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Applying a Targeted Label-free Approach using LC-MS AMT Tags to Evaluate Changes in Protein Phosphorylation Following Phosphatase Inhibition

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Abstract

In order to identify phosphoproteins regulated by the phosphoprotein phosphatase (PPP) family of S/T phosphatases, we performed a large-scale characterization of changes in protein phosphorylation on extracts from HeLa cells treated with or without calyculin A, a potent PPP enzyme inhibitor. A label-free comparative phosphoproteomics approach using immobilized metal ion affinity chromatography and targeted tandem mass spectrometry was employed to discover and identify signatures based upon distinctive changes in abundance. Overall, 232 proteins were identified as either direct or indirect targets for PPP enzyme regulation. Most of the present identifications represent novel PPP enzyme targets at the level of both phosphorylation site and protein. These data can be used to define the underlying signaling pathways and events regulated by the PPP family of S/T phosphatases.

Keywords

Label-free quantitation; Targeted MS/MS; AMT tag pipeline; Comparative phosphoproteomics; Immobilized metal ion affinity chromatography (IMAC); Mass spectrometry; 20 μ m ID monolithic column; Phosphoprotein phosphatase (PPP) family; Ser/Thr protein phosphatase; Calyculin A

INTRODUCTION

In eukaryotes important functions ranging from metabolism and gene expression to cell cycle regulation and cellular stress responses are governed by reversible phosphorylation at specific serine, threonine, and tyrosine protein residues. Together with protein kinases, phosphatases control the phosphorylation status of proteins in the cell. Members of the phosphoprotein phosphatase (PPP) structural family of S/T protein phosphatases, which includes protein phosphatase 1 (PP1), protein phosphatase 2A (PP2A) and others, control the cellular function of a wide variety of proteins. This may occur by direct dephosphorylation of a protein or indirectly via regulation of other kinases, phosphatases, or protein partners. Malfunctions of

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PPP enzymes have been implicated in numerous disease states,¹⁻³ underscoring the importance of defining cellular targets for these enzymes. However, our knowledge of substrates and pathways regulated by PPP enzymes has lagged behind that of protein S/T kinases. Unlike many kinases, the substrate specificity for this family of phosphatases is not defined by consensus sequences within each substrate, but is instead controlled in a complex manner by regulatory subunits and protein-protein interactions within the cell.^{4, 5} Identification of substrates and downstream effectors for PPP enzymes is thus best addressed in the context of the cell.

Qualitative global phosphoproteomics studies typically focus on cells experiencing a single defined condition,⁶⁻¹⁰ however this offers little information about the relevance of a phosphoprotein in the context of a particular pathway or cell response. Regulatory pathways can be more effectively probed with quantitative and comparative approaches that measure changes between the resting and treated states. Comparative quantitative studies of the phosphoproteome have thus far been rare and typically involve the use of stable isotopic labeling e.g. with either ³D-methanol or ¹³C, ¹⁴N-SILAC or iTRAQ reagent¹¹⁻¹⁴. Mann and colleagues recently combined triple-encoding SILAC and phosphopeptide enrichment techniques for identification and quantitation of phosphorylation sites as a function of stimulus, time, and subcellular location.¹⁴ However, isotopic labeling can be costly and may increase the sample complexity.

Label-free approaches for quantitative proteomics have been hindered primarily by technical problems such as non-linear responses and strongly biased detection of co-eluting species due to ionization suppression effects, especially in the circumstance of complex biological matrixes. Recently, our lab developed and applied small ID (10 and 20 μ m i.d.) silica-based monolithic capillary columns for more quantitative and sensitive proteomics measurements and showed that low nL/min LC flow rates for ESI-MS improved quantitation based upon the more uniform signal intensities obtained for different peptides and good linear correlations between MS responses and peptide amounts.^{15, 16} With this development, in the present study we employed a targeted and label-free approach to identify phosphopeptides whose abundance changed in response to treatment with an inhibitor of the PPP family of phosphatases. Abundance profiles of features from replicate LC-MS analyses of phosphopeptides derived from untreated and calyculin A-treated HeLa cells were created using recently developed alignment and clustering algorithms.¹⁷ Features up-regulated in treated samples were then targeted for fragmentation in LC-MS/MS analyses. This analysis identified 583 unique phosphopeptides representing 416 unique proteins. Of these, 285 phosphopeptides representing 232 proteins appeared to be regulated by PPP enzymes. The majority of these represent new PPP targets.

EXPERIMENTAL PROCEDURES

Cell culture and treatment

HeLa cells were grown in Dulbecco's Modified Eagle Medium with high glucose (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (Clontech, Mountain View, CA) and penicillin/streptomycin (Invitrogen) at 37°C in 5% CO₂. At optimal confluency, cells were treated with DMSO vehicle (control), 10 nM or 20 nM calyculin A for 15 min at 37°C and harvested for SDS-PAGE or protein extraction.

SDS-PAGE and western blotting

Cells were lysed in (Tris-buffered saline (TBS) with 2% SDS, 5 μ M zinc acetate, 1 mM sodium orthovanadate, 2 mM β -glycerophosphate, 1 mM PMSF, EDTA-free protease inhibitor cocktail (Roche Applied Science, Indianapolis, IN), 100 nM microcystin) and boiled for 5 min. Protein

concentrations were determined using the BCA protein assay kit (Pierce, Rockford, IL) with BSA as standard. Samples were diluted in SDS gel loading buffer (25 mM Tris-HCl pH 6.8, 12 mM EDTA, 2% SDS, 160 mM sucrose, 0.1% bromophenol blue, 2% β -mercaptoethanol), and 25 μ g of each sample were subjected to SDS-PAGE using either a 6% (for the phospho-T blot) or a 12% (for elongation factor 2 (EF2) blots) resolving gel. Proteins were transferred to nitrocellulose membranes (Schleicher and Schuell Bioscience) for 1 h at 100 V in 50 mM Tris, 39 mM glycine, 0.03% SDS, 20% methanol (pH 9). The membranes were incubated in blocking buffer (5% non-fat milk, 0.1% Tween-20 in TBS) for 1 h at room temperature. The phospho-T blot was incubated with phospho-T primary antibody (1:500, Cell Signaling Technology, Danvers, MA) in TBS containing 0.1% Tween (TBST) and 5% BSA overnight at 4°C, and bound antibody detected with goat anti-rabbit IgG secondary antibody conjugated to horseradish peroxidase (1:5000, Cell Signaling Technology) and chemiluminescence (ECL plus Western Blotting Detection System, Amersham Biosciences, Pittsburgh, PA). Because the antibodies for total and phospho-T56 EF2 were both from rabbit, a single blot containing two lanes for each sample was prepared. A lane for each sample was probed for either total or phospho-T56 EF2 together with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a loading control. The section of the blot probed with phospho-T56 EF2 primary antibody (1:500) was incubated overnight at 4°C. Other portions of the blot were incubated with total EF2 primary antibody (1:500) or GAPDH primary antibody (1:16000, Ambion, Austin, TX) for 1 h at room temperature. Blots were washed with TBST, and bound phospho-T56 or total EF2 antibody detected with goat anti-rabbit secondary antibody conjugated to IR-680 dye (1:10000, Molecular Probes, Eugene, OR), while GAPDH antibody binding was visualized with goat anti-mouse secondary antibody conjugated to IR-680 dye (1:10000, Molecular Probes). Signals were quantified using an Odyssey infrared imaging system (Li-Cor Biosciences, Lincoln, NE). Fluorescent signals for phospho- and total EF2 were normalized to their respective GAPDH signals, then the ratio of phospho-T56 EF2: total EF2 was calculated.

Protein extraction, digestion and methylation

For each sample, protein was extracted using Trizol reagent (Invitrogen, Inc.) according to the manufacturer's instructions with modifications as described.¹⁸ The final protein pellet was resolubilized in 6 M guanidine hydrochloride. Two batches of cells (from 5 nearly confluent 100 mm plates) were independently treated and harvested, followed by protein extraction. Proteins from the two independent biological samples were pooled. Protein concentration was determined and the extracts were stored at -80°C until further use. Samples were diluted 10-fold with 50 mM NH_4HCO_3 (pH 7.4) and digested with modified trypsin (Promega, Madison, WI) at a trypsin/protein ratio of 1:20 at 37 °C for 4 h, then for an additional 16 h at room temperature after treatment with a second aliquot of trypsin. Proteolysis was terminated by acidifying the reaction mixture with glacial acetic acid (HOAc). The tryptic digest was desalted using a SPE C18 column (Supelco, Bellefonte, PA), dried under vacuum, then methylated according to the published protocol⁶ with the exception that the 1 h methylation reaction was repeated a second time to ensure complete methylation.

IMAC

Methylated peptides from each sample (~1.5 mg) were resuspended in 600 μ L of 0.1% HOAc/MeCN/MeOH (1:1:1), and loaded onto Fe^{+3} -activated IMAC MacroTrap cartridges (3 mm i.d. $\times$ 8 mm length) (Michrom, Auburn, CA) custom packed with POROS 20 MC resin (Applied Biosystems, Foster city, CA). The IMAC cartridges were washed first with 300 μ L of 25/74/1 MeCN/ H_2O /HOAc containing 100 mM NaCl, then with 300 μ L 0.1% acetic acid, and were eluted with 150 μ L of 50 mM Na_2HPO_4 (pH~8.5). Eluted peptides were immediately acidified with glacial HOAc to pH~4.

Quantitative LC-MS analysis by LC-FTICR

To achieve ultra-sensitive comparative phosphoproteomics measurements, aliquots of IMAC-enriched samples were analyzed using a 20 μm i.d. silica-based monolithic column (~20 nL/min) coupled to an 11 Tesla FTICR¹⁹. Samples were loaded onto a reversed phase pre-column (360 μm O.D. $\times$ 75 μm I.D.) packed with 5 cm of YMC 5-15 μm C18 resin (Waters Inc., Milford, MA), washed with 0.1% HOAc, then the pre-column was connected to an analytical HPLC silica-based monolithic column (360 μm O.D. $\times$ 20 μm I.D., 40 cm long) integrated with a laser-pulled electrospray ionization emitter tip (2-4 μm in diameter). The HPLC gradient (A = 100 mM HOAc in water, B = 80% MeCN/100 mM HOAc in water) was 0 – 40% B for 180 min, 40 – 60% B for 20 min, 60 – 100% B for 15 min, 100 – 0% B for 10 min, and 0% B for an additional 120 min to re-equilibrate the column. Replicate LC-FTICR analyses were performed for treated (5 analyses) and control (4 analyses) samples.

Data analysis pipeline

The analysis pipeline used for this study is outlined in Figure 1. Features from all the LC-FTICR analyses were collapsed into mass, elution time and abundance values using the Decon2LS and VIPER software packages (<http://ncrr.pnl.gov/software/>).²⁰ After each analysis was aligned to one baseline dataset to correct for mass and elution time variation, abundance profiles of common features with similar mass and elution time were collated into a master list. Each group of common features is referred to as a cluster. The mass and elution time of each cluster was calculated as the median of the mass and elution time of each set of grouped features.¹⁷

Two target lists were created from this master list by selecting those clusters showing greater abundance in treated samples relative to control samples at two different levels of statistical significance, using z-score thresholds of 1 and 0.5 respectively, then entered into the LTQ instrument XCalibur control software (ThermoElectron, San Jose, CA) as inclusion lists to prioritize the acquisition of MS/MS spectra for features that were up-regulated in treated samples. Because the software does not allow specification of LC elution times in the inclusion list, we partitioned the LC-MS/MS experiment into 27 segments of 10 min intervals each. The normalized elution times (NETs) (defined in Supplementary Material) of the clusters in the target lists were scaled into the time segments and each cluster put into its respective segment; clusters at segment boundaries were entered into straddling segments. Elution times of the clusters were adjusted for expected variability for the analysis by first performing screening analyses in normal data-dependent mode on samples and realigning the clusters to the screening analyses. An additional dead time correction was made by manually entering an adjustment based on the m/z of three ions we observed in the screening analysis.

We acquired MS/MS spectra for the 10 most abundant species in the inclusion list after each MS scan, with up to 3 MS/MS spectra allowed for each species (to provide the highest quality merged spectra). In addition, we performed normal data-dependent analyses for the 10 most abundant species from each MS scan for any time segment that lacked inclusion list features to maximize the possibility of identifying peptides of changing abundance. For each target list, two replicate analyses were performed for the control and treated sample. HPLC conditions used for these analyses were identical to those for LC-MS analyses, except that a 360 μm O.D. $\times$ 100 μm I.D. pre-column packed with 5 cm of YMC 5-15 μm C18 resin (Waters Inc., Milford, MA) and an analytical HPLC silica-based monolithic column (360 μm O.D. $\times$ 50 μm I.D., 40 cm long) integrated with a laser-pulled electrospray ionization emitter tip (2-4 μm in diameter) were utilized.

The targeted LC-MS/MS results were analyzed by SEQUEST using static methylation on D, E and the C terminus, as well as dynamic phosphorylation of S, T and Y residues. Tandem MS

peaks were generated by extract_msn.exe, part of the SEQUEST software package. The human International Protein Index (IPI) database (Version 3.05 containing 49,161 protein entries, available on line at www.ebi.ac.uk/IPI) was combined with its reversed sequences and used in the SEQUEST search. LC-MS clusters were identified by aligning the clusters to the LC-MS/MS database of results and matching clusters to peptides based on mass and elution time tolerances. The matching was done while controlling the false discovery rate (FDR) as described in Supplementary Materials.

To measure the probability of correct phosphorylation site localization in each identified peptide that passed the FDR, a probability-based score, the AScore, was calculated for each phosphorylation site based on the presence of site-determining ions in MS/MS spectra, according to Gygi's recently published approach.²¹

RESULTS

Establishing calyculin A treatment conditions

To define conditions for elevating phosphorylation, HeLa cells were treated with various doses of calyculin A for increasing amounts of time. Phosphorylation was elevated with 10-20 nM calyculin A treatment as early as 15 min (Fig 2). To minimize potential toxicity from exposure to this potent phosphatase inhibitor, treatment with 10 nM calyculin A for 15 min was chosen to prepare samples for proteomics analysis.

Identification of phosphopeptides using the global targeted approach

1351 clusters were identified with a constraint of 5% FDR at 2.3 standard deviations (Mahalanobis distance) of mass and elution time variability. This corresponded to an elliptical region with a mass tolerance of 12 ppm and a NET tolerance of 0.06. As a result, 583 phosphopeptides were identified. The XCorr threshold was 2.65 for charge 3+ peptides and was 2.01 for charge 2+ peptides. These peptides are listed in Supplementary Table 1. Spectra for all 583 phosphopeptides and their SEQUEST identification information are included in the SpectrumLook Software Package (see Supplementary Materials).

Quantitation of protein phosphorylation

A *t*-test was performed at a significance level of 5% on the set of identified clusters after performing a log base 2 transformation of the intensities. 184 phosphopeptides were found to be differentially abundant, with 145 exhibiting up-regulation in the treated condition (listed in Supplementary Table 2A). Figure 3 shows the clustergram of peak areas of the phosphopeptides inferred as being differentially abundant from the *t*-test.

A number of phosphopeptides that were either undetected or detected only once in one condition, but were found three or more times in the other condition, were also considered to have changed significantly: 90 phosphopeptides were observed 3-5 times in treated samples, but were not observed or observed only once in control (referred to as C1T3), whereas 11 phosphopeptides were found in control samples 3-4 times, but were detected only once or not at all in treated samples (referred to as C3T1). These phosphopeptides, together with their abundance information, are included in Supplementary Table 2B.

Table 1 summarizes the number of identified phosphopeptides for different types of MS analyses (5% FDR), and the number in the final phosphopeptide lists after peak matching of the clusters to the AMT tag database (5% FDR, 583 phosphopeptides, listed in Supplementary Table 2C). From Table 1, it is clear that the greatest number of changing phosphopeptides were identified in the targeted analyses of treated samples. In normal data-dependent analyses of control and treated samples, although 218 phosphopeptides were identified, only 60 (standard

t-test plus C1T3 peptides) of these showed significant up-regulation in the treated sample. Similarly, only 54 phosphopeptides up-regulated in response to calyculin A treatment were detected in targeted analyses for control samples. In contrast, 189 up-regulated phosphopeptides were identified from targeted analyses of treated samples. This indicates the effectiveness of using the targeted approach to identify changed features (in this study, up-regulated features) in treated samples compared to the use of normal data-dependent analysis. Even though all features in the target lists were chosen based on their up-regulation, some down-regulated features and C3T1 peptides were also identified. This is not surprising, since we programmed the MS/MS analysis in such a way that when the targets were not available during a given time interval, the LTQ instrument acquired MS/MS for the 10 most abundant peptides in its normal data-dependent mode of operation to identify more phosphopeptides. Because we limited our target lists to up-regulated features, the real number of down-regulated phosphopeptides occurring as a function of calyculin A treatment is likely to be much greater.

Validation of peptide identification cut-off criteria

Manual confirmation of phosphopeptides that passed the 5% FDR filter from several datasets was also performed. This manual confirmation verified that the estimated FDR of the phosphopeptides was similar to that observed above. Figure 4 shows the MS/MS spectrum of a phosphopeptide from a known PP2A target, EF2.^{22, 23} Manual confirmation of this spectrum verified the EF2 phosphopeptide sequence, and an increase in EF2 phosphorylation at this site as a result of calyculin A treatment was confirmed by quantitative western blot analysis (Fig 5). Despite this, the EF2 phosphopeptide change failed the 5% FDR criterion used for filtering the phosphopeptides in our initial data analyses to build the AMT tag database for peak matching. In addition, several other phosphopeptides, including some from known PPP enzyme targets such as stathmin²⁴ and cytochrome P450 C17A1,²⁵ which failed to pass the FDR filter were also shown to be correct based on manual confirmation. Overall, this indicates that the 5% FDR filter applied in this study is conservative and underestimates the number of changed phosphoproteins.

For phosphopeptides that passed the 5% FDR filter we occasionally observed that, even though the peptide sequence was correct, our manual analysis indicated that the phosphorylation site assignment was incorrect. In some cases the phosphorylation site can be determined from the b- and y-ions associated with a specific phosphorylated residue and/or the corresponding neutral loss ions; however, in some cases, especially for peptides with multiple phosphorylation sites, assignment can be problematic. Thus, the effective 5% FDR refers to the peptide sequence and number of phosphorylation sites, but does not reflect the FDR for phosphorylation site identification. In cases where more than one phosphorylation site assignment is possible, additional confirmation is required. For this purpose, we calculated the ambiguity score AScore²¹ to estimate the confidence in the phosphorylation site assignment based on the presence of related site-determining ions in MS/MS spectra. Supplementary Table 3 lists the AScores for each ambiguous phosphorylation site. An AScore greater than 19 was considered a confident assignment of a particular phosphorylation site.²¹

Identification of known and novel targets for regulation by PPP enzymes

A number of proteins with phosphorylation elevated by calyculin A treatment are well-established direct or indirect targets of PPP enzymes. These are listed in Table 2, together with a small sample of the novel targets for regulation identified in this study. For cyclin-dependent protein kinase (CDK1), PP1 regulatory subunit 12, and heterogenous ribonucleoprotein (HnRNP)-K, the sites of phosphorylation seen to increase following calyculin A treatment have all been previously documented as either direct or indirect targets for PPP enzymes, or sensitive to PPP enzyme blockers (see Table 2 for refs).

Vimentin is a cytoplasmic intermediate filament protein that undergoes a complex pattern of phosphorylation by multiple kinases. Calyculin A treatment caused an apparent increase in vimentin-derived peptides containing p-S4, p-S55 and p-S458. Elevated ^{32}P incorporation into the residues corresponding to S55 and S458 of vimentin from hamster-derived BHK cells following calyculin A treatment has been reported previously.²⁶ Other studies have also shown that PPP enzyme blockers elevate vimentin phosphorylation and that PP2A is associated with vimentin.²⁷⁻³⁵

Plectin, which links various cytoskeletal proteins with each other and with other targets, also undergoes elevated phosphorylation by treatment with PPP enzyme inhibitors,³⁶ however, specific sites have not been identified. In the present study, a peptide containing p-S4386 was elevated, indicating that this site is a target for PPP enzyme regulation.

The actin binding protein, cortactin, is phosphorylated by activation of the Extracellular signal-regulating kinase (ERK) MAP kinase pathway, most likely by ERK itself.³⁷ Although the effect of PPP blockers on cortactin has not been reported, ERK is inactivated by PP2A and is activated by okadaic acid treatment.³⁸ Two cortactin residues identified in this study, T401 and S418, are followed by proline, the minimal requirement for an ERK phosphorylation site. Serine 418 is phosphorylated *in vivo* following growth factor stimulation and by ERK *in vitro*.³⁷ Although ERK phosphorylation of T401 was not observed *in vitro*, an increase in phosphothreonine at an unidentified site was shown to occur in a growth factor-stimulated, MAP-kinase/ERK kinase (MEK)-dependent manner.³⁷ We also observed elevated phosphorylation of cortactin residue Y421, which can be attacked by the tyrosine kinases Src³⁹ and Abl;⁴⁰ thus elevated phosphorylation of this site may reflect indirect regulation of one of these tyrosine kinases by PP2A³⁸ or another member of this family.

HnRNP-A1, which plays a role in mRNA splicing, is phosphorylated by numerous kinases *in vitro* and contains multiple phosphorylation sites.⁴¹ The phosphorylation of HnRNP-A1 was shown to be elevated by cell treatment with another PPP enzyme blocker okadaic acid,⁴²⁻⁴⁴ although the sites of phosphorylation influenced by okadaic acid were not mapped in these studies. In the present study, S5 and S362 exhibited elevated phosphorylation following calyculin A treatment. Phosphorylation of hnRNP-A1 S5 was reported in calyculin A-treated murine cells⁴⁵, however in this study no comparison was made between untreated and treated cells. Our results demonstrate that phosphorylation at both S5 and S362 is influenced by PPP enzyme activity.

The B subunit of chromatin assembly factor 1 (CAF-1B) undergoes a cycle of phosphorylation and dephosphorylation during S phase chromatin assembly⁴⁶ that involves multiple phosphorylation events.⁴⁷⁻⁴⁹ This is thought to involve cyclin-dependent protein kinases and PP1 and to be required for nucleosome assembly concomitant with DNA synthesis.⁵⁰ Phosphorylation site(s) in CAF-1B relevant to nucleosome assembly have not been identified. Our study revealed two residues in CAF-1B, S429 and T433, whose phosphorylation was increased by calyculin A. Each of these residues is followed by a proline, consistent with the possibility that they are cyclin kinase-dependent phosphorylation sites.

It should be noted that while specific PPP family members have been implicated in directly or indirectly controlling the phosphorylation status of these protein targets for each of the cases described above, other calyculin A-sensitive PPP family members may also be involved in controlling the sites identified in these proteins.

Novel targets for PPP regulation identified in this study also represent proteins with a wide variety of functions and residing in numerous subcellular compartments. All targets (285 unique phosphopeptides) regulated by PPP enzymes are listed in Supplementary Table 2A and 2B (*t*-test, C1T3 and C3T1). Cases in which tyrosine phosphorylation was altered clearly

represent indirect action of PPP enzymes. However, for those cases in which phosphorylation was up-regulated the effect of PPP enzymes may be either direct or indirect.

DISCUSSION

This study presents a novel label-free and global targeted approach for comparative phosphoproteomics applied to the analysis of proteins subject to regulation by PPP-type S/T protein phosphatases. The confirmation of phosphorylation sites in proteins already known to change as a function of PPP enzyme inhibitor treatment, such as T14 and Y15 in CDK1 and S284 in hnRNP-K, validates our proteomics strategy for identifying PPP enzyme targets. We also identified calyculin A-sensitive sites within known protein targets for PPP enzymes for which specific sites have not previously been defined (e.g. plectin). Other calyculin A-sensitive phosphorylation sites found in this study represent sites identified in various global phosphoproteomic screens,^{7, 10, 45, 51, 52} such as T457 in DNA Repair Protein XRCC1, for which no functional information about regulation by phosphorylation is currently available. Our study confirms the identification of these sites and further shows that their phosphorylation status is influenced by PPP family phosphatases. This new information, together with knowledge of the kinase attacking these sites and the protein function, will assist in formulating hypotheses about the signaling pathways and regulatory events involving these phosphoproteins. Other sites identified in this study include phosphorylation sites not previously reported in proteins that have established or unknown functions. Identification of novel phosphorylation sites and their regulation by a PPP family member provides an important new clue concerning the regulation of these proteins. Altogether, 232 known and novel protein targets for PPP enzymes were identified. We estimate that over 95% of these represent new phosphorylation sites and protein targets.

To date, only one other study has compared phosphoproteins from samples treated with or without a PPP enzyme inhibitor. In this study of phosphorylation changes in neurons induced by okadaic acid, a strategy involving isolation of phosphoproteins, followed by ICAT labeling was employed. These investigators also found that the majority of peptides did not change in phosphorylation status and identified 75 unique peptide pairs yielding 13 proteins with changed phosphorylation status.⁵³ Although several other global phosphoproteomic studies used a PPP inhibitor to elevate phosphorylation, no comparison between treated and untreated samples was performed to determine whether phosphorylation sites were subject to PPP enzyme regulation.^{45, 54, 55} Despite the large number of novel targets for PPP enzyme regulation revealed in this study, many well-established targets were not identified. This may be due to the fact that phosphorylation of some targets requires activation of specific signaling pathways, the nature of the ion fragmentation processes, or to limitations in sensitivity, dynamic range or from ion suppression by more abundant species. In addition, sample preparation steps including the Trizol extraction procedure, IMAC and the use of trypsin are factors that can influence protein or peptide recovery.

The success of the present strategy is due to the inclusion of several technical, analytical and informatics advances. Building on our earlier study demonstrating the effects of LC column dimensions and flow rate on quantitation,¹⁶ in the present study we employed a long (40 cm) monolithic column with a small I.D. (20 μ m),¹⁵ operated at ~20 nL/min, providing higher separation power compared with shorter columns (15 cm or less) and greater sensitivity compared to the higher LC flow rates employed in other studies.^{10, 11, 13} This provided more reliable, sensitive and comprehensive quantitation results. The targeted analyses themselves were only possible through the extension of the AMT tag strategy²⁰ employed for this analysis where LC-MS datasets were first aligned to each other to detect clusters which showed interesting abundance profiles. The identification of the clusters themselves was performed using the traditional AMT tag approach by collating the LC-MS/MS results together.

In this study we encountered several challenges, both in performing the experiment and in analyzing the results. The construction of inclusion lists was challenging because some instrument control software does not provide a mechanism for use of LC elution times with the inclusion list. Even when a mechanism is provided for specification of elution time, LC-MS separation times are generally not perfectly reproducible, and none provide for automated correction of the elution times of inclusion lists to account for chromatographic variability in the current experiment. Such capabilities will be essential if targeted experiments are to be successful on a regular basis in high throughput settings.

Targeted studies, such as ours, based on feature profiling, can increase analyses efficiency, sensitivity and throughput by focusing tandem MS capabilities on peptides of interest. It can greatly facilitate studies in which the amount of sample is limited and the objective is to identify changed features under different biological conditions without the need to comprehensively characterize all the features, most of which are often not related to the biological stimulus of interest. This is particularly helpful for changes occurring in low abundance proteins. This approach also minimizes the number of fractionation or chemical modification steps, which can be time consuming and cause sample losses, as well as the extensive analyses required with other approaches to obtain a similar level of identification for features exhibiting differential abundance. In this study, we performed targeted analyses for both significantly changed features and features displaying only modest up-regulation in treated samples. According to our results, targeted analysis could be further improved by focusing on only significantly changed features such as those with $z > 1$. Further success can be achieved by combining the profiling strategy with “smart” mass spectrometry which would combine on-line alignment algorithms and intelligent on-line acquisition of relevant, informative spectra using selective application of MSⁿ or ETD capabilities when sufficient sensitivity exists.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

The data analysis pipeline used for this study. Replicate LC-MS analyses were performed on control and treated samples and accurate mass and time features discovered in each sample. The datasets were aligned to each other to correct for mass measurement and elution time drifts. Similar features from all experiments were grouped together into one cluster if their mass and elution times were similar by using single linkage clustering. As a result a feature master list was created, with each cluster having an abundance in an experiment equal to the intensity of the feature in that experiment that was mapped to the cluster. The abundance profile of features in a cluster was used to select those clusters whose features were up-regulated in treated samples as compared to control samples, after normalization was performed. The m/z and elution time of these clusters were used to perform targeted LC-MS/MS analyses. Clusters in the master list were then aligned and peak matched against peptides identified by LC-MS/MS analyses using an automated method that controlled the FDR of the process by an extension of the decoy database method to include the use of elution time tolerances.

Figure 2. Elevation of protein phosphorylation in response to calyculin A treatment. Samples treated with DMSO vehicle control (0 nM), 10 nM or 20 nM calyculin A were subjected to SDS-PAGE and western blot analysis for phospho-T. The migration of molecular weight standards is marked.

Figure 3. Clustergram of peak areas for phosphopeptides inferred as being differentially abundant between the four control (labeled C1 to C5) and five treated samples (labeled T1 to T5). We can see several different clusters showing different patterns of differential expression. Prominently, we see several clusters showing up-regulation in treated samples in the upper half of the clustergram, while the lower section shows clusters showing up-regulation in control samples.

72	129	272	373	529	676	857	986	1087	1276
A	G	E	T	R	F	T*	D	T	R
1276	1204	1147	1004	903	747	600	419	290	189

Figure 4.
Manual confirmation of the MS/MS spectrum for a phosphopeptide derived from EF2.

Figure 5.

Elevation of EF2 phospho-T56 levels in response to calyculin A treatment. Samples from vehicle control (0 nM) and 10 nM calyculin A treatment were subjected to SDS-PAGE and western blot analysis for phospho-T56 EF2 or EF2. GAPDH was monitored as a loading control. Blots were quantified using fluorescent secondary antibodies and Li-Cor fluorescence based imaging and the ratio of treated to control showed a 3-fold increase in phospho-T levels following treatment with 10 nM calyculin A.

TABLE 1

Number of phosphopeptides identified in different types of MS analyses.

MS analyses	Phosphopeptides	Up-regulated phosphopeptides in the treated sample (<i>Standard t-test/CIT3</i>)
Total	583	145/91
Control & Treated Data-dependent analysis	218	38/22
Control targeted analysis total	233	41/13
Treated targeted analysis total	407	118/71

TABLE 2
Examples of Phosphopeptides Exhibiting Calyculin A-induced Changes

SWISS/ PROT	Protein name	Phosphopeptide	T/C#	phospho- residue	phospho- residue identified previously *	Protein phosphorylation increased by PPP enzyme blockers
O43823	A Kinase Anchor Protein (AKAP) 8	VDS EGDFS **	0.2	S323	--	--
Q8IZP0	Abl-Interactor 1	LGSQHS PGR *	CIT3	S328	10	--
Q01518	Adenyl cyclase-associated protein 1	SGPKPFSAPKQTS PSPK *	3.7	S224 S307	--	--
		SGPKPFSAPKQTS S **	1.5	T306	--	
		SGPKPFSAPKQTS PS **	0.8	S307		
Q13112	Chromatin Assembly Factor 1 Subunit B	TQDPSS PGTI **	2.0	S309 S429	- 10	Yes
Q14247	Cortactin	AKTQT PPVSPAPQPTTEERLPSS **	17.1	T433	--	--
		TQT PPVSPAPQPTTEERLPSSPVY **	10.1	T401 S418 T401 Y421	10 10, 37 10 39, 40, 51, 56	
P06493	Cyclin-dependent Protein Kinase 1	LPSS PVYEDAASFK *	CIT3	S418	10	Yes
		IEKIGEGT Y **	0.4	T14	57-59	
		IEKIGEGT Y **	0.5	Y15 T14	57-59	
P18887	DNA Repair Protein XRCC1	TKPTQAAGPS SPQKPPTPEET **	15.5	Y15 S446	--	--
Q9NRY4	Glucocorticoid Receptor DNA Binding Factor 1	TSFSVGS DDELGPRI *	CIT3	T457 S1179	-- 10	--
P09651	Heterogenous Nuclear Ribonucleoprotein A1	NQGGYGGSSSS SSYGSGR *	5.7	S362	60	Yes
P61978	Heterogenous Nuclear Ribonucleoprotein K	SES PKEPEQLR *	5.8	S5	45 (mouse ortholog)	Yes
		RDYDDMS PR *	42.0	S284	7, 10, 61, 62	
Q8WUF5	Inhibitor of Apoptosis Stimulating Protein of P53	DYDDMS PR *	171	S284	--	--
		AGS PRGS **	C3T1	S183		
O60716	p120 Catenin	HYEDGYPGGSDNYGS LSR *	CIT3	S187	--	--
Q9BTU6	Phosphatidylinositol 4 Kinase Type II	SSS ESYTQSFQSR *	10.7	S230 S462	10	--
P20020	Plasma membrane Ca ²⁺ transporting ATPase 1	VAAAAGSGPS PPGS **	1.9	S47	--	--
		IEDS EPHIPLDDTI **	8.3	S51 S1193	--	--
Q01814	Plasma membrane Ca ²⁺ transporting ATPase 2	SATSSSPGS PIHSLETS **	CIT3	T1203 S1234	--	--
		SATSSSPGSPHSLETS L *	CIT3	S1242 S1242	--	--

SWISS/ PROT	Protein name	Phosphopeptide	T/C#	phospho- residue	phospho- residue identified previously*	Protein phosphorylation increased by PPP enzyme blockers
Q15149 O14737	Plectin Programmed Cell Death Protein 5	SSS VGSSSSYPISPAVSR* VMDS DEDDDY*	2.9 7.8	S4386 S118	7, 10 10	Yes --
O14974 Q14738	Protein phosphatase 1 Regulatory subunit 12A Protein Phosphatase 2A, 56 Kda regulatory subunit, delta isoform	RKVMDS DEDDDY* RST QGVTLTDLQEAEK* PS*NS*T* PPPTQLSKIKY*S* GGPQIVK	7.7 C1T3 C1T3	S118 T696 S60	63-75 76	Yes --
P29803	Pyruvate Dehydrogenase E1 component alpha subunit	Y HGHSMSPDGV**	1.7	S62 T63 Y74 S75 Y289	-- -- 76 40	--
P46060	Ran GTPase Activating Protein 1	ILDPNTGEPAPVLS SPPPADVSTFLAFPS**	C1T3	S300 S427/S428†	77-79 10, 55 (for S428)	--
Q14155 Q81WW6	Rho Guanine Nucleotide Exchange Factor 7 Rho-GTPase Activating Protein 12	MS GFYQGLPTTGMTITK* ATT PPNQGRPDS**	C1T3 0.5	S442 S340 T231	10, 55 -- 45(mouse ortholog), 52	-- -- --
P17275	Transcription Factor JunB	SRDAT PPVS**	3.2	S240 T255	-- 10	--
P08670	Vimentin	S VSSSSSYR* DGQVINETS QHHDDLE* SLYASS PGGVYATR	80.7 11.9	S259 S4 S458 30.7	-- -- -- S55	Yes 26,55, 80-84

† site may be one of two sequential residues
C1T3-peptide observed ≤ 1 time in control sample, but ≥ 3 times in treated sample
C3T1- peptide observed ≥ 3 times in control sample, but ≤ 1 time in treated sample
* indicates no report, number indicates reference for residues previously identified