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Phosphoproteomic Analysis of Liver Homogenates

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Summary

Regulation of protein function via reversible phosphorylation is an essential component of cell signaling. Our ability to understand complex phosphorylation networks in the physiological context of a whole organism or tissue remains limited. This is largely due to the technical challenge of isolating serine/threonine phosphorylated peptides from a tissue sample. In the present study, we developed a phosphoproteomic strategy to purify and identify phosphopeptides from a tissue sample by employing protein gel filtration, protein SAX (strong anion exchange) and SCX (strong cation exchange) chromatography, peptide SCX chromatography and TiO₂ affinity purification. By applying this strategy to the mass spectrometry-based analysis of rat liver homogenates, we were able to identify with high confidence and quantify over four thousand unique phosphopeptides. Finally, the reproducibility of our methodology was demonstrated by its application to analysis of the mammalian Target of Rapamycin (mTOR) signaling pathways in liver samples obtained from rats in which hepatic mTOR was activated by refeeding following a period of fasting.

Keywords

Signal transduction; liver; mass spectrometry; phosphoproteins; ion exchange chromatography; metal oxide chromatography

¹⁵To assess the reproducibility of our phosphopeptide enrichment strategy in the context of *in vivo* studies, we compared LC/MS runs from three control biological replicates as well as four technical replicates obtained from each biological replicate. LC/MS retention time alignments of multiple datasets were carried out to correct for chromatographic shifts between experiments. Eighty-two percent of the phosphopeptides were identified in all three control biological replicates, while only 13% and 5% of the phosphopeptides were identified in two and one of the control biological replicates, respectively. This high degree of reproducibility, and the detection of over four thousand phosphopeptides, was dependent on the performance of the technical replicates.

¹⁶The final data set derived from three biological control replicates contained 3,231 nonredundant phosphorylation sites representing 1,400 proteins and 4,238 phosphopeptides at a 1% false discovery rate. Eighty-five percent of all phosphorylation sites represented serine phosphorylation. Thirteen percent represented phosphorylated threonine residues, and 2% were phosphorylated tyrosine residues.

¹⁷Identification of 5 known rapamycin-sensitive phosphorylation sites supported the reliability of our analysis. In addition, we identified 62 rapamycin-sensitive candidate phosphorylation events. A high number of these candidate phosphorylation sites were found on proteins known to be involved in transcription, translation or cell growth, or known to interact with mTOR itself.

1. Introduction

A primary goal of systems biology is to understand the control of complex signaling pathways in the physiological context of an organism. Our studies have utilized the laboratory rat and, more specifically, analysis of signaling in liver. The liver is the largest vital organ in vertebrates and it has been widely used as a model for the study of regeneration, development, drug metabolism, and signal transduction pathways. Only five previously published studies have aimed to characterize the phosphoproteome in liver tissue and none of these examined drug-sensitive phosphorylation events (1-5). Of these studies, only one explored the reproducibility of their findings (6).

Recent advances in the technology of mass spectrometry (MS) have made possible the quantitative analysis of dynamic phosphorylation events. However, these newly developed techniques have some limitations, particularly in the analysis of global serine/threonine phosphorylation. In the last few years, several MS studies have been conducted on tyrosine-phosphorylation changes following various stimuli to cell signaling (7-9). In these cases, the enrichment of tyrosine phosphorylated peptides has been carried out by peptide immunoprecipitation with antibodies specific to tyrosine phosphorylation sites. The lack of equivalent antibodies against phosphoserine and phosphothreonine precludes this approach for studying these far more prevalent phosphorylation events.

To meet the goal of profiling the liver phosphoproteome, we had to refine and adapt the available methods and ascertain the reproducibility of our analyses. In effect, we combined several standard methods for protein and peptide fractionation. This allowed us to reduce sample complexity, thereby improving the sensitivity of LC/MS analysis (10, 11). In addition to traditional methods, a newer strategy, the enrichment of phosphopeptides by using metal oxide chromatography (MOC), was employed. In the MOC approach, phosphorylated peptides are enriched based on their affinity for metal oxides, including zirconium dioxide (12), titanium dioxide (13) and aluminum hydroxide (14). For our experiments, we used the more recently developed methodology of titanium dioxide (TiO₂) chromatography in the presence of lactic acid.

Most phosphoproteomic studies focusing on signal transduction have been conducted using cell lines. In addition, identification of the greatest number of phosphorylation changes has been given primary importance over reproducibility of the analyses. "Label-free" quantitation has been substituted by some investigators as an alternative to quantitative methods that employ isotope labeling (15). However, data on the reproducibility of label-free quantitation for tissue analysis are lacking. We therefore used the approach of analyzing multiple technical and biological replicate samples using a phosphoproteomic platform that employs automated desalting and reversed-phase separation of peptides in a highly consistent and reproducible manner. All loading steps and column elutions are accurately replicated with computer controls (16).

The use of this experimental approach has enabled us to identify more than four thousand unique phosphopeptides from rat liver with high reproducibility on an LTQ-Fourier-transform ion cyclotron resonance (FTICR) mass spectrometer. To assess the applicability of our proteome-wide analysis of cell-signaling pathways and drug treatment effects *in vivo*, we extended our studies to the analysis of a particular signaling pathway that involves the mammalian Target of Rapamycin (mTOR).

2. Materials

2.1 Animal Studies, Preparation of Liver Homogenates and Gel Filtration Chromatography

1. Adult male Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA) that were of age 35-40 days.
2. Scalpels, scissors, forceps.
3. Liquid nitrogen
4. Buffer A: 16 mM β -glycerophosphate, pH 7.4, 150 mM NaCl, 100 μ M sodium orthovanadate, 10 mM NaF, 1 mM EGTA, and 5 mM EDTA. Store at 4°C.
5. Glass-Teflon homogenizer (Wheaton Science Products, Millville, NJ)
6. Bradford Protein Assay (Bio-Rad, Hercules, CA).
7. Buffer B: 10 mM Tris, pH 8.6, 100 μ M sodium orthovanadate, 10 mM NaF, 0.5 mM EGTA, 2 mM EDTA.
8. Sephadex G-25 Coarse (GE Healthcare Bio-Sciences Corp., Piscataway, NJ.)
9. 2.5 $\times$ 20 cm Econo-column chromatography column (Bio-Rad).

2.2 Ion Exchange Separation of Proteins

1. Q Sepharose Fast Flow beads (GE Healthcare Bio-Sciences Corp.)
2. SP Sepharose Fast Flow beads (GE Healthcare Bio-Sciences Corp.)
3. 1.5 $\times$ 5 cm Econo-column chromatography columns (Bio-Rad)
4. Buffer C: 10 mM Tris, pH 7.3, 100 μ M sodium orthovanadate, 10 mM NaF, 0.5 mM EGTA, 2 mM EDTA.

2.3 Protein Reduction, Alkylation, and Digestion

1. Urea, electrophoresis grade (Fisher Scientific, Pittsburgh, PA)
2. 1 M dithiothreitol
3. 550 mM iodoacetamide
4. 10 mM Tris, pH 8.0
5. Sequencing grade modified trypsin (Promega, Madison, WI)
6. Trifluoroacetic acid (TFA)
7. Oasis HLB extraction cartridges (Waters Corp., Milford, MA)
8. SpeedVac Concentrator

2.4 Strong Cation Exchange Fractionations of Peptides

1. Ammonium formate, 99% (Acros Organics, Belgium)
2. 0.1% formic acid (Acros Organics), 30% acetonitrile HPLC grade (Fisher Scientific)
3. Buffer D: 5 mM ammonium formate, pH 2.65, 30% acetonitrile (pH adjusted with formic acid)
4. Buffer E: 1.2 M ammonium formate, pH 2.65, 30% acetonitrile (pH adjusted with formic acid)

5. 1 ml Resource S column (GE Healthcare Bio-Sciences Corp.)

2.5 Titanium Dioxide Chromatography

1. Titansphere® Phos-TiO Kit (GL Sciences, Torrance, CA)
2. 1% ammonium hydroxide (Sigma) in water
3. 1% ammonium hydroxide in 40% acetonitrile
4. 5% pyrrolidine in water

2.6 Automated MS Analysis

1. 0.1% acetic acid in water
2. 5 µm Monitor C18 particles
3. Solvent A (aqueous buffer): 0.1 M acetic acid in distilled water
4. Solvent B (organic buffer): 0.1 M acetic acid in acetonitrile
5. 0-70% 0.1 M acetic acid in acetonitrile
6. Linear Trap Quadrupole-Fourier Transform [LTQ-FT] (Thermo Fisher Scientific, Waltham, MA)
7. C18 analytical column (360 µm outer diameter × 75 µm inner diameter; fused silica with 12 cm of 5 µm Monitor C18 particles and an integrated 4 µm ESI emitter tip fritted as described previously (17))
8. Xcalibur for mass spectrometer instrument data analysis (Thermo Fisher Scientific)
9. Bioworks software using the SEQUEST algorithm (Thermo Fisher Scientific)
10. High Throughput Autonomous Proteomic Pipeline (HTAPP) (18)
11. PeptideDepot software, available from <http://peptidedepot.com> (19)

3. Methods

The approach used for phosphoproteomic profiling of liver homogenates involved the development of methods for homogenate preparation, protein fractionation and peptide fractionation (Fig. 1). The first step in our methodology focuses on the preparation of liver homogenates containing soluble liver proteins (*see Note*¹) and removing phospholipids from these preparations. This is achieved by passing liver homogenates through a Sephadex G-25 gel filtration column. In order to fractionate the final MS sample to achieve greater sensitivity in phosphopeptide detection (*see Note*²), we followed gel filtration chromatography with a series of protein ion exchange chromatography steps (Fig. 2). First, strong anion exchange (SAX) gradient chromatography is used to fractionate the crude homogenate into 3 fractions. Strong cation exchange (SCX) chromatography is used to

¹The homogenization buffer described here results only in the extraction of soluble proteins. Since the presence of detergents in samples complicates MS analysis, extension of our methods to lipid-soluble proteins will require the application of additional methods for removal of detergent. One such method has been described by Lu et al. (23). As an alternative approach, livers can be homogenized in a buffer containing 8M urea to extract both cytoplasmic and membrane-associated proteins (11). Furthermore, tryptic peptides derived from liver homogenates can be fractionated using reverse phase chromatography at intermediate pH in place of ion exchange chromatography (24).

²Very low abundance proteins or proteins with a low stoichiometry of phosphorylation could presumably go undetected in our MS analysis due to sensitivity limits of the LTQ-FTICR mass spectrometer and the suppression of ionization efficiency from the addition of negatively charged phosphorylation groups detected in positive ion mode. With advances in technology, we anticipate that the sensitivity of peptide detection will increase substantially.

fractionate the SAX flow-through into 2 additional fractions. After trypsin digestion of the five liver homogenate ion exchange fractions (*see Note*³), the resultant peptides are desalted by solid-phase extraction and separated by peptide SCX chromatography (Fig.3). Finally, phosphopeptides recovered from SCX fractions by TiO₂ chromatography in the presence of lactic acid are identified by LC/MS/MS.

3.1. Animal Studies, Preparation of Liver Homogenates and Gel Filtration Chromatography

1. Liver tissue should be obtained under appropriate conditions that minimize stress to the rat. Harvested liver tissue should be immediately flash frozen in liquid nitrogen and stored at -70°C until further processing (*see Note*⁴).
2. Bring tissue to balance on dry ice and weigh frozen tissue, being careful not to let it thaw (*see Note*⁵).
3. Add 10 ml of ice-cold Buffer A (*see Note*⁶) for every gram of liver and mince tissue in beaker with scissors.
4. Homogenize tissue at 700 rpm for 20 sec on ice using a glass homogenizer fitted with a Teflon plunger. Repeat this process seven times.
5. Centrifuge the resulting homogenate at $1,086 \times g$ for 15 min at 4°C .
6. Transfer the supernatants to new tubes and centrifuge at $100,000 \times g$ for 1 hr at 4°C .
7. Discard the resulting pellets and retain the supernatants. Perform a protein assay to determine protein concentration and use the remaining homogenate for gel filtration, or store at -70°C .
8. Dilute liver homogenate 1:3 in ice-cold Buffer B. At 4°C , load
9. Apply 25 ml of diluted homogenate to the 80 ml Sephadex G-25 Coarse column equilibrated in Buffer B (*see Note*⁷).
10. Elute the column with 80 ml Buffer B by gravity flow. Discard the first 30 ml that comes off the column and collect the next 30 ml flow-through in a clean tube. Freeze the flow-through at -70°C .

3.2. Ion Exchange Separation of Proteins

1. Set up 3 ml Q-Sepharose and SP-Sepharose columns at 4°C .
2. Apply 50 mg (approximately 30 ml) of homogenate protein to the Q-Sepharose Fast Flow column at 0.8 ml/min.
3. Collect flow-through and wash the column with 5 ml of Buffer C at 0.8 ml/min. Collect Buffer C wash, and combine with the flow-through. Designate this as the Q-Sepharose flow-through fraction.

³Identification of some phosphorylation sites requires treatment with proteases other than trypsin due to a suboptimal density of tryptic sites in a given protein. More specifically, proteins with too few or too many tryptic sites to yield phosphopeptides of optimal length will require the use of alternative proteases.

⁴If two samples are to be compared, the experimental design should be appropriate for paired analyses. That is, control and experimental samples should be obtained, prepared, processed, and analyzed in parallel. This approach allows for the statistical analyses of the data to also be done in a paired manner.

⁵It is essential that all homogenates and ion exchange fractions be maintained at 4°C to prevent proteolysis and protein dephosphorylation.

⁶Prepare all buffers and solutions in deionized water and stored at 4°C unless otherwise stated.

⁷Protein recovery during the gel filtration step was determined by protein quantification and gel electrophoresis to be greater than 90%.

4. Elute the column at 0.8 ml/min with 5 ml of Buffer C containing 150 mM NaCl. Collect this eluate as a single fraction in a clean tube.
5. Elute the column at 0.8 ml/min with 5 ml of Buffer C containing 300 mM NaCl. Collect this eluate as a single fraction in a clean tube.
6. Elute the column at 0.8 ml/min with 7.5 ml of Buffer C containing 1 M NaCl. Collect this eluate as a single fraction in a clean tube.
7. Equilibrate the SP-Sepharose column with Buffer C.
8. Apply the Q-Sepharose flow-through/wash (pH adjusted to 7.3, if necessary, using HCl) to the SP-Sepharose column.
9. Collect flow-through and wash column with 5 ml of Buffer C at 0.8 ml/min. Collect the Buffer C wash, and combine with the flow-through as the SP-Sepharose flow-through fraction.
10. Elute proteins from the SP-Sepharose column at 0.8 ml/min with 10 ml of Buffer C containing 1 M NaCl. Collect this eluate as a single fraction in a clean tube.
11. Measure protein concentrations in five liver homogenate fractions using Bradford reagent (*see Note*⁸).

3.3. Protein Reduction, Alkylation, and Digestion

1. Denature proteins in the ion exchange fractions by adding urea to each to a final concentration of 8 M.
2. Reduce the sample for 1 hr at 56°C by addition of 1M DTT to a final concentration of 1 mM DTT (*see Note*⁹).
3. Alkylate the samples by adding iodoacetamide to a final concentration of 5 mM then incubating for 1 hr at room temperature in the dark (*see Note*⁹).
4. Dilute the resulting reduced and alkylated samples four-fold with 10 mM Tris, pH 8.0, and incubate overnight at 37°C with sequencing grade modified trypsin at a 1:100 protease:protein ratio.
5. Acidify the resulting tryptic peptides with trifluoroacetic acid (TFA) to pH 2.7. Clarify the peptide mixture by centrifugation at $1000 \times g$ for 5 min.
6. Desalt the clarified peptide supernatants using Oasis HLB extraction cartridges according to the manufacturer's directions. Dry the desalted samples using a SpeedVac Concentrator or equivalent (*see Note*¹⁰).

3.4. Strong Cation Exchange (SCX) Fractionation of Peptides

1. Reconstitute the dried samples in 1 ml of Buffer D and centrifuge them for at $12,000 \times g$ for 8 min at 4°C.
2. Equilibrate a 1 mL Resource S SCX column with Buffer D.
3. Using an HPLC system, apply the peptide mixture to the Resource S column and separate the peptides using the following protocol: flow rate, 0.5 ml/min; Buffer D

⁸As expected, gel electrophoresis of the five protein ion exchange fractions showed different patterns of protein expression but similar protein content (Fig. 3).

⁹Dithiothreitol and iodoacetamide solutions should be prepared fresh on the day of use.

¹⁰Sarstedt Eppendorf tubes should be used to minimize sample loss during sample drying in SpeedVac.

for 20 min; linear ammonium formate gradient, using Buffers D and E, from 5 mM to 1.2 M over 44 min. Entire process yields 32 fractions, 1 ml each.

4. Estimate peptide content of each fraction by measuring the absorbance at 280 nm (see Note ¹¹).
5. Pool fractions to obtain two pools of approximately four 1 ml fractions each (see Note ¹²). Split each combined fraction pool into four microfuge tubes to speed the drying process. Dry the fraction pools in SpeedVac Concentrator and store at -70°C .

3.5. Titanium Dioxide Chromatography

1. Dissolve the peptides in each SCX pool in 400 μl of 0.1% formic acid, 30% acetonitrile. Separate each of these samples into four 100 μl samples for the performance of technical replicates.
2. Enrich phosphopeptides using the Titansphere Phos-TiO Kit following the manufacturer's protocol up to the elution step. In this protocol, lactic acid is included in the loading buffer to reduce the interaction between acidic non-phosphopeptides and titanium dioxide. (see Note ¹³).
3. Elute phosphopeptides from the Phos-TiO tips by first applying 25 μl of 1% ammonium hydroxide in water. Then treat the tips with 25 μl of 1% ammonium hydroxide in 40% acetonitrile. Finally, elute with 50 μl of 5% pyrrolidine in water.
4. Dry all samples in a SpeedVac Concentrator and store at -70°C .

3.6. Automated MS Analysis

1. Dissolve dry phosphopeptide-enriched mixtures in 0.1% acetic acid in water and elute from the nano HPLC reverse phase column into the mass spectrometer with a gradient of 0-70% 0.1 M acetic acid in acetonitrile developed over 30 min at a flow rate of 1.8 $\mu\text{l}/\text{min}$. Perform static peak parking as previously described by flow rate reduction from 200 nl/min to 20 nl/min at the elution time previously determined from a BSA peptide scouting run (20). Apply electrospray voltage of 2.0 kV in a split flow configuration and collect spectra in positive ion mode, essentially as described (21). Perform one full MS scan in the Fourier Transform (from m/z 400 to 1800) followed by 9 data-dependent MS/MS spectra in the linear ion trap from the 9 most abundant ions. Dynamically exclude selected ions for 30 sec and screen for charge-states of +1, +2 and +3. Use previously described settings for FTMS and ion trap mass spectrometry scans (22).
2. MS/MS spectra were automatically searched against separate mouse, hamster and rat National Center for Biotechnology Information (NCBI) non-redundant protein databases using the Sequest algorithm provided with Bioworks 3.2. Quantitative data were automatically calculated by PeptideDepot for every assigned peptide in all LC/MS runs based on exact precursor mass and retention time (see Notes ^{14,18}).

¹¹ Amino acids with aromatic rings absorb UV light at 280 nm. Although absorbance reading at 280 nm does not yield a precise measure of peptide content in solution, it is a convenient way to determine relative peptide abundance.

¹² Peptide SCX peak 1 was shown by MS analysis to be highly enriched with phosphopeptides and successfully separated from the more complex, positively-charged multivalent peptides that eluted later in the peptide SCX peak 2.

¹³ The lactic acid-treated Titansphere Phos-TiO kit was shown by MS analysis to provide an improved recovery of phosphopeptides when compared to Fe^{3+} immobilized metal affinity chromatography (IMAC) or loose TiO_2 beads in the presence of 2,5-dihydroxybenzoic acid.

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¹⁴The applicability of our method to analysis of drug treatment effects and cell-signaling was determined by fasting followed by refeeding, a well characterized rat model for mTOR activation (25). This model had been previously employed in our laboratory to study liver growth in the absence of hepatocyte proliferation (26). During a period of food deprivation lasting 48 hours, liver mass and protein content decrease by approximately one quarter and one third, respectively. Within one hour of refeeding, marked activation of mTOR Complex 1 (mTORC1) occurs, as assessed by phosphorylation of ribosomal protein S6 and S6 kinase. Prior to refeeding, injection of rapamycin, a specific mTORC1 inhibitor, prevents this activation while injection of vehicle (DMSO) has no effect. (26). Thus, this model is suitable for studies on mTORC1 signaling in liver tissue.

¹⁸We observed similar proportions of enhanced and reduced phosphorylation in response to rapamycin. Gene ontology analysis showed over-representation of mTOR pathway related proteins among rapamycin-sensitive phosphopeptide candidates.

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Fig. 1.
Experimental approach to the phosphoproteomic profiling of liver homogenates.

Fig. 2.

Patterns of protein expression in five protein ion exchange fractions. The panel on the *left* shows the strategy for fractionation of liver homogenate proteins and peptides. This strategy, including the analysis of four technical replicates per fraction, resulted in forty samples per homogenate. A rat liver homogenate was subjected to SAX and SCX chromatography. The panel on the *right* shows a Coomassie-stained gel of the resulting fractions. Those derived from SAX chromatography (flow through [FT] and 0.15 M, 0.3 M and 1M NaCl eluates) and SCX chromatography (flow through and 1M NaCl eluate) are shown following the lanes containing molecular weight markers (M) and 50 μ g of the unfractionated liver homogenate (H). The numbers to the left of the stained gel represent molecular weight in kilodaltons.

Fig. 3.

Peptide SCX chromatography separation at pH 2.65 of a liver homogenate fraction. The graph shows an analysis of a single 300 mM NaCl SAX fraction subjected to peptide SCX chromatography. For this representative sample fractionation and all other analyses, digested peptides (10 mg) were applied to a Resource S SCX column and eluted with isocratic ammonium formate (5 mM) followed by a 5 mM to 1.5 M ammonium formate gradient. Peptide content of each fraction was estimated by measuring the absorbance at 280 nm. Two fraction pools containing the highest peptide amounts (designated as Peaks 1 and 2) were recovered from each column run.