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Novel Mass Spectrometric Method for Phosphorylation Quantification Using Cerium Oxide Nanoparticles and Tandem Mass Tags

Weitao Jia¹, Armann Andaya¹, and Julie A. Leary^{1,*}

¹Department of Molecular and Cellular Biology, University of California, Davis, One Shields Avenue, Davis, California 95616

Abstract

The stoichiometry of protein phosphorylation significantly impacts protein function. The development of quantitative techniques in mass spectrometry has generated the ability to systematically monitor the regulation levels of various proteins. This study reports an integrated methodology using cerium oxide nanoparticles and isobaric tandem mass tag (TMT) labeling to assess absolute stoichiometries of protein phosphorylation. This protocol was designed to directly measure the dephosphorylation levels for a known phosphorylation site therefore allowing for quantification of phosphosites. Both the accuracy and precision of the method were verified using standard peptides and protein tryptic digests. This novel method was then applied to quantify phosphorylations on eukaryotic initiation factor 3H (eIF3H), a protein integral to overall eukaryotic protein translation initiation. To date, this is the first report of assessment of protein phosphorylation quantification on eIF3.

INTRODUCTION

Reversible protein phosphorylation/dephosphorylation plays a significant role in regulating a wide range of cellular processes, from cell proliferation, growth, and division, to protein-protein interactions, protein stability, signal transduction, and apoptosis^{1–3}. Recently, large-scale mass spectrometry-based proteomic analyses have been used to both characterize this widespread post-translational modification and to map novel protein phosphorylation sites^{4–5}. Subsequent to identification of these novel sites, determination of stoichiometric level may reveal those biologically impactful sites. As phosphosites are subject to differing levels due to varying kinase/phosphatase interactions, protocols aimed at determining phosphosites and quantifying phosphorylation levels are imperative to the overall understanding of protein biology.

Protocols incorporating stable-isotope labeling with mass spectrometry have produced datasets that illustrate protein phosphorylation changes under a variety of conditions. To date, several recognized methods are used to quantify phosphorylation levels: stable isotope labeling with amino acids in cell culture (SILAC)⁶, isotope-coded affinity tag (iCAT)⁷, isotope tags for relative and absolute quantitation (iTRAQ)⁸, mTRAQ (a developed variation of iTRAQ)⁹, tandem mass tagging (TMT)^{10–12}, ¹⁸O labeling¹³, and use of deuterium formaldehyde to dimethylate free amines¹⁴. Each of these large-scale methods, however, are better suited for quantifying differences arising from varying biological

*Corresponding author: Julie A. Leary jaleary@ucdavis.edu.

Supporting Information Available. Supplementary figures and tables. This material is available free of charge via the Internet at <http://pubs.acs.org>.

conditions, such as diseased versus non-diseased states, and are not directly configured to quantify absolute phosphorylation levels. Because of biological complexities, direct comparisons between different samples may represent only regulation levels and not the important yet subtle intricacies concerning absolute phosphorylation stoichiometry. For example, the fractional difference between 25% and 100% and that of 0.1% and 0.4% is 4; however, their absolute difference differs dramatically from 75% to 0.3%, respectively. Therefore, assessment of the absolute phosphorylation stoichiometries may provide a more comprehensive understanding of biological function.

Quantitative measurements of phosphorylation in relation to protein complexes and/or proteomes are far from routine. Biochemical methods such as radiolabeling of ^{32}P or western blotting have been traditionally used to quantify phosphorylation levels^{15–16}. These two methods, however, are time-consuming, and for western blotting, there exists a necessity for both *a priori* knowledge of the phosphosite as well as antibody specificity for proper quantification.

Mass spectrometry is now the preferred method to measure protein phosphorylation levels via two primary approaches: label-free^{17–19} or stable-isotope labeling^{7, 12, 20}. Label-free protocols quantify phosphorylation based on the ratio of ion signal intensities of phosphopeptides and the corresponding non-phosphorylated peptides (such is the case when using iQEM, isotope-free quantitation of the extent of modification)¹⁸. However, this approach often incorrectly assumes that phosphopeptides, and their nonphosphorylated counterparts, have negligible ionization differences. Selected reaction monitoring (SRM) mass spectrometry in combination with synthetic peptide standards addresses some, but certainly not all, of the inherent complications of the label-free approach²¹. Concerning stable-isotope labeling, absolute quantitation (AQUA) measures the phosphorylation level in one protein and/or defines the absolute levels of every protein expressed by an organism²². Phosphorylation stoichiometries are measured by comparing the absolute abundance of the phosphorylated and nonphosphorylated form for each peptide. Unlike the label-free approach, ionization discrepancies are unlikely to impair quantification using AQUA. However, only previously established phosphosites can be analyzed, and the analyses of multiple phosphosites can become financially burdensome.

Recently, mass spectrometry based strategies have emerged that use phosphatase treatment in combination with both specific and differential stable-isotope labeling methods to quantify phosphorylation. The dephosphorylation that results from phosphatase treatment is then followed by deuterium²³ or ^{18}O labeling²⁴. For deuterium labeling, a D_5 or D_0 -propionyl group can label the N-termini while ^{18}O labeling involves incorporation via trypsin and ^{18}O -enriched water. Measurement of the ion ratio of D_5 - D_0 or ^{18}O - ^{16}O peptide pairs via matrix assisted laser desorption/ionization mass spectrometry (MALDI-MS), can measure phosphorylation stoichiometries based on the ion signal increase of the dephosphorylated peptide relative to its non-dephosphorylated counterpart.

In addition to global quantitative phosphoproteome analysis, specific protein targeted quantification methods such as SILAC established by Mann and colleagues have been used to monitor a minimum of three different ratios representing protein/phosphopeptide/unphosphopeptide changes and were able to determine site-specific stoichiometry of targeted proteins in Hela S3 cells²⁵. Recently, iTRAQ labeling was used to measure the phosphorylation status of Fibroblast Growth Factor Receptor 3²⁶ by measuring a single ratio relating phosphatase-treated and mock-treated samples. A similar strategy used deuterioformaldehyde to chemically label phosphatase-treated samples and phosphorylation stoichiometries of proteins from *Saccharomyces cerevisiae* grown through mid-log phase were obtained via the LTQ-Orbitrap²⁷. However, additional inadvertent enzymatic reactions

may result from the use of impure commercial phosphatases^{28–29} leading to erroneous quantification measurements as alkaline phosphatase is seldom frequently isolated separate from other unwanted proteases. Since specific chemical cleavage of the phosphosite is desired, in lieu of non-specific proteolytic cleavage, cerium oxide has been shown to specifically dephosphorylate phosphopeptides³⁰.

Herein, we have developed an analytically optimized cerium oxide nanoparticles based, time-efficient and cost-effective protocol allowing for phosphorylation quantification within or between proteins under various biological conditions. Accuracy and precision measurements were verified, and the method was used to measure the dephosphorylation levels for a prior established phosphorylation site. As phosphoproteins, and subsequently phosphopeptides, occur naturally at low abundance, a mixture exists of peptides with and without phosphorylation. Taking advantage of this aspect, we subsequently dephosphorylated all phosphopeptides using cerium oxide nanoparticles resulting in a mixture of dephosphorylated phosphopeptides with their unphosphorylated counterparts. After labeling the mixture with the heavy isoform of stable isotopic tandem mass tag (TMT), we combined this with the native phosphopeptide sample previously labeled with the TMT light isoform. The mixed sample can then be used to quantify phosphorylation by indirectly measuring native phosphorylation levels via mass spectrometry. By using this method, we successfully quantified the phosphorylation level of Ser183 in eIF3H, a site of phosphorylation which has a significant role in the oncogenic process of cancer cells.

MATERIALS AND METHODS

Chemicals and Materials

Triethylammonium bicarbonate, Tris(2-carboxyethyl)phosphine hydrochloride, iodoacetamide, ammonium hydroxide, cerium oxide nanopowder (<25nm particle size), α -Cyano-4-hydroxycinnamic acid, and bovine serum albumin (BSA) were purchased from Sigma-Aldrich (St. Louis, MO). Sequencing grade trypsin was obtained from Promega (Madison, WI). TMT-duplex isobaric label reagents (TMT-2¹²⁶ and TMT-2¹²⁷, 0.8 mg vials) were purchased from Thermo Fisher Scientific Inc. (Rockford, IL). Phosphopeptide standards (500 pmol each) were obtained from Protea Biosciences (Morgantown, WV). Amicon Ultra-0.5 mL centrifugal filters (10K MWCO) and Amicon Ultra-4mL centrifugal filters (30K MWCO) were obtained from Millipore (Billerica, MA). All other HPLC grade chemicals were purchased from Thermo Fisher and used without further purification.

Purification of eIF3 protein complex and tryptic digestion of eIF3

The eIF3 protein complex was purified following previously published protocols³¹ (see supporting information). Briefly, 20 μ L of 1 mg/mL eIF3 complex was purified from 400mL postnuclear HeLa cell lysate (~150 g of cells) through ultracentrifugation with subsequent separation via chromatography. A coomassie stained SDS-PAGE of the eIF3 preparation is provided in Figure S-1.

Filter aided sample preparation (FASP)³² obtained the tryptic digestion of eIF3 (see supporting information). A 0.5 pmol standard peptide mixture (DRVYIHPF, IKNLQSLDPSH, DFNKFHTFPQTAIGV, DRVpYIHPF, IKNLQpSLDPSH and DFNKFHpTFPQTAIGV, 0.5 pmol each, from Protea Biosciences) was added to the combined filtrates/digests, split equally into two tubes, and flash frozen into liquid nitrogen. The digest was lyophilized and stored at -80 °C until further analysis.

Dephosphorylation of phosphopeptides

Half of the eIF3 tryptic digest was dephosphorylated using cerium oxide nanopowder as previously described with optimizations³⁰. 100 μ L of 100 μ g/ μ L cerium oxide nanopowder slurry suspended in 100 mM aqueous ammonium hydroxide (pH8.5) was sonicated for 5 min and mixed with 20 pmol of the phosphopeptide mixture (DRVpYIHPF, IKNLQpSLDPSH and DFNKFHpTFPQTAIGV), or 10 μ g eIF3 digest, and then incubated for 30 min on a Thermomixer (Eppendorf) at room temperature. After 14,000 *g* centrifugation, the dephosphorylated peptides were eluted with 100 μ L 100 mM ammonia hydroxide in 50% acetonitrile for 10 min. The supernatants were combined, flash frozen in liquid nitrogen and lyophilized. Samples were stored at -80°C until further analysis.

TMT labeling of peptides

Both standard peptides and tryptic digests were labeled with TMT-duplex according to the manufacturer's protocol (see supporting information). For efficiency range assessment of TMT labeling, trypsin digested BSA was labeled with TMT-duplex then mixed in a series of ratios as follows: 1:1, 1:2, 1:3, 1:5, 1:7, 1:10, 1:15, 1:20, 1:30, 1:50, 1:70 and 1:100 (TMT¹²⁶:TMT¹²⁷). 400–600 femtomole was loaded onto an online C18 column for LC-MS/MS analysis. Three independent replicates were analyzed in establishing method efficiency.

To assess for proper stoichiometric phosphorylation, a standard phosphopeptide mixture (DRVpYI-HPF, IKNLQpSLDPSH, DFNKFHpTFPQTAIGV, 0.5 pmol each, from Protea Biosciences) along with its non-phosphorylated counterparts (Protea Biosciences) were mixed in varying amounts (1:9, 1:1 and 9:1). With subsequent dephosphorylation of half of the sample, samples were TMT labeled and stoichiometry assessed via direct-infusion nanoESI into a LTQ-OrbitrapXL or by MALDI-TOF/TOF analysis. Three independent replicates were carried out.

Mass spectrometric analyses

Nano-RPLC tandem mass spectrometric analyses were performed on a LTQ-OrbitrapXL mass spectrometer (ThermoFisher, San Jose, CA) equipped with an ADVANCE nanospray ion source (Bruker-Michrom, Auburn, CA). MALDI-TOF/TOF measurements were acquired on a 4700 Proteomics Analyzer (ABSciex, Foster City, CA) with parameters described in supporting information. Direct-infusion nanoESI was performed on the LTQ-OrbitrapXL (see supporting information).

Database searches and peptide quantification

After acquisition of LC-MS/MS data, RAW files were converted to MGF files and searched using MASCOT2.2 (MatrixScience) against an in-house database (see supporting information). Searches were performed with tryptic specificity allowing for three missed cleavages and a mass tolerance of 15 ppm in MS mode and 0.2 Da in MS/MS mode. The quantification method was set as "TMT-2plex" and instrument setting set to "ESI-trap". Possible structure modifications allowed were protein N-terminal acetylation, carbamidomethylation of Cys, oxidation of Met and phosphorylation of Ser, Thr, and Tyr. All identified peptides have an individual MS/MS ion score greater than 19 (*p* value <0.05). Ratios for peptide quantification were reported by MASCOT and validated manually. Phosphorylation site stoichiometries were directly calculated according to the ratio of TMT¹²⁷/TMT¹²⁶ $((1-1/\text{ratio}) \times 100\%)$. Values less than 1% were assigned to 1%.

RESULTS AND DISCUSSION

An overview of our protocol is shown in Figure 1. This method was designed to directly measure the dephosphorylation levels based on *a priori* knowledge of the site to be

investigated (either from previous scientific experiments, database and/or literature). Due to the naturally low occurring abundance of phosphoproteins, and subsequently phosphopeptides, there inherently exists a mixture of peptides with and without phosphorylation. Taking advantage of this aspect, we subsequently dephosphorylated all remaining phosphopeptides and thus produced a mixture of dephosphorylated phosphopeptides with their corresponding unphosphorylated counterpart.

Using this general procedure, our samples were first trypsin digested. Identical 10 μ g peptide aliquots were subjected to either cerium oxide nanoparticle based dephosphorylation treatment or processed as is. Subsequent to this reaction, peptides in the cerium oxide treated sample were labeled with the heavy isoform of stable isotopic tandem mass tag, TMT-2¹²⁷. The corresponding untreated sample was labeled with the light isoform, TMT-2¹²⁶. Both labeled samples were equally combined resulting in a 1:1 ratio. This combined sample was then sequenced, identified, and ultimately quantified using two mass spectrometry platforms, either MALDI-TOF-TOF or nano-LC-LTQ-OrbitrapXL. For sample mixtures not requiring prior chromatographic separation, MALDI-TOF-TOF was used to assess phosphorylation levels. For all other samples, nano-LC-MS/MS on the LTQ-OrbitrapXL was used.

Native unphosphorylated peptides, labeled with TMT¹²⁶, and their dephosphorylated counterpart, labeled with TMT¹²⁷, have isobaric masses. The resulting mixture of peptides was then used to quantify protein phosphorylation levels. Analyzing the subsequent tandem mass spectra, the ratio of TMT¹²⁷ to TMT¹²⁶ (Fig. 1), allows for the evaluation of the overall levels of phosphorylation using the following equation: $((1 - 1/r) \times 100\%)$; where $r = \text{ratio of TMT}^{127}:\text{TMT}^{126}$. This protocol thus allows direct measurement of dephosphorylation levels via tandem mass spectrometry and consequently indirectly measures native phosphorylation stoichiometries.

Evaluation of cerium oxide nanoparticles directed dephosphorylation efficiency and assessment of TMT labeling efficiency

Since this protocol relies heavily on a complete dephosphorylation method, we both tested and subsequently optimized the dephosphorylation procedure using cerium oxide nanoparticles. Previous studies have employed an enzymatic approach at dephosphorylation which may be problematic with typically purchased impure enzymes that may inadvertently cause erroneous and unreliable quantification measurements. Dephosphorylation using chemical³⁰ means (such as with cerium oxide nanoparticles) rather than enzymatic treatments has proven to be more specific and complete at removing phosphate groups from phosphoserine, phosphothreonine, and phosphotyrosine.

MALDI-TOF-TOF analysis of a phosphopeptide mixture (DRVpYIHPF, IKNLQpSLDPSH and DFNKFHpTFPQTAIGV, 1 pmol each), incubated for 30 minutes with cerium oxide nanoparticles was sufficient to completely dephosphorylate the peptides by 100% (Fig. 2a-b). This illustrates both the efficiency of dephosphorylation using cerium oxide nanoparticles and also its ability to dephosphorylate all three of the common mammalian phosphorylation sites (Ser, Thr and Tyr). Subsequent to dephosphorylation, we labeled the dephosphorylated phosphopeptides with the TMT-duplex. After 1 hr incubation, all three peptides were 100% labeled (Fig. 2c). The peptide DRVYIHPF had only one possible TMT labeling site (Asp at N-term), and a corresponding mass shift of 225 Da between the peptide with and without TMT-duplex label was detected. Regarding peptides IKNLQSLDPSH and DFNKFHFTFPQTAIGV, they contain two potential TMT labeling sites (N-term and Lys). As anticipated, each peptide demonstrated the appropriate 450 Da mass shift. These data suggest adequate optimization of the first step of our quantification protocol.

A possible concern in the dephosphorylation protocol using cerium oxide nanoparticles may be sterics and the ability to successfully dephosphorylate multi-phosphorylated peptides containing phosphosites in close proximity to one another. We therefore investigated the use of cerium oxide on two multi-phosphorylated peptides, a tri- (TRDI-pY-ETD-pY-pY-RK) and tetra- (RELEELNVPGEIVE-pS-L-pS-pS-pS-EESITR) phosphopeptide. Within 30 min of treatment with cerium oxide nanoparticles, the tri- and tetra-phosphopeptide showed mass losses of 240 and 320 Da, respectively, at 100% efficiency (Fig. 3A, 3B and Fig. S-2). These mass losses correspond to complete dephosphorylation for these multi-phosphorylated peptides. Thus, cerium oxide nanoparticles proved viable and completely dephosphorylate both singly and multiply phosphorylated peptides independent of proximity of the phosphate groups to one another.

Competency of TMT-duplex labeling on either dephosphorylated peptides or native unphosphorylated peptides

In order to assess possible bias for preferential peptide binding to either of the TMT-duplex tags, we independently labeled both native unphosphorylated peptides and dephosphorylated (via cerium oxide nanoparticles) phosphopeptides with both TMT¹²⁶ and TMT¹²⁷ and then pooled the peptides in varying ratios (Fig. 3C, 3D). In a mixture of 1 part native unphosphorylated peptides labeled with TMT¹²⁶ to 5 parts dephosphorylated peptides labeled with TMT¹²⁷, the mass spectra illustrated a ratio of 21:100 (Fig. 3E). Likewise, peptides incorporated in the reverse ratio of 5:1 (5 parts dephosphorylated peptides labeled with TMT¹²⁶ to 1 part native unphosphorylated peptides TMT¹²⁷) revealed a mass spectrum ratio of 100:22 resulted (Fig. 3F). These findings suggest negligible bias exists between peptides to either of the TMT-duplex reagents. This same experiment was also assessed using direct-infusion nanoESI on the LTQ-OrbitrapXL and yielded similar results, thus showing no preference to mass spectrometry platform.

Evaluation of TMT-duplex labeling efficiency using the nano-LC-LTQ-OrbitrapXL

Although MALDI-TOF-TOF proved adequate for simple peptide mixtures, our nano-LC-LTQ-OrbitrapXL system has been routinely used for identifying and subsequently quantifying significantly more complex samples. In order to ascertain the dynamic range of TMT-duplex labeling with the nano-LC-LTQ-OrbitrapXL system, we used the standard native protein bovine serum albumin (BSA). BSA first underwent a standard reduction, alkylation, trypsin digestion and was split into two halves, one half labeled with TMT¹²⁶ and the other with TMT¹²⁷. The two labeled halves were mixed in a series of differing ratios, 1:1, 1:2, 1:3, 1:5, 1:7, 1:10, 1:15, 1:20, 1:30, 1:50, 1:70 and 1:100 (TMT¹²⁶:TMT¹²⁷). Three independent replicates obtained the average ratios as 1:0.92, 1:2.12, 1:2.94, 1:5.50, 1:7.84, 1:9.60, 1:13.5, 1:21.6, 1:28.1, 1:52.5, 1:66.5 and 1:97.0 respectively (Fig. S-3). Because the experimental points do not follow a linear trend, a linear curve was not expected. However, the average deviations were within 12% at all ranges and TMT-duplex labeling did not discriminate across the ranges. These results from high accuracy nanoLC-LTQ-OrbitrapXL analyses with HCD mode indicated consistent accurate measurements throughout the varying ratios of the TMT-duplex reagent. In addition, we optimized our LC-MS/MS parameters prior to this evaluation, as previous reports^{33–35} have suggested optimal conditions may vary from instrument to instrument.

Analysis of premixed phosphopeptides mixture on MALDI-TOF-TOF and LTQ-OrbitrapXL

We next tested our method to quantify phosphorylation levels on more complex samples by using a mixture incorporating several phosphopeptides. The phosphopeptides DRVpYIHPF, IKNLQpSLDPSH and DFNKFHpTFPQTAIGV, along with their non-phosphorylated counterparts, were mixed in varying amounts (1:9, 1:1 and 9:1, indicating 10%, 50%, and 90% phosphorylation abundance levels, respectively). For each independent combination of

phosphopeptides, half of the sample was dephosphorylated using cerium oxide nanoparticles and the other half was untreated. Both halves were labeled with TMT-duplex and the stoichiometry was assessed with either direct-infusion nanoESI into an LTQ-OrbitrapXL or analyzed by MALDI-TOF-TOF. Analysis of the MALDI-TOF-TOF mass spectra of dephosphorylated IKNLQpSLDPSH showed TMT¹²⁷/TMT¹²⁶ ratios of 47:100, 90:100 and 8.5:100 which corresponded to the premixed samples at ratios of 1:1, 1:9 and 9:1 of phosphopeptide along with its unphosphorylated isoform, respectively (Fig. 4). Quantitative results attained for the other phosphopeptides (DRVpYIHPF and DFNKFHpTFPQTAIGV) were similar to that for IKNLQpSLDPSH. Three independent replicates were carried out for this analysis (Table S-1).

We also performed our analysis of a complex mixture of phosphopeptides using the nanoESI LTQ-OrbitrapXL (Fig. 5). Ratios of TMT¹²⁷/TMT¹²⁶ were obtained at 49:100, 91:100 and 10:100 (Fig. 5C, 5D and 5E), corresponding to premixed samples at ratios of 1:1, 1:9 and 9:1 of phosphopeptide along with its unphosphorylated isoform. Peptides DRVpYIHPF and DFNKFHpTFPQTAIGV produced the same results with data shown in supporting information. Three independent replicates were carried out for this evaluation analysis (Table S-1).

These data indicate that our cerium oxide nanoparticles-based phosphorylation stoichiometry protocol is sufficiently robust to be effectively and accurately applied to differing mass spectrometry platforms such as the MALDI-TOF-TOF and LTQ-OrbitrapXL.

Quantification of phosphorylation of eIF3H

Lastly, we used our method to quantify biologically meaningful information. Eukaryotic initiation factor 3 (eIF3), an approximately 800 kDa hetero-multimeric protein complex composed of thirteen individually distinct subunits is integral to the process of protein translation. Protein translation can be subdivided into three distinct phases: initiation, elongation, and termination. During the heavily regulated process of translation initiation, eIF3, binds to the 40S ribosome thus preventing the formation of the 80S ribosomal complex signifying the initial stages of translation initiation. Subsequent to 40S binding, other initiation factors, (such as eIF1, eIF5, and eIF4G) then bind to eIF3 and the 40S ribosome eventually creating a 48S pre-initiation complex which can then bind RNA to initiate translation initiation. The eIF3 protein provides a necessary and central role in initiation and our group has previously published the identification of novel phosphorylation sites of this protein³¹. Studies conducted by Blenis have shown that eIF3 acts as a dynamic scaffold for mTOR and S6K1³⁶. Of the thirteen subunits of eIF3, phosphorylation has been implicated on the three largest subunits, eIF3A, eIF3B, and eIF3C³⁷ as well as eIF3J, a subunit whose presence dictates RNA binding³⁸. Although multiple sites of phosphorylation can be found on the aforementioned eIF3 subunits³¹, only one site of phosphorylation was reported for the subunit, eIF3H. Interestingly, through site-directed mutagenesis studies, replacement of Ser183 with alanine induces a slow growth phenotype in cancer cells and thus implicates phosphorylation as a regulatory factor in cancer growth³⁹. This is a general feature of eIF3H, as high levels also affect translation, proliferation, and a number of malignant phenotypes of CHO-K1 and HeLa cells and, most significantly, of a primary prostate cell line. Furthermore, increased levels of phosphorylation on Ser183 for eIF3H have been shown to potentially enhance the oncogenic process in cancer cells³⁹. We therefore analyzed the levels of phosphorylation of eIF3H in the cervical cancer cell line HeLa. Indeed, high levels of phosphorylation, 70%, were observed for Ser183 which is in agreement with previous biological experiments (Fig. 6). To date, this represents the first report of quantification on this integral translation initiation factor.

CONCLUSIONS

We have described a protocol that measures the absolute abundance of phosphorylation. Unlike previous reports which have used enzymatic removal of phosphosites which may inadvertently generate side reactions detrimental to quantification procedures, we have optimized the use of chemical cerium oxide nanoparticles and describe in detail its use to effectively remove phosphorylation and thus enable accurate quantification of these highly dynamic posttranslational modifications. By using TMT-duplex labeling technology, we illustrated the applicability of our method to differing mass spectrometry platforms. Currently our method is not suited to quantify phosphorylation stoichiometries on multi-phosphorylated sites within a single peptide; however, it can provide useful information as to the phosphorylation dynamics contained within such domains which may further encourage future directed studies. With our optimized quantification method, we quantified the absolute phosphorylation level of Ser183 as high as 70% on human eIF3H derived from cancer cells grown in log phase. To date, this is the first report of quantification of phosphorylation on eIF3. Ongoing studies are underway to investigate the levels of phosphorylation not only for eIF3H, but the other phosphorylated subunits of this eukaryotic initiation factor as well as other biologically relevant proteins. Ultimately, this protocol provides an accurate alternative method to easily quantify phosphorylation and should be of great use to researchers interested in biological aspects of protein regulation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.
Schematic overview using cerium oxide nanoparticles and tandem mass tags to measure protein phosphorylation levels.

Figure 2.

MALDI-TOF mass spectra of 1 pmol phosphopeptide mixture (DRVpYIHPF [$M+H$] $^{+}$ 1126.5 m/z , IKNLQpSLDPSH [$M+H$] $^{+}$ 1331.6 m/z and DFNKFHpTFPQTAIGV [$M+H$] $^{+}$ 1801.8 m/z ,) (A) mass spectrum of original phosphopeptide mixture, (B) mass spectrum of phosphopeptide mixture incubated for 30 min with cerium oxide nanoparticles (at room temperature) and (C) mass spectrum of (B) followed by 1 hr TMT-duplex labeling.

Figure 3.

MALDI-TOF mass spectra of 1 pmol triphosphopeptide (TRDI-pY-ETD-pY-pY-RK, [M+H]⁺ 1862.7 m/z) (A) spectrum of the triphosphopeptide, (B) spectrum of triphosphopeptide incubated for 30 min with cerium oxide nanoparticles. And MALDI-TOF mass spectra of 1 pmol TMT-duplex labeled dephosphorylated peptide (DLDVPIGRFDRRSVAE, Mw: 2111.1 Da) and the native unphosphorylated isoform were mixed in varying ratios. (C) precursor ion of the [M+H]⁺ 2337.3 m/z (with TMT-duplex label on N-term Asp); (D) tandem mass spectrum of 2337.3 m/z; (E) the low mass range (120.0–135.0 m/z, for illustration purposes) of the tandem mass spectra from (D) illustrating a ratio of 1:5 for TMT¹²⁶ labeled native unphosphorylated peptide to TMT¹²⁷ labeled dephosphorylated

isoform, (F) ratio 5:1 for TMT¹²⁶ labeled dephosphorylated peptide with TMT¹²⁷ labeled unphosphorylated isoform. Asterisks (*) indicates the TMT-duplex labeling sites on the peptide. Phosphorylation site stoichiometries were calculated according to the ratio of TMT¹²⁷/TMT¹²⁶ $((1-1/\text{ratio}) \times 100\%)$.

Figure 4.

MALDI-TOF-TOF analysis of phosphorylation levels of a standard phosphopeptide (IKNLQpSLDPSH) mixed in varying amounts with its nonphosphorylated counterpart. The ratio of mixing phosphorylated with unphosphorylated samples were 1:1, 1:9, and 9:1. (A) mass spectrum of precursor ion of *I*KNLQSLDPSH (1702.0 m/z, IKNLQSLDPSH with TMT-duplex labels on Lys and N-term Ile); (B) tandem mass spectrum of singly charged *I*KNLQSLDPSH from (A); the low mass range (120.0–135.0 m/z, for illustration purposes) of the tandem mass spectra from premixed IKNLQpSLDPSH and IKNLQSLDPSH in (C) ratio 1:1, (D) 1:9 and (E) 9:1. Asterisks (*) indicates the TMT-duplex labeling sites on the peptide. Phosphorylation site stoichiometries were calculated according to the ratio of TMT¹²⁷/TMT¹²⁶ ((1-1/ratio)×100%).

Figure 5.

Direct-infusion nanoESI LTQ-OrbitrapXL analysis of phosphorylation levels of a standard phosphopeptide (IKNLQpSLDPSH) mixed in varying amounts with its nonphosphorylated counterpart. Ratio of mixing of phosphorylated with unphosphorylated were 1:1, 1:9, and 9:1. (A) mass spectrum of precursor ion of $I^*KNLQSLDPSH$ (851.49 m/z , IKNLQSLDPSH with TMT-duplex labels on Lys and N-term Ile); (B) tandem mass spectrum of doubly charged $I^*KNLQSLDPSH$ from (A); the low mass range (125.3-127.7 m/z , for illustration purposes) of the tandem mass spectra from premixed IKNLQpSLDPSH and IKNLQSLDPSH in (C) ratio 1:1, (D) 1:9 and (E) 9:1. The normalized collision energy for HCD was set at 36%. Asterisks (*) indicates the TMT-duplex labeling sites on the peptide.

Figure 6.

Assessment of the phosphorylation stoichiometry of Ser183 in eIF3H subunit. (A) precursor mass scan of *DFSPEAL*K using Orbitrap; (B) MS/MS scan of the m/z 678.89 ion by HCD, inset shows the zoomed in low mass range (125.3–128.0 m/z) containing the TMT-duplex reporter ions 126.13 and 127.13 m/z ; (C) For illustration purposes, the relative abundance range 0–20% of MS/MS scan from (B) is shown. Asterisks (*) indicates the TMT-duplex labeling sites on the peptide.