples. The reaction conditions established for β-elimination and EDT labeling of denatured protein mixtures are described in detail elsewhere.[9] Briefly, 150 μL of H_2O, 185 μL of DMSO, 35 μL of ethanol, 35 μL of acetonitrile, 5 μL of 250 mM EDTA (pH 8.0), 45 μL of 5 M NaOH, and 11 μL of either EDT-D_0 or EDT-D_4 were used to perform β-elimination/EDT addition. After addition of 200 μL of reaction solution to the lyophilized protein samples, they were incubated for 1 h at 55 °C, and then the reaction quenched by neutralizing with acetic acid. The protein samples labeled with EDT-D_0 or EDT-D_4 were combined and desalted by gel filtration using a prepacked PD-10 column containing Sephadex G-25 (Amersham Pharmacia Biotech, Piscataway, NJ) equilibrated with 0.1 M NH_4HCO_3, pH 8.2. The protein and sulfhydryl content were monitored by using the bicinchoninic acid (BCA) assay[10] and 5,5′-dithiobis(2-nitrobenzoic acid) assay,[11] respectively. The EDT-D_0/D_4-labeled protein was either (1) biotinlyated or (2) lyophilized and stored at −80 °C until biotinylation could be performed.

The lyophilized EDT-labeled proteins were resuspended in 1 mL of 0.1 M NH_4HCO_3, pH 8.2, denatured by adding GdnHCl to 6 M, reduced with TCEP-HCl, and the EDT-labeled residues were biotinylated by adding a 5 molar excess of iodoacetyl-PEO-biotin with constant stirring in the dark for 90 min at ambient temperature. The PhIAT-D_0/D_4-labeled proteins were desalted into 0.1 M NH_4HCO_3, pH 8.2, and digested using sequencing grade-modified trypsin (Promega, Madison, WI) overnight at 37 °C using a 1:50 (w:w) trypsin-to-protein ratio. Tryptic activity was quenched by boiling the sample followed by the addition of phenylmethanesulfonyl fluoride to a final concentration of 1 mM.

Avidin Affinity Purification of PhIAT-Labeled Peptides. The PhIAT-labeled peptides were purified by affinity chromatography using ImmunoPure immobilized monomeric avidin from Pierce (Rockford, IL) using between 1.2 and 1.5 mL of avidin slurry packed in a glass Pasteur pipet containing a glass wool plug. Before loading the sample, irreversible biotin binding sites were blocked as per the manufacturer's instructions. The sample containing the PhIAT-labeled peptides was added to the column and permitted to incubate for 30 min at ambient temperature. The column was stringently washed using 15 mL of each of the following: 2× PBS (0.2 M sodium phosphate, 0.3 M NaCl, pH 7.2), 1× PBS, and 0.1 M NH_4HCO_3, pH 8.2. Elution of the PhIAT-labeled peptides was performed using 2 mL of 30% acetonitrile/0.4% TFA with the recovery of PhIAT-labeled peptides quantified using the BCA assay as previously discussed.[9] After lyophilization, the PhIAT-labeled peptides were stored at −20 °C.

Capillary Reversed-Phase HPLC−MS and HPLC−MS/MS Analysis. The HPLC system consisted of a Gilson model 321 pump and 235P autoinjector both controlled via Unipoint System software (Gilson Inc., Middleton, WI). The reversed-phase separa-

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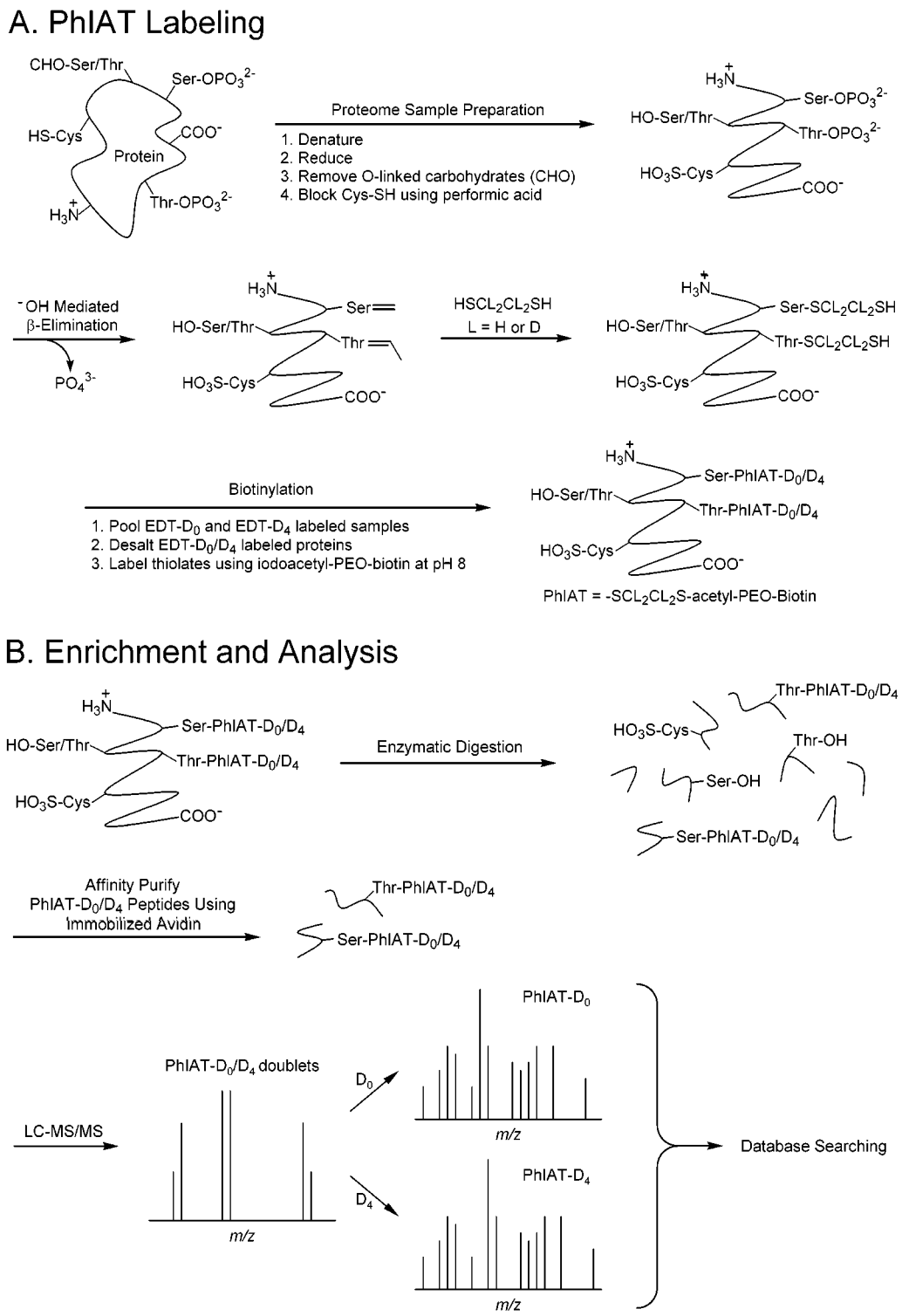

Figure 1. Phosphoproteome analysis using phosphoprotein isotope-coded affinity tags. The first phase (A) involves PhIAT labeling the phosphoseryl (Ser-OPO_3^{2-}) and phosphothreonyl (Thr-OPO_3^{2-}) residues. After O-linked carbohydrates are removed and the cysteine amino acid residues converterd into cysteic acid via performic acid oxidation, the phosphate groups are removed via hydroxide ion-mediated β-elimination and PhIAT labeled as shown. In the second phase (B), the proteins are digested and the sample is enriched for PhIAT-labeled peptides by avidin affinity chromatography. This enriched mixture is then analyzed by capillary reversed-phase LC−MS/MS with subsequent database searching to determine peptide sequence and site(s) of phosphorylation.

tion was performed using a 3-μm Jupiter C_{18} stationary-phase (Phenomenex, Torrence, CA) capillary (Polymicro Technologies Inc., Phoenix, AZ) column, 65 cm length × 150 μm i.d. The mobile phase consisted of 0.2% acetic acid and 0.05% TFA in water (A) and 0.1% TFA in 90% acetonitrile/10% water (B). Mobile phases were degassed on-line using a vacuum degasser (Jones Chromatography Inc., Lakewood, CO). Mobile-phase flow from the Gilson 321 pump, 300 μL/min, was split through a capillary microtee assembly (Upchurch Scientific, Oak Harbor, WA) before the autoinjector. Flow through the column was measured as 1.5 μL/

min. After a sample volume of 10 μL of 1.0 μg/μL labeled peptides was injected onto the reversed-phase capillary HPLC column, the mobile phase was held at 100% A for 10 min. Gradient elution was performed by increasing the mobile-phase composition to 100% B over 90 min. The composition was held at 100% B for 20 min and then reequilibrated with 100% A prior to the next injection.

LC−MS analysis was performed using a hybrid quadrupole time-of-flight (TOF) mass spectrometer (QStar hybrid Quadrupole TOF System, Applied Biosystems − MDS Sciex, Toronto, Canada) equipped with an Applied Biosystems−MDS Sciex MicroIonSpray ESI interface. Typically, a 10-μL injection of 0.1−0.5 μg/μL labeled peptides was used in the analysis. The instrument was run in the TOF-only mode (m/z 400−2000) and ESI conditions were as follows: spray voltage, 5.5 kV; nebulizing gas, 8; curtain gas, 35. No makeup liquid was used.

LC−MS/MS analysis was performed using an LCQ ion trap mass spectrometer (Thermo Finnigan, San Jose, CA). The LC capillary was coupled to the mass spectrometer using an in-house manufactured ESI interface using a spray voltage of 3.0 kV, a capillary temperature of 150 °C, a capillary voltage of 5 V, and a tube lens offset of 0 V. To identify the eluting peptides, the ion trap mass spectrometer was operated in a data-dependent MS/MS mode, in which a full-scan mass spectrum was followed by three MS/MS scans. The precursor (or parent) ions were dynamically selected for collision-induced dissociation (CID) based on their intensity in the MS scan. The normalized collision energy was set at 35%.

Peptide Identification. Peptides were identified by the database-searching program Sequest[12] (Thermo Finnigan), which searched the MS/MS spectra against the complete bovine protein database included with the program (12 474 entries). All of the spectra were analyzed twice: once using a dynamic mass modification on seryl and threonyl residues (i.e., both modified and unmodified forms of the residues) equal to the additional mass of the light isotopic version of the complete PhIAT label (490.7 Da) and then performed again for the heavy version (494.7 Da).

RESULTS

Enrichment and Identification of Phosphopeptides from *β*-Casein. The PhIAT approach outlined in Figure 1 was used to label equivalent amounts of β-casein using either EDT-D_0 or EDT-D_4. In the first stage of the procedure (Figure 1A), the phosphate groups of seryl and threonyl residues are removed by hydroxide ion-mediated β-elimination to produce thiolate-reactive sites (i.e., α,β-unsaturated double bonds). When the relative phosphorylation states of peptides from two distinct samples are compared, these thiolate-reactive sites can be modified with either the light (EDT-D_0) or heavy (EDT-D_4) isotopic version of EDT. Once the samples are stable isotope labeled, they are combined and modified with the biotinylated reagent, iodoacetyl-PEO-biotin. After the proteins are digested with trypsin (Figure 1B), the PhIAT-labeled peptides are affinity isolated using immobilized avidin and analyzed by capillary reversed-phase LC−MS/MS. Identification of the peptides isolated from the PhIAT-labeled β-casein samples is performed using automated database searching. The casein sequences and the sites of phosphorylation that were elucidated with the PhIAT approach agree with those previously documented[13] and listed in the SwissProt and PIR databases.

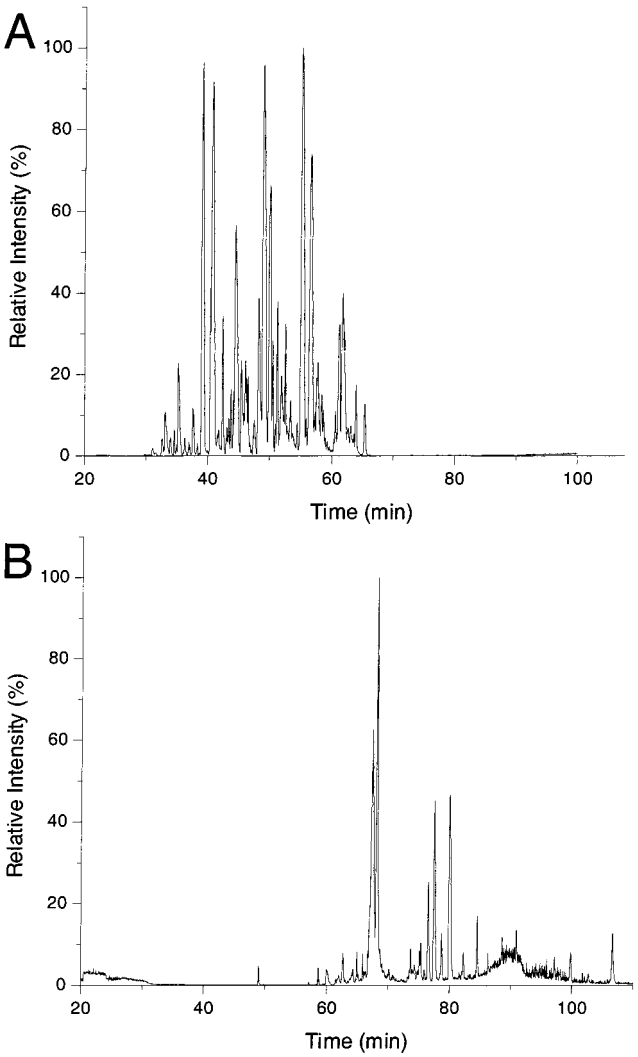

Figure 2. Chromatographs of β-casein tryptic peptides and isolated PhIAT-labeled phosphopeptides. The base peak chromatographs shown are for (A) a tryptic digest of β-casein and (B) PhIAT-D_0/D_4-labeled β-casein phosphopeptides enriched by affinity chromatography using immobilized avidin. The LC−MS analysis was performed using a 10-μL injection of a 1.0 μg/μL sample with the LCQ ion trap operating in profile mode.

The enrichment of phosphopeptides afforded by PhIAT labeling is clearly demonstrated when the chromatographs presented in Figure 2 are examined. It is evident that the baseline chromatograph for the mixture of peptides produced by the tryptic digestion of unlabeled β-casein is more complex than the one generated using the PhIAT approach outlined in Figure 1. Although the gradients used for both separations may be slightly different, the retention times for the PhIAT-labeled peptides are increased relative to the unlabeled forms. This is likely a direct result of replacing the polar phosphate group with the more hydrophobic PhIAT label. The gradient used in these LC−MS acquisitions was also used for the LC−MS/MS analyses.

The MS/MS spectra of the β-casein phosphopeptide IEKFQp-SEEQQQTEDELQDK labeled with the light (PhIAT-D_0) and

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Table 1. Results of Database Searching the Product Ions Using Sequest with the Data Acquired from the LC−MS/MS Analysis of the Tryptic Digest of PhIAT-D_0/D_4-Labeled *β*-Casein

peptide[a]	protein	calcd mass[b]	obsd mass	charge state	Xcorr[c]	ΔCn[d]	ions[e]	PhIAT label (*)[f]
K.IEKFQS*EEQQQTEDELQDK.I	*β*-casein	2843.2	2842.9	3	5.117	0.416	30/72	D_0
K.IEKFQS*EEQQQTEDELQDK.I		2847.2	2848.4	3	4.778	0.342	28/72	D_4
K.TVDMES*TEVFTK.K	α_{S2}-casein	1877.2	1876.8	2	2.763	0.190	13/22	D_0
K.TVDMES*TEVFTK.K		1881.2	1880.6	2	3.044	0.363	15/22	D_4
K.VPQLEIVPNS*AEERLHSMK.E	α_{S1}-casein	2668.2	2670.1	3	2.238	0.135	24/72	D_0
K.YKVPQLEIVPNS*AEER.L		2366.8	2367.9	3	3.826	0.316	28/60	D_4
K.[SC]*QAQPTTMAR.H[g]	*κ*-casein	1684.1	1683.5	2	2.476	0.425	14/20	D_0
K.[SC]*QAQPTTMAR.H[g]		1684.1	1684.8	2	1.758	0.109	12/20	D_0

[a] The amino acid residues appearing before and after the periods correspond to the residues proceeding and following the peptide in the protein sequence. [b] Average peptide mass. [c] Cross-correlation score of the peptide is based on the "fit" of the MS/MS data to the theoretical distribution of ions produced for the peptide. [d] Difference between the top two Xcorr scores for the given peptide. [e] The total number of b and y ions identified/theoretical. [f] PhIAT label, −S−CL_2CL_2−S-acetyl-PEO-biotin where L is H (D_0) or D (D_4) [g] Based on the MS/MS data, the PhIAT-labeling site could only be narrowed down to the SC dipeptide segment.

heavy (PhIAT-D_4) isotopic versions of EDT are shown in Figure 3A and B, respectively. Comparison of the y^{2+} ion (doubly protonated y ion) series displayed in Figure 3 shows that there is a difference of 2 between the m/z values of the PhIAT-D_4- and PhIAT-D_0-labeled peptide fragments. Cleavage at the peptide bonds flanking the PhIAT-labeled Ser residue (formerly a pSer residue) produces y_{13}^{2+} and y_{14}^{2+} species that differ by m/z 288.7 and 290.8, corresponding to 577.4 and 581.6 Da, which are in good agreement with the calculated average masses for PhIAT-D_0- and D_4-modified seryl residues of 577.7 and 581.7 Da, respectively. Not only does the PhIAT label permit quantitation based on the isotopic label, it allows for unequivocal identification of the Ser residue as the site of phosphorylation. Although the Thr residue is capable of being phosphorylated, the m/z values of the y_{13}^{2+} ions (and subsequent y_i^{2+} ions in the series where $i \leq 12$) indicate that the Thr residue was not labeled and, hence, not phosphorylated in the original sample.

The other sites of *β*-casein O-phosphorylation are located at the N-terminal tryptic peptide RELEELNVPGEIVEpSLpSpSpSEESITR. Detection of this peptide containing four PhIAT tags by the LCQ ion trap mass spectrometer used in these studies would require detection of the $[M + 3H]^{3+}$ (m/z 1589.6) or the $[M + 4H]^{4+}$ (m/z 1192.5) ion to due to the instrument's limited operational range (m/z 150−2000). While the PhIAT-D_0/D_4 pair for this peptide was not detected using the LCQ ion trap, the $[M + 3H]^{3+}$ species was observed using the Qstar TOF mass spectrometer. This extreme example illustrates the ability of the PhIAT strategy to label multiple adjacent phosphorylated residues.

Identification of Low-Level Phosphopeptides. In addition to detecting all known phosphopeptides from *β*-casein, phosphopeptides originating from other members of the casein family were also identified from the commercial sample of *β*-casein used in this study. A summary of the top Sequest scores for each PhIAT-D_0/D_4-labeled peptide pair observed in this analysis is presented in Table 1. Interestingly, the α_{S2}-casein phosphorylated peptide TVDMEpSTEVFTK was identified. The MS/MS spectra for both the PhIAT-D_0- and PhIAT-D_4-labeled versions of this peptide are shown in Figure 4. As expected, many similarities exist between the two spectra. Ions containing the PhIAT labels differ by m/z values of 2 and 4 for the +2 and +1 ion series, respectively, while product ions not containing a label have the same m/z value in the two spectra. The mass difference between the y_6^+ and y_7^+ fragments of the light and heavy PhIAT-labeled phosphopeptides is 577.1 and 581.3 Da, indicating that the seryl residue is the site of the PhIAT modification. These mass differences are very similar to those obtained for the modified seryl residue of the *β*-casein phosphopeptide shown in Figure 3.

The MS/MS spectra for another pair of phosphopeptides identified as originating from α_{S1}-casein are shown in Figures 5 and 6. In this example, the phosphorylation state was identified by a PhIAT-D_0/D_4 pair in which the PhIAT tag was attached to the same residue but the peptides contained a missed cleavage site. In Figure 5, the phosphopeptide VPQLEIVPNpSAEERLHSMK was PhIAT-D_0 labeled within the NSA peptide segment. The loss of this segment produces a difference of m/z 381.3, or 762.6 Da, between the b_8^{2+} and b_{11}^{2+} ions, in good agreement with the calculated value of 763.0 Da for this sequence containing a PhIAT-modified seryl residue. Although ions differing in mass corresponding to the S-PhIAT-D_0 were not detected, only the Ser residue of the NSA segment is capable of being PhIAT labeled, provided that it had been previously phosphorylated. The inability to detect the $[M + 3H]^{3+}$ PhIAT-D_4 counterpart is readily explained by examining the precursor MS scan shown as the inset of Figure 5. Because the data-dependent MS acquisition used in these experiments only selected the three most abundant peaks from the precursor scan, the PhIAT-D_4 peak, although present, was the fourth most abundant and hence not selected for tandem MS. The MS/MS spectrum of a shorter PhIAT-D_4-labeled peptide YKVPQLEIVPNpSAEER from α_{S1}-casein shown in Figure 6 contains the same pSer residue discussed above. In this case, the PhIAT label can be unequivocally assigned to the Ser residue based on the 581.6 Da between the y_4^+ and y_5^+ ions. The $[M + 3H]^{3+}$ PhIAT-D_0 counterpart was detected in the precursor MS scan but not selected for tandem MS for the same reason as described for the example in Figure 5.

Stable Isotopic Labeling of Phosphopeptides. Since it is directly reflective of a phosphoprotein's activity, it would be advantageous to be able to reliably quantify differences in the relative phosphorylation state of a protein obtained from two distinct sources. The PhIAT strategy provides this capability through the use of light (EDT-D_0) and heavy (EDT-D_4) versions of EDT. The mass spectrum of the PhIAT-labeled α_{S2}-casein

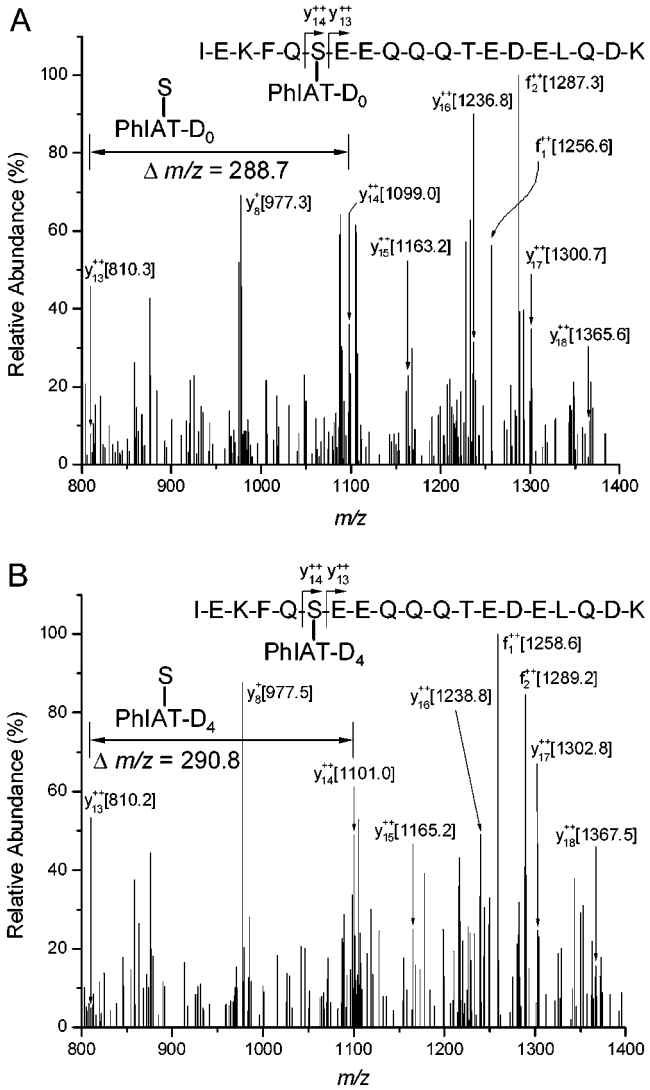

Figure 3. Tandem mass spectrometry identification of β-casein phosphopeptides. The MS/MS spectra of a phosphopeptide from β-casein modified and isolated via the PhIAT strategy using the (A) light and (B) heavy isotopic linkers are shown. In this LC−MS/MS experiment, both labeled versions of the phosphopeptide were identified in the enriched mixture. The Ser residue containing the PhIAT label is identified by the loss of *m*/*z* 288.7 and 290.8 between the y_{13}^{2+} and y_{14}^{2+} ions of the PhIAT-D_0- and PhIAT-D_4-labeled peptide, respectively.

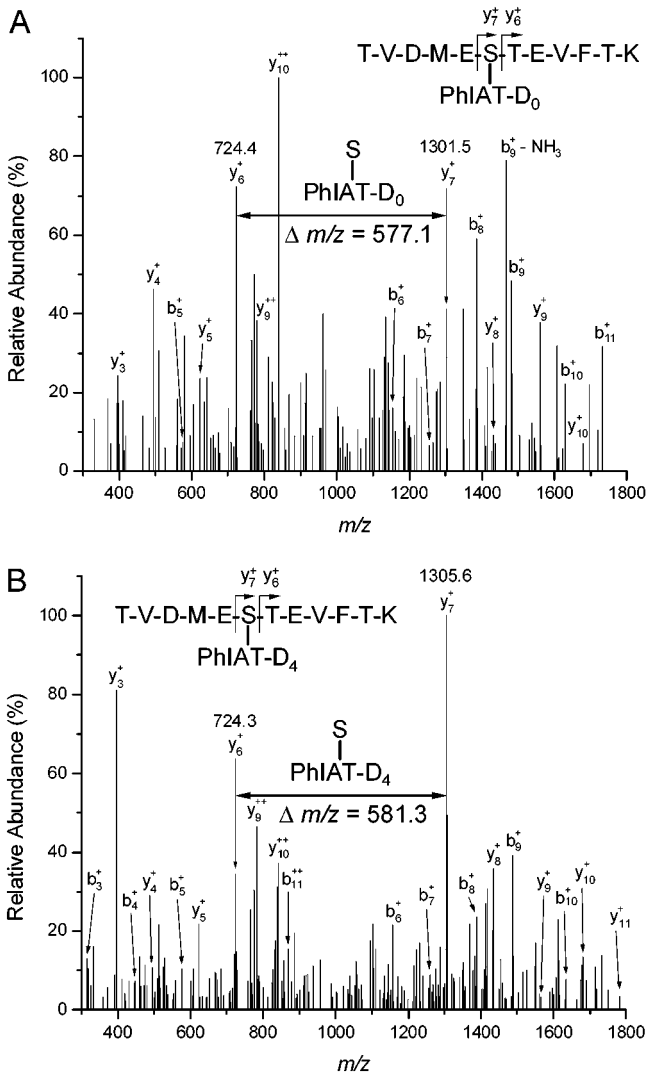

Figure 4. Tandem mass spectrometry identification of α_{S2}-casein phosphopeptides. The MS/MS spectra of the modified phosphopeptide affinity isolated via the PhIAT strategy labeled with the (A) light and (B) heavy isotopic linkers are shown. Both labeled versions of the α_{S2}-casein phosphopeptide were identified by LC−MS/MS analysis of the affinity-isolated mixture starting from a protein sample whose content was >95% β-casein. The Ser residue containing the PhIAT label is identified by the loss of *m*/*z* 577.1 and 581.3 between the y_6^+ and y_7^+ ions of the PhIAT-D_0- and PhIAT-D_4-labeled peptide, respectively.

phosphopeptide TVDMEpSTEVFTK is shown in Figure 7. Notably, the *m*/*z* difference between the monoisotopic $[M + 2H]^{2+}$ ions at *m*/*z* 938.91 and 940.91 is in direct agreement with the expected 4-Da difference resulting from PhIAT labeling of one sample with EDT-D_0 and the other with EDT-D_4. The relative intensities of the peaks for the PhIAT-D_0- and PhIAT-D_4-labeled versions of this low-level phosphopeptide reflect the expected equal abundances of the phosphoproteins in the two separate solutions, which were prepared by initially splitting a sample of β-casein into two equal aliquots.

To further illustrate the quantitative ability of the PhIAT method, EDT-D_0 and EDT-D_4 were used to label samples containing stoichiometric concentrations of β-casein in ratios of 1:1, 5:1, and 10:1. As shown for the MS spectra in Figure 8, the detected PhIAT-labeled peptides reflect the stoichiometric conversion of the phosphorylated residues present in the protein sample. Manual integration of these MS spectra produced ratios of 1.0:1, 4.9:1, and 9.8:1, suggesting that significant changes in relative phosphorylation can be labeled and detected using the PhIAT approach. However, more rigorous experiments are required to fully characterize the ability of PhIAT to quantify relative phosphorylation states.

Further examination of PhIAT-D_0/D_4 peptide pairs was performed by generating the extracted ion chromatograph (XIC) of each isotopically labeled peptide. The XIC for each $[M + 2H]^{2+}$ peak at *m*/*z* 938.91 and 940.91 is shown in Figure 7B. Each peptide appears as a pair of peaks presumably due to the formation of diastereomers (i.e., epimers) upon Michael addition of EDT. The hydroxide ion-mediated β-elimination of the phosphate moiety from pSer generates an α,β-unsaturated double bond, $XYC_\alpha{=}C_\beta H_2$,

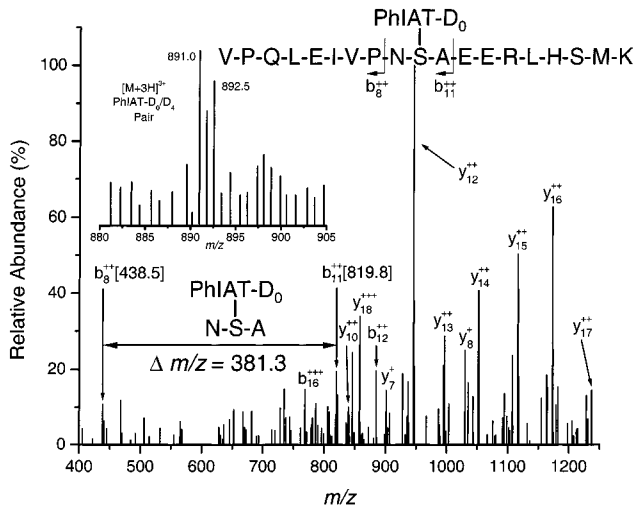

Figure 5. Tandem mass spectrometry identification of α_{S1}-casein phosphopeptide labeled with PhIAT-D_0. The MS/MS spectrum of the α_{S1}-casein-modified phosphopeptide labeled with PhIAT-D_0 and affinity isolated via the PhIAT strategy is shown. The fragmentation pattern displayed is the result of CID of the $[M + 3H]^{3+}$ ion at *m*/*z* 891.0. As shown in the inset, the precursor MS scan displays the PhIAT-D_4-labeled peptide (*m*/*z* 892.5), however, this peak was the fourth most abundant in the MS scan and was not selected due to the settings employed for the tandem MS analysis.

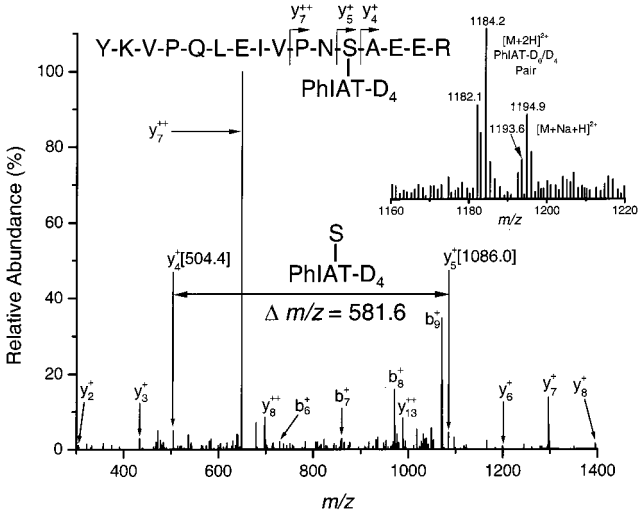

Figure 6. Tandem mass spectrometry identification of α_{S1}-casein phosphopeptide labeled with PhIAT-D_4. The MS/MS spectra of the α_{S1}-casein modified phosphopeptide labeled with PhIAT-D_4 and affinity isolated via the PhIAT strategy is shown. The fragmentation pattern displayed is the result of CID of the $[M + 3H]^{3+}$ ion at *m*/*z* 789.9. Although the PhIAT-D_0 version of the peptide was not selected for tandem MS, the inset displays the $[M + 2H]^{2+}$ PhIAT-D_0/D_4 doublet in the precursor MS scan, confirming the presence of both versions of the peptide.

where X and Y are both chiral, provided that the pSer was not at the carboxy or amino terminus of the protein. If a carbanion mechanism is involved, EDT nucleophilic addition at the β-carbon results in an α-carbanion that can abstract a solvent-derived proton that may lead to epimerization, depending the conformation of X and Y prior to the protonation step. Diastereomers could also result if the pathway involves the tautomerization of an enol intermediate. Based on the XICs in Figure 7B, it appears that the formation of one diastereomer is preferred. Closer inspection of

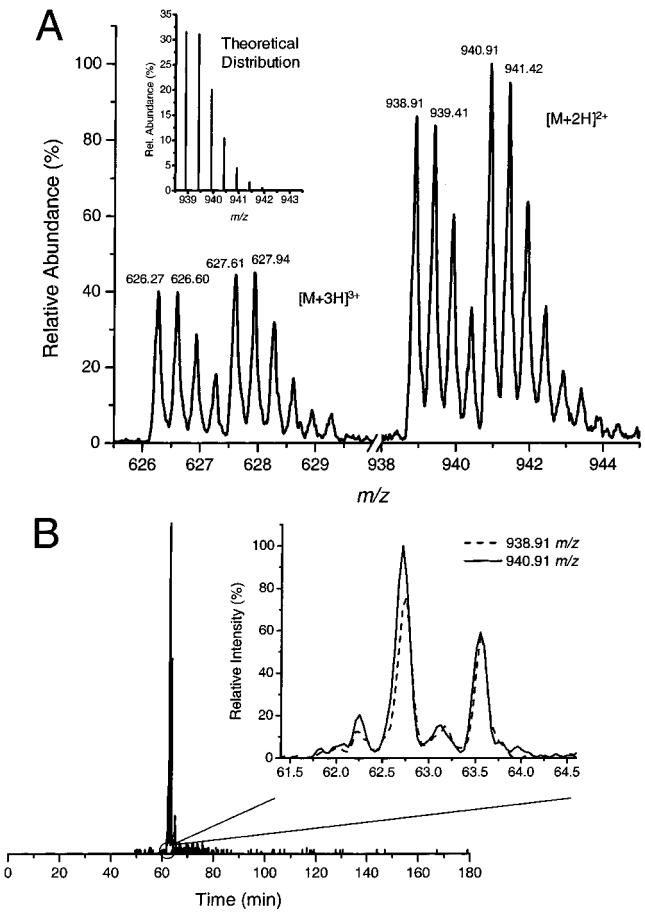

Figure 7. PhIAT-D_0/D_4 labeling of TVDMEpSTEVFTK from α_{S2}-casein. (A) The mass spectrum showing the $[M + 2H]^{2+}$ and $[M + 3H]^{3+}$ peaks for the peptide labeled with PhIAT-D_0 and PhIAT-D_4, with the inset illustrating the theoretical isotopic distribution of the PhIAT-D_0-labeled peptide. Equal amounts of the β-casein protein sample were labeled with either EDT-D_0 or EDT-D_4, combined, and biotinylated. The PhIAT-D_0/D_4 peptides were affinity isolated using immobilized monomeric avidin and analyzed by LC coupled to a hybrid quadrupole-TOF mass spectrometer. (B) The ion chromatographs for the *m*/*z* values of 938.91 and 940.91 correspond to the PhIAT-D_0 and PhIAT-D_4 species, respectively.

the XICs indicates that the PhIAT-D_4 isotopomer elutes slightly earlier (~2.5 s) than the PhIAT-D_0 species, an isotope effect due the presence of four alkyl C−D bonds. Results in our laboratory have shown a similar isotopic elution effect for peptides labeled with the light (D_0) and heavy (D_8) isotopic versions of the ICAT reagents (unpublished observations). While this differential elution of the light and heavy isotopic labeled peptides is not great enough to cause a significant difficulty in identifying or quantifying the peptide pairs, nevertheless, it shows the need for independent integration of the light and heavy forms to provide accurate isotope ratio determinations.

DISCUSSION

Although altering a cell's environment may result in changes in gene expression, other effects, such as changes in protein phosphorylation without a concomitant change in protein expression, may occur as well.[14−16] These changes in protein phospho-

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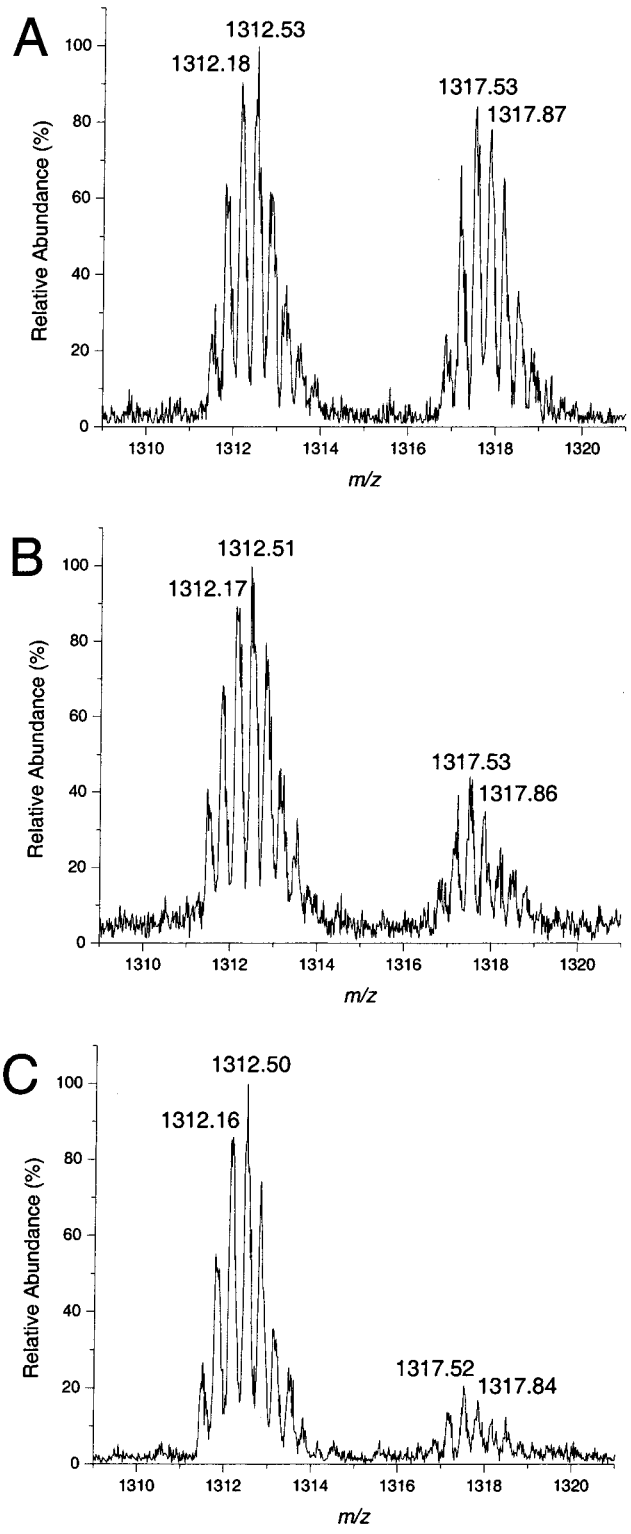

Figure 8. Stoichiometric conversion of phosphorylation states using PhIAT-D_0/D_4 labeling. The mass spectra shown are for the β-casein peptide RELEELNVPGEIVEpSLpSpSpSEESITR in which two of the phosphoseryl residues are modified with PhIAT and the other two with only EDT. Samples of β-casein containing ratios of (A) 1:1, (B) 5:1, and (C) 10:1 were labeled with EDT-D_0:EDT-D_4, combined, biotinylated, affinity isolated using immobilized avidin, and analyzed by LC−MS using a hybrid quadrupole-TOF mass spectrometer.

rylation play a key role in the control of a wide range of biological functions, accentuating the need for techniques capable of quantifying changes in the relative phosphorylation states, as well as relative abundances, of proteins. One of the major difficulties in identifying phosphopeptides is the lack of a method to efficiently extract this class of peptides from complex mixtures. Because phosphoproteins are usually present at low concentrations, accurate identification and quantitation are extremely difficult. The PhIAT approach was developed to overcome these analytical impediments by providing the ability to enrich and quantify phosphopeptides by targeting O-linked phosphorylated sites with a stable isotopically distinct biotin tag that can be affinity purified using immobilized avidin.

Identification of Phosphopeptides from β-Casein. The unambiguous identification of the low-level phosphopeptides from α_{S1}- and α_{S2}-casein shows the ability of the PhIAT strategy to effectively enrich mixtures for phosphopeptides. The content of the commercially available sample used in this study is estimated to be greater than 95% β-casein with the known impurities comprising less than 5% of the total sample, including both α- and κ-caseins (personal communication Sigma). These data represent, to the best of our knowledge, the first report of phosphorylated peptides from α_{S1}- and α_{S2}-casein being identified from a commercial β-casein. It is important to note that the κ-casein peptide SCQAQPTTMAR was also identified by PhIAT. However, with the present data, the site of modification could only be determined to be within the SC dipeptide. Moreover, there is no additional documented evidence to indicate that the seryl residue is phosphorylated. These results suggest that the cysteinyl sulfhydryl is susceptible to β-elimination and may have been modified under the reaction conditions used. In studies using more complex mixtures of proteins (e.g., proteomes), it would be necessary to block any free thiolate groups prior to PhIAT labeling. The preferred method would be oxidation of the cysteinyl residues to cysteic acid, since alkylated sulfhydryls may also be susceptible to β-elimination.

For a positive identification of a PhIAT-D_0/D_4 peptide pair emanating from labeling equivalent samples of β-casein, both isotopic forms should be detected. This can be accomplished, for example, by obtaining an acceptable identification (e.g., Sequest score) for each isotopic form or alternatively an acceptable identification for one isotopic form with verification of its isotopic partner from the corresponding precursor mass spectrum. Because of the highly alkaline conditions used for β-elimination, it is possible for base hydrolysis of the peptide to occur; thus, the Sequest searches were performed without an enzyme cleavage constraint. However, all the peptides identified with appreciable cross-correlation scores were tryptic fragments (Table 1). Each form of the PhIAT label was searched separately with a differential tag mass for the seryl and threonyl residues of 490.7 Da for PhIAT-D_0 and 494.7 Da for PhIAT-D_4. Without an enzyme cleavage constraint imposed during the Sequest search, several additional peptides were tentatively identified, but further examination of the data showed these to be false positives. Thus, the application of an enzyme cleavage constraint or identification of the isotopic pair minimizes erroneous assignments due to systematic errors involved in such database searching.

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The alternative proteolytic PhIAT-labeled peptides listed in Table 1 seem to suggest that the PhIAT label may affect trypsin substrate specificity. However, any predilection toward incomplete digestion, especially if the label occurs near a Lys/Arg residue in primary sequence, will be equally shared between the light and heavy forms of the PhIAT-labeled peptides and not impact quantitation. In fact, highly glycosylated proteins present more of a problem for tryptic digestion because the size of the sugar can prevent trypsin from accessing a substantial fraction of Lys/Arg residues present on the protein substrate, resulting in a significant number of missed cleavages. Even for unmodified proteins, the close proximity of neighboring Lys/Arg residues within the primary sequence may result in incomplete digestion by trypsin because the peptides generated after the first initial amide bond scission may have a lower binding affinity and preclude further digestion. Nevertheless, the nuances of tryptic digestions do not affect the overall ability to identify and quantify phosphoproteins using the PhIAT approach.

Assessing the Identification of PhIAT-Labeled Peptides. When the MS/MS spectra of the PhIAT-labeled peptides from the β-casein sample are compared, several characteristic features are evident. Fragment ions of significant intensity that do not correspond to ions of traditional peptide fragmentation are observed. An example is shown in Figure 3 where the fragment ions are labeled as f_1 and f_2. Based on the m/z values of the f_1 and f_2 ions, the m/z differences of 2 between the ions generated by the PhIAT-D_0- and PhIAT-D_4-labeled peptides indicate that these peaks correspond to doubly protonated species that still retain the isotopic portion of the label. Nevertheless, the results of the database search using the tandem MS data suggest that the PhIAT label generally remains intact during CID of the peptide. The stability of the PhIAT tag during CID enables the localization of the site of phosphorylation to be identified from the MS/MS spectrum. Future studies will examine the CID characteristics of PhIAT-labeled peptides and the PhIAT reagent.

Many studies have used immobilized metal affinity chromatography (IMAC) to enrich for phosphopeptides.[17,18] IMAC has also been coupled directly to MS to identify phosphopeptides from mixtures.[19] Since IMAC exploits the affinity of the first-row transition metals (e.g., Fe^{3+}) for functional groups containing oxygen, sulfur, and nitrogen, it is also capable of binding sulfonated peptides or more likely those containing a large number of Asp and Glu residues and other contaminates through nonspecific interactions, thus contaminating the phosphopeptide pool and complicating downstream analyses. In addition, the absolute identification of phosphoseryl- and phosphothreonyl-containing peptides isolated using IMAC by tandem MS is more difficult since the phosphate group is extremely labile under the CID conditions typically employed for peptide fragmentation, resulting in the loss of either H_3PO_4 or HPO_3.[20] Although partial loss of the phosphate moiety can be tolerated, it prevents phosphopeptides from being quantified with the precision and accuracy necessary to study protein phosphorylation at the proteome level.

While the use of the biotin tag in conjunction with immobilized avidin chromatography is designed to selectively isolate only the PhIAT-modified peptides, a small number of unmodified peptides were also identified in the tandem MS analysis of the derivatized sample. All of these unmodified peptides were hydrophobic, typically contained proline as the second residue, and eluted late in the reversed-phase HPLC separation. The hydrophobic nature of these peptides is consistent with the role of hydrophobic interactions in the binding of biotin to avidin.[21−23] None of these peptides interfered with the observation and identification of the phosphopeptides previously discussed.

The chemistry employed to PhIAT label phosphorylated sites can also result in the modification of O-glycosylated sites. Sites of O-glycosylation are known to undergo β-elimination, resulting in groups that can be reactive toward EDT. While this may initially be seen as a disadvantage, there are methods to affinity isolate either phosphorylated (i.e., immunoaffinity) or glycosylated peptides (i.e., lectin chromatography) so that these peptide classes could be enriched prior to PhIAT analysis. In addition, if only phosphorylated sites were of interest, the sample could be treated with glycanases to remove O-linked carbohydrate groups or alternatively phosphatases could be used to remove phosphate groups enabling the analysis of glycosylated sites. These well-established protocols potentially allow the identification and quantification of both O-phosphorylation and O-glycosylation sites within a protein sample using the same chemistry.

Assessing Quantitation of Phosphorylation Using the PhIAT Approach. Accurate quantitation of the D_0/D_4 isotope ratios of PhIAT-labeled peptides is highly dependent on the resolution of the MS measurements and can benefit from analyses using TOF or Fourier transform ion cyclotron resonance mass spectrometers, especially if small changes in phosphorylation are being examined. As shown in Figure 7, the isotopic peak from each pair could be used to generate an XIC and the diastereotopic peaks integrated. However, due to the partial overlap between the isotopomers of the PhIAT-D_0 and PhIAT-D_4 peptides for $[M + 2H]^{2+}$ ions, the PhIAT-D_4 peak area will contain contributions from the PhIAT-D_0 peptide. If the number of PhIAT labels was greater than one or the number of deuteriums incorporated within the PhIAT label was increased, there would be no overlap between each PhIAT-D_0 and PhIAT-D_4 species, as is the case for the peptide in Figure 8. To account for isotopomer overlap, a reference isotope ratio (a reference proteome containing a 1:1 labeling of the control and perturbed sample at time $t = 0$) would be used to normalize the isotope ratio measurements between the control and perturbed samples at times $t > 0$. Additional experiments are required to determine the most reproducible and reliable method for quantitation and whether it can be performed using XICs generated from tandem MS data or requiring a separate MS acquisition.

The identification and quantitation of phosphoproteins using the PhIAT approach can be improved by using a "double isotopic labeling" strategy. For an experiment containing a control protein

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sample (e.g., healthy cell) and a perturbed protein sample (e.g., cancerous cell), equivalent amounts of protein can be labeled with PhIAT-D_0 and PhIAT-D_4 for the control and perturbed samples, respectively, and pooled. Another labeling is also performed using PhIAT-D_4 to label the control and PhIAT-D_0 to label the perturbed sample. In this manner, if one isotopomer is missed in the D_0/D_4 labeling, it may be detected in the D_4/D_0 labeling and limit the amount of time required to manually search though MS precursor spectra for labeled pairs. Due to the phosphorylation/dephosphorylation events occurring within cells upon an environmental perturbation or manifestation of a diseased state, only one form of the phosphopeptide may be detected in each labeling. Hence, this double PhIAT-labeling strategy will help to verify phosphoproteins that are not present as pairs in the combined control/perturbed samples.

Other Applications of the PhIAT-Labeling Strategy. Although the PhIAT-labeling strategy was developed for analyzing O-linked phosphoproteins on a proteome-wide basis, it can be used to study individual phosphoproteins of interest to determine and quantify the site(s) of phosphorylation. It can also be used to monitor in vitro kinase- and phosphatase-mediated signaling cascades. For conventional proteomic studies, proteins can be PhIAT labeled, separated by 2D-PAGE, and detected by antibodies specific for biotin. In addition, bands identified as PhIAT-labeled proteins from electrophoretic separations could be excised and affinity isolated using immobilized monomeric avidin. If the entire protein is not desired, in-gel digestion with trypsin, followed by avidin affinity chromatography, could be used to enrich only the PhIAT-labeled peptides. For quantitation of phosphoproteins from cell cultures, cells can be grown in either ^{14}N- or ^{15}N-enriched media[24,25] and the samples combined and PhIAT labeled using only EDT-D_0, an alternative to performing separate EDT-D_0 and EDT-D_4 labeling reactions. In this manner, quantitation of phosphorylation is performed by measuring the $^{14}N/^{15}N$ isotope ratio and likely represents a more accurate method for quantitating phosphoproteins in systems amenable to metabolic labeling.

CONCLUSIONS

While studies to date have predominantly focused on the measurement of changes in relative protein abundances between two distinct proteomes, characterization at this level of protein expression is only one of many possible differences that can be observed. Perhaps as important, particularly in higher eukaryotic systems, are measures of the changes in relative protein phosphorylation states. The measurement of changes in protein phosphorylation has been hampered by effective means to accurately quantify and identify phosphopeptides in mixtures containing all other peptide classes. The PhIAT strategy provides a means to alleviate both difficulties. The differential isotopic labeling used in the PhIAT reaction scheme allows relative phosphopeptide abundances to be measured. Additionally, the biotin group of the PhIAT reagent allows isolation of modified phosphopeptides using immobilized avidin. Although a few unlabeled, hydrophobic peptides were also isolated, the enrichment afforded the ability to identify α_{S1}- and α_{S2}-casein phosphopeptides from a mixture whose content was greater than 95% β-casein. Effective enrichment should increase the dynamic range of global phosphopeptide measurements and the throughput of relative phosphorylation state determinations.

ACKNOWLEDGMENT

We thank the United States Department of Energy Office of Biological and Environmental Research and the National Cancer Institute (Grants CA86340 and CA93306) for support of portions of this research. Pacific Northwest National Laboratory is operated by Battelle Memorial Institute for the U.S. Department of Energy under contract DE-AC06-76RLO 1830.

Received for review June 12, 2001. Revised manuscript received August 31, 2001. Accepted October 5, 2001.

AC015528G