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Review Article

Proteomic Methodologies in Protein Phosphorylation - ∂

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ABSTRACT

The main focus of discovery biology for the past few years has been in the areas of diabetes, cardiovascular, cancer, inflammation and anti-infective. One of the key out of box approach for combating diabetes has been the regulation of key protein AMP kinase. For cancer therapy, several of the signal transduction kinases like ERK kinase, MAP kinase, Aurora kinase etc have been used as key protein targets. Kinases are enzymes that regulate downstream pathways by post translational modification (phosphorylation) of target proteins. Thus the detection of phosphorylation state of a protein is very important in these studies. Classically kinase assays were and are performed using radiolabeled P^{32} or P^{33} antibodies that have serious disadvantages. The regulation of proteins at the gene and mRNA level can be very well studied by genomics technology but the post translational modification of proteins can be studied exclusively by proteomic strategies. In view of the tremendous importance of kinases to discovery research, a non-radioactive, non-antibody, high throughput and safe assay for detection of in vivo phosphorylation of proteins -the proteomic way is proposed.

INTRODUCTION

Since the pioneering work of Ed Fisher and Ed Krebs (who were awarded the Nobel Prize in 1992), phosphorylation of proteins has been widely recognized as central to the regulation of most aspects of cell function and cell physiology. Phosphorylation is a reversible post-translational modification, in which a phosphoryl group is covalently attached to or removed from the hydroxyl moiety of serine, threonine or tyrosine residues. This in turn regulates such processes as enzyme activity, protein-protein interactions, subcellular distribution, and stability and degradation. Protein phosphorylation/dephosphorylation systems consist of at least three components:

a. Phosphoproteins, which alter their properties during phosphorylation/dephosphorylation;

b. Protein kinases, which transfer a phosphate group from a nucleotide triphosphate (often ATP) to specific serine, threonine, or tyrosine residues on phosphoproteins (Figure 1)

c. Protein phosphatases, which dephosphorylate phosphoproteins to return the particular protein phosphorylation system to its initial state (Figure 1)

The stoichiometry of phosphorylation of a given site is controlled by the relative activities of a cell's reserves of protein kinases (Approximately 2000 protein kinases are encoded by the human genome [1]) and phosphatases (200 phosphatases [2]). About 40% of cellular proteins may be phosphorylated during some stage of growth and differentiation. Many proteins are multiply phosphorylated on scores of sites (250,000 distinct sites) [3].

Phosphorylation is a widespread post-translational modification governing signal propagation [4]. Indeed phosphorylation is an efficient mean to control cell response to internal and external cues: it is rapid, taking as little as a few seconds, it does not require new proteins to be synthesized or degraded and can be easily reverted. Protein phosphorylation plays a key role in controlling a variety of cellular processes, such as migration, proliferation, apoptosis, differentiation, metabolism, organelle trafficking, immunity, learning and memory [5-7]. Thus, it is not surprising that aberrant phosphorylation profiles correlate with disease conditions such as cancer, diabetes and neurodegenerative or inflammatory disorders [8-10]. Eukaryotic protein phosphorylation typically occurs on serine, threonine or tyrosine residues. Olsen et al. have found the distribution of *p*Ser, *p*Thr, *p*Tyr sites in the human proteome to be around 79.3%, 16.9% and 3.8% respectively [11]. Furthermore approximately 17 000 proteins have at least one annotated residue in the Phosphosite database [12]. Indeed protein kinases are one of the largest gene family in eukaryotes, making up about 2% of the genome [12-14].

It is often very difficult to map phosphorylation sites in substrate proteins due to low copy number (picomole to subpicomole quantities) In addition, most phosphoproteins are phosphorylated on more than one site and phosphorylation of any given site is often sub stoichiometric. The serious constraints of low phosphopeptide yield and stoichiometry make it essential to have analytical methods that can preferentially detect and analyze phosphopeptides.

The ability to assay the state of phosphorylation of specific proteins is of great utility in the quest to establish the function of a given protein (Table 1). Such assays are also critical for the identification of drugs that can influence the phosphorylation, and hence the function of specific proteins.

Phosphoproteome profiling, by use of proteomic technology is the only way that provides a snapshot of the level of numerous small reactions within a cell and how those levels change under different physiological, pharmacological and toxicological conditions in one go. Many HTS techniques that determine the activity of protein kinases *in vitro* are useful in the development of small molecule kinase inhibitors, but do not address underlying mechanistic concerns or efficient *in vivo* targeting. Observing protein phosphorylation as a key post-translational modification of proteins in pathway signaling and in protein degradation, in a high throughput manner is fundamental to understanding the mechanism of action of lead molecules and whether they target signaling pathways of interest. Currently earmarked as metabolic profiling, phosphoprotein profiling is an important area for development that is actively being applied to several studies in drug discovery and drug development by high throughput proteomic technologies.

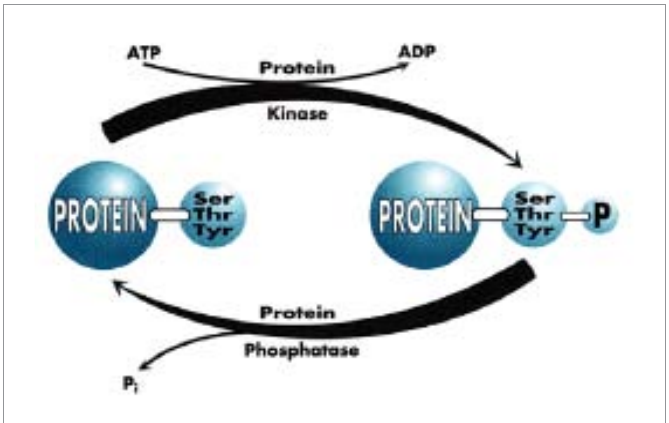

Figure 1:

Table 1: Detection of phosphoproteins.

Method	Advantages	Disadvantages
Autoradiography-incorporation of ^{32}P or ^{33}P [4]	• Very sensitive • Widely used • quantitative	• Limited samples • Bio-Hazardous material
immunoblotting and immunoprecipitation-specific antibodies [5]	• Highly specific • Very sensitive • quantitative	• potential steric hindrances • false positive signals • not for global use
Antibody microarrays	• Highly Specific • High throughput • quantitative	• False positive signals • Not cost effective • technical support required
2DE-Fluorescence-In Gel Staining [6]	• Very simple • Very sensitive • High throughput • Quantitative • Global application	• Complex Image analysis • Does not identify phosphoprotein
Mass Spectrometry-MALDI-TOF/LC-MS/MS [7,8,9]	• Phosphosite identification • Identification ofphosphoprotein • Very sensitive • High throughput	• Highly Technical • Phosphoprotein should be in pure form.

Study of phosphorylation by proteomics approach is a step-wise process and the various steps are outlined below.

- Isolation, separation and detection of Phosphoproteins.
- Identification of phosphorylation state of phosphopeptides by MALDI and stoichiometric determination of phosphorylation.
- Identification of site of phosphorylation of phosphoproteins of interest by LC MS/MS.
- Phosphoproteome profiling of normal and treated samples.
- Phosphoprotein arrays for high throughput screening for drug efficacies.

MATERIALS AND METHODS

Phosphoproteome of cells consists of a pool of proteins containing phosphotyrosine, phosphothreonine or phosphoserine sites. These are isolated directly from cellular protein extracts with a phospho-specific chromatography step or detected by phosphor specific stain and are identified by tandem mass spectrometry. Applying this proteomic approach to several cell systems shows it can be used to identify activated protein kinases and their phosphorylated substrates without prior knowledge of the signaling networks that are activated, a first step in profiling normal and disease signaling networks. The general work flow of phosphorylation analysis by proteomics and mass spectrometry is shown (Figures 2 & 3).

Sample preparation and 2D gel electrophoresis

2-DE analysis samples are mixed with rehydration solution containing 8 M Urea, 2% CHAPS, 2.8 mg/ml DTT (Sigma-Aldrich, Steinheim, Germany), 0.5% IPG Buffer (pH 3-10) (Amersham Biosciences, Uppsala Sweden) and a small amount of bromophenol blue. For analytical gels add 150 µg of protein to a final volume of 450 µl for each sample. Incubate samples for approximately 15 min at room temperature in order to completely solubilize and denature the proteins. Centrifuge samples at 13000 rpm for 10 min and thereafter load onto 24 cm 3-10 non-linear IPG strips (Amersham Biosciences, Uppsala, Sweden). Perform In-gel rehydration and isoelectric focusing (IEF) over night (~60000 Vh) using Ettan IPGphor Isoelectric Focusing System (Amersham Biosciences, Uppsala, Sweden). After IEF, store the strips at -70°C until analysed. Equilibrate the IPG strips in SDS equilibration buffer (75 mM Tris, 6 M Urea, 30% glycerol, 2% SDS and 0.002% bromophenol blue (Sigma-Aldrich, Steinheim, Germany)) for 2 × 15 min. Add DTT (10 mg/ml) (Sigma-Aldrich, Steinheim, Germany) to the first and iodoacetamide (25 mg/ml) (Sigma- Aldrich, Steinheim, Germany) to the second equilibration step. After equilibration, load the strips onto laboratory-made 12.5% acrylamide second dimension gels. Perform SDS-PAGE at constant effect (10 W/gel) for about 4 h and 30 min using the Ettan DALT II system (Amersham Biosciences).

Sequential staining and detection

Fix the second dimension gels in 30% ethanol and 10% acetic acid overnight and wash them 4 × 30 min in 20% ethanol and stain with the fluorescent dye ruthenium II tris-bathophenantroline disulfonate (1 µM) for about 6 h. Thereafter de stain the gels in 40% ethanol and 10% acetic acid overnight and wash with double distilled water for about 4 × 30-60 min [15]. Perform all incubation and washing steps with gentle agitation. Keep the gels in dark in double distilled water at 4°C until scanned. Scan using a robotic system together with a 9410 Typhoon scanner (488 nm laser) from Amersham Biosciences [16] and analyze the gel images using the computer soft wares Image master 2D Platinum (Amersham Biosciences) and Ludesi 2D Interpreter (Ludesi AB, Lund, Sweden). Then calculate volume in each spot as integrated optical density over the spot's area. Express the amount of protein in each spot as %VOL (ppm), that is the volume for the spot divided with the total volume for all spots in the gel.

Alternatively, the SDS-PAGE gels can be stained specifically for phosphoproteins by phosphoprotein specific stains. The gels are normally excited at 550nm and the emission at 580nm is detected by phosphor imager. The intensity is proportional to three orders of magnitude to the number of phosphates present. The gels are destained and stained for silver staining to detect total protein. The gels are scanned by a densitometer scanner and the images analyzed by 2-d gel analysis software. The relative quantities between total protein and phosphoproteins will give the extent of phosphorylation of the specific proteins.

Silver staining protocol

Incubate the gel in Fixer (40% ethanol, 10% acetic acid, 50% H2O) for 1 hr. Wash the gel in H2O for at least 30 min. Sensitize the gel in 0.02% sodium thiosulfate (0.04 g Na2 S2O3, 200 ml H2O) for only 1 min. Wash gel in H2O for 3 x 20 sec. Incubate gel for 20 min in 4°C cold 0.1% silver nitrate solution (0.2 g AgNO3, 200 ml H2O, 0.02% formaldehyde (add 40 µL 35% formaldehyde just before use). Wash the gel in H2O for 3 x 20 sec. Place the gel in a new staining tray. Wash the gel in H2O for 1 min. Develop the gel in 3% sodium carbonate (7.5 g Na2CO3 in 250 ml H2O), 0.05% formaldehyde (add 125 µL 35% formaldehyde just before use). Change developer solution immediately when it turns yellow. Terminate when the staining is sufficient. Wash the gel in H2O for 20 sec. Terminate staining in 5% acetic acid for 5 min. Leave the gel at 4 °C in 1% acetic for storage. Prior to MS analysis the gel is washed in water for 3 x 10 min to ensure complete removal of acetic acid.

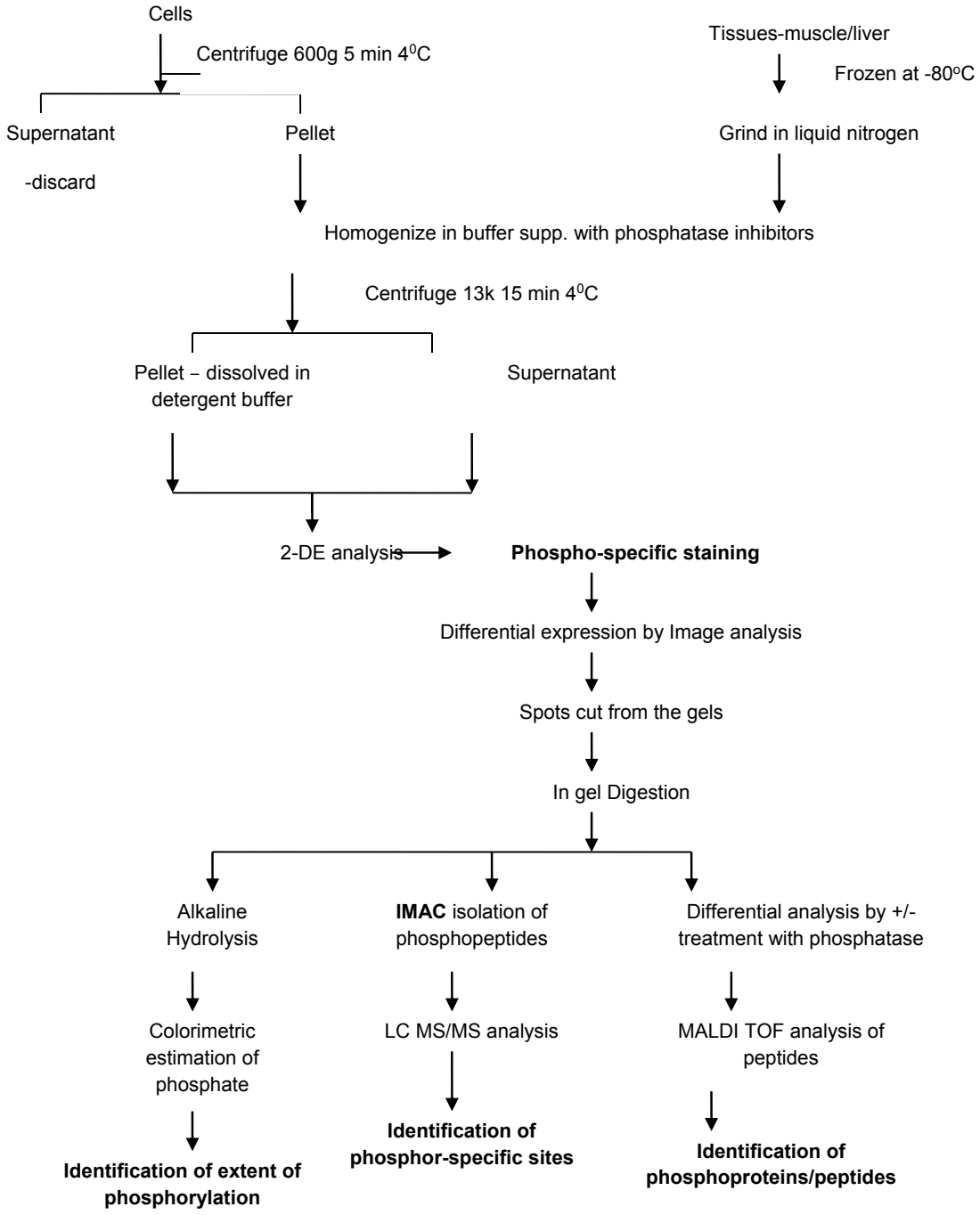

Figure 2: Phosphoprotein isolation, detection and identification by using Phosphospecific staining

Spot excision and in-gel digestion

Capture an image of the gel. Excise protein band using a clean scalpel and cut into 1 × 1 mm2 cubes. Transfer gel pieces to micro centrifuge tube washed with 50% methanol. If using Silver stain, de stain gel pieces before proceeding to next step. Add 100% methanol for 5 min to dehydrate gel pieces. Add sufficient volume of methanol to cover gel pieces. Remove 100% methanol and add 30% methanol for 5 min to rehydrate gel pieces. Remove 30% methanol and wash gel pieces twice for 10 min per wash with water (HPLC-grade). Wash gel pieces three times for 10 min per wash with 30% acetonitrile in 100 mM ammonium bicarbonate. Dry gel pieces in vacuum centrifuge (speed vac) for 15 min. Gel pieces will become opaque when dry. Add 10 mM DTT (enough volume to cover gel pieces) and let incubate for 1 h at 60°C to reduce protein disulfide bonds. Briefly centrifuge gel pieces and remove liquid. Add 55 mM iodoacetamide (enough volume to cover gel pieces) and let incubate for 45 min at room temperature in the dark to alkylate cysteine residues. Briefly centrifuge gel pieces and remove liquid. Wash gel pieces for 15 min with 100 mM ammonium bicarbonate. Remove liquid. Shrink gel pieces with 100% acetonitrile. Remove liquid. Dry gel pieces in vacuum centrifuge (speed vac) for 15 min. It is critical to ensure that gel pieces are completely dry to facilitate the absorption of trypsin into the gel

Figure 3: Phosphoprotein isolation, detection and identification by using Phosphospecific Affinity chromatography.

pieces in the next step. Rehydrate the gel pieces in trypsin solution at 4°C (on ice) for 45 min. Trypsin solution: 1.5 ng/µL trypsin in 50 mM ammonium bicarbonate. Add adequate volume of trypsin solution to completely cover gel pieces. Remove any remaining trypsin solution and add sufficient volume of digestion buffer to completely cover gel pieces. Let incubate at 37°C overnight. Briefly centrifuge gel pieces and transfer supernatant to another micro centrifuge tube washed with 50% methanol. Add 50 mM ammonium bicarbonate to cover gel pieces and incubate at 37°C with shaking for 15 min. Add volume of acetonitrile equal to volume of 50 mM ammonium bicarbonate utilized and incubate at 37°C with shaking for 15 min. Centrifuge gel pieces and add supernatant to the supernatant collected in earlier steps. Extract peptides: Add acetonitrile/5% formic acid (50:50) to gel pieces and incubate at 37°C with shaking for 15 min. Remove supernatant and combine with supernatant collected in earlier steps.

In-solution trypsin digestion

Use at least 50 µg protein for in-solution digestion. Protein sample should ideally be free of any detergents prior to in-solution digestion and LC-MS/MS. Perform buffer exchange with 100 mM ammonium bicarbonate using Micro con centrifugal filter devices. Be aware of the Molecular Weight Cut-Off (MWCO) for the filter devices. Optimum volume of the sample following buffer exchange is the volume that yields a protein concentration of ~0.1-1.0 µg/µL. Add methanol to 40% to denature protein. Add DTT to 5 mM final concentration to reduce protein disulfide bonds. Vortex and incubate for 30 min at 37°C. Alkylate cysteine residues by addition of iodoacetamide to 10 mM final concentration. Vortex and incubate for 1 h at room temperature in the dark. Stop alkylation reaction by adding DTT to 20 mM final concentration. Vortex and incubate for 1 h at room temperature. Add trypsin to sample at a ratio of 1 trypsin: 50 protein

and let incubate overnight at 37°C. Stop enzymatic digestion by acidifying with addition of acetic acid to 5% final volume. Dry sample in speed vac.

Immobilized Metal Affinity Chromatography (IMAC) for phosphopeptide isolation

Materials: PHOS-Select Iron Affinity Gel (Sigma P9740), SigmaPrep Spin column (Sigma SC1000), Milli-Q Water, HPLC Grade Acetonitrile, HPLC Grade Glacial Acetic Acid, Reagent Grade Ammonium Hydroxide

Reagent Grade Hydrochloric Acid (HCl), *p*H Test Strips

Reagents: Sample Acidifying Solution: 1M HCl = 82.5µl concentrated HCl in 1ml water.

Equilibration/Wash Solution: 250mM acetic acid in 30% acetonitrile = 144µl acetic acid in 10ml 30% acetonitrile/water.

Elution Solution: 150mM ammonium hydroxide in 25% acetonitrile = 102µl ammonium hydroxide in 10ml 25% acetonitrile/water. NOTE: Prepare fresh immediately before use with ammonium hydroxide from a tightly sealed bottle that has been stored at 4C.

Procedure:

A. Remove PHOS-Select Gel from freezer and allow to warm to room temperature. If already in solution, adjust sample pH to 2.5-3.0 with 1M HCl, if necessary, and add acetonitrile to ~30% (v/v). If sample is dry, reconstitute in Equilibration/Wash solution and adjust pH to 2.5-3.0 with 1M HCl if necessary. NOTE: The total volume of sample to be subjected to IMAC should be between 100 and 400µl.

B. Rinse a spin column by adding 400µl Equilibration/Wash Solution and mixing end over end for 5 minutes. Spin at 10000 rpm for 30 seconds in micro centrifuge and discard liquid.

C. Invert PHOS-Select Gel bottle several times to form a uniform suspension and measure an appropriate amount into a rinsed spin column. This is best accomplished using a pipette tip that has had the bottom trimmed off to allow the gel to enter the tip. The binding capacity of the gel is 1nmole/µl of packed gel.

D. Equilibrate gel by adding 500µl Equilibration/Wash Solution, vortex and spin at 10000 rpm for 30 seconds in micro centrifuge. Do this three times discarding the liquid from the collection vial.

E. Add sample to the equilibrated gel, vortex and mix end over end for a minimum of 1 hour for efficient binding of the phosphopeptides to the gel.

F. Spin at 10000 rpm for 30 seconds in micro centrifuge and discard liquid (unphosphorylated peptide fraction) in collection vial unless needed for further analysis.

G. Wash gel by adding 200µl Equilibration/Wash Solution to the gel, vortex and spin at 10000 rpm for 30 seconds in micro centrifuge. Discard liquid in collection vial or pool with the unphosphorylated peptide fraction if saving for further analysis. Repeat for a total of two washes.

H. Remove residual Equilibration/Wash Solution by adding 500µl water to the gel, vortex and spin at 10000 rpm in micro centrifuge for 30 seconds. Discard liquid in collection vial. Repeat for a total of two rinses.

I. Elute phosphopeptides by adding 200-400µl Elution Solution, vortex and mix end over end for 5 minutes. Spin at 10000 rpm for 30 seconds in micro centrifuge and save the liquid in the collection vial (phosphorylated peptide fraction) for subsequent mass spec analysis.

Liquid chromatography and mass spectrometry

Here we present a generic phosphoproteomics workflow based on a two-stage enrichment of phosphopeptides from digests of whole cell/tissue lysates. Phosphopeptides are enriched using strong cation exchange and TiO 2 chromatography, and their analysis is performed in the LTQ-Orbit rap mass spectrometer. This strategy was successfully used in the previously reported global qualitative and quantitative studies of eukaryotic [11] and prokaryotic [17] protein phosphorylation.

Materials: HPLC solvent "A": 0.5% acetic acid (Fluka, Cat. No. 45731) in water.

HPLC solvent "B": 0.5% acetic acid, 80% acetonitrile in water.

HPLC loading solvent: 1% trifluoroacetic acid, 2% acetonitrile in water.

Stage tips [18,19]: Empore C8 Disk (Varian, Cat. No. 12145002).

Reversed phase material for nano-HPLC column [20]: Reprosil-Pur C18-AQ, 3 µm (Dr. Maisch, Cat. No. r13.aq).

METHODS

Liquid chromatography

1. The Liquid Chromatography (LC) part of the analytical LC-MS system described here consists of an Agilent 1200 Series nanoflow LC system (Agilent Technologies) comprising a solvent degasser, a nanoflow pump, and a thermostated micro-autosampler with an 8- µL injection loop.

2. Pack an analytical column in a 20-cm fused silica emitter (Proxeon Biosystems, 75- µm inner diameter with a 5- µm laser-pulled tip), with a methanol slurry of reverse-phase C18 resin at a constant helium pressure (50 bar) using a bomb-loader device (Proxeon Biosystems), as described previously [20].

3. Connect the packed emitter (C18 RP HPLC column) directly to the outlet of the 6-port valve of the HPLC autosampler through a 20-cm long, 25- µm inner diameter fused silica transfer line (Composite Metals) and a micro Tee-connector (Upchurch) ("liquid junction" connection)

4. Load 6 µL of the tryptic phosphopeptide mixture using the HPLC autosampler onto the packed emitter at a flow of 500 nL/min (typical back-pressure of 130–200 bar) for 20 min using 2% of HPLC solvent B.

5. After loading, by-pass the sample loop, reduce the flow-rate to 200 nL/min and increase the HPLC solvent B content to 10%.

6. Separate and elute the bound peptides with a 90 min linear gradient from 10 to 30% of HPLC solvent B. Wash-out

hydrophobic peptides by linearly increasing the HPLC solvent B content to 80% over 15 min.

Mass Spectrometry

1. All mass spectrometric experiments discussed here are performed on an LTQ orbitrap "XL" or "Classic" mass spectrometer (Thermo Fisher Scientific) connected to an Agilent 1200 Series nanoflow LC system (Agilent Technologies) via a nanoelectrospray LC-MS interface (Proxeon Biosystems)
2. Operate the mass spectrometer in the data-dependent mode to automatically switch between MS and MS/MS using the Tune and Xcalibur 2.4 software package.
3. Use the following settings in the "Tune" acquisition software: (a) FT full scan: accumulation target value 1E6; max. fill time 1,000 ms (b) FT MS n: accumulation target value 5E4; max. fill time 500 ms. IT MS n : accumulation target value 5E3; max. fill time 150 ms
4. For accurate mass measurements enable the "Lock mass" option in both MS and MS/MS mode in the Xcalibur software [21]. Use the background Polydimethylcyclosiloxane (PCM) ions generated from ambient air (e.g., m/z = 445.120025) for internal recalibration in real time. For single SIM scan injections of the lock mass ion into the C-trap, set the lock mass "ion gain" at 10% of the target value of the full mass spectrum. If the fragment ion measurements are performed in the orbitrap, use the PCM ion at m/z = 429.088735 (PCM with neutral methane loss).
5. In the "Xcalibur Instrument Setup" create a data-dependent acquisition method in which full scan MS spectra, typically in the m/z range from 300 to 1,800, are acquired by the orbitrap detector with resolution R = 60,000.
    a. For high-accuracy and full mass range measurements of fragment ions, set the data-dependent MS 2 of the three most intense multiply charged ions to be measured in the FT analyzer (orbitrap) at the resolution of R = 15,000 and enable the HCD option (Higher-energy C-trap dissociation). Set the first mass in mass range to m/z = 80. This will allow for low mass reporter ions such as immonium ions to be identified [22].
    b. For fast-scanning and high-sensitivity but low resolution measurements of fragment ions, set the data-dependent MS 2 of the five most intense multiply charged ions to be measured in the linear ion trap. Enable the preview mode for FTMS master scans to perform data-dependent MS 2in-parallel with the full scan in the orbitrap. Set the fragmentation mode to CID and enable the Multi-Stage Activation (MSA) fragmentation option by which the neutral loss species at 97.97, 48.99, or 32.66 m/z below the precursor ion will be successively activated for 30 ms each [23].
6. Standard acquisition method settings:
    a. Electrospray voltage, 2.4 kV
    b. No sheath and auxiliary gas flow.
    c. Ion transfer (heated) capillary temperature, 150°C.
    d. Collision gas pressure, 1.3 mTorr.
    e. Dynamic exclusion of up to 500 precursor ions for 60 s upon MS/MS; exclusion mass width of 10 ppm.
    f. Normalized collision energy using wide-band activation mode; 35% for both CID and HCD.
    g. Ion selection thresholds: 1,000 counts for CID and 10,000 counts for HCD.
    h. Activation q = 0.25; Activation time = 30 ms.

Database Searching and Phosphorylation Site Identification

- The raw MS/MS fragmentation data must be processed using database search engine software such as commercially available Sequest (Thermo Fisher Scientific) or Mascot (Matrix Science, Ltd.) at http://www.matrixscience.com/. These software programs contain algorithms to extract MS/MS data files for querying protein databases such as the annotated all-species Swiss-Prot (http://www.uniprot.org/downloads) or species specific databases from IPI (http://www.ebi.ac.uk/IPI/). Alternatively, one can use freeware search engines such as XTandem! (http://www.thegpm.org/tandem/) or Andromeda within Max Quant software environment (http://www.maxquant.org/).
- Once downloaded, it is recommended that the protein database is also set up as a decoy database. This can be either a database of reversed sequences or random sequences and is usually concatenated with the target (forward) database. This allows one to calculate a False Discovery Rate (FDR) in order to statistically evaluate the search results.
- In the search engine, use the following parameters:
    a. Enzyme: Trypsin (cleavage at C-term of Lys (K)/Arg (R)
    b. Precursor mass tolerance: ≤ 15 ppm for a well-calibrated mass spectrometer
    c. Differential modifications: oxidation + 15.9949 on Met (M); phosphorylation + 79.9663 on Ser (S)/Thr (T)/Tyr (Y)
    d. Fragment ion tolerance: ≤ 15 ppm for high mass accuracy MS/MS (HCD, TOF, Orbitrap) and ~ 0.08 Da for low mass accuracy MS/MS (ion trap CID, quadrupole CID)

NOTE: Alternatively, one can use a single entry database with no enzyme specificity. This is useful when enzymes other than trypsin or combinations of enzymes were used for digestion of the protein; however, more rigorous validation is required.

Notes

1. All solvents in this protocol should be prepared with high purity deionized Milli-Q water of the resistivity 18.2 MΩ cm (Millipore Q-Gard 2 cartridge). This deionized water is referred to as "water" in the text. Denaturation, reduction and alkylation buffers can be frozen as stock solutions at −20°C.
2. The entire protocol is optimized for protein amounts from 2

to 20 mg. During cell lysis and protein extraction, keep the salt content at a minimum since it may interfere with the SCX chromatography. If needed, precipitate proteins with acetone or chloroform/methanol prior to analysis.

3. Do not heat-up the samples during protein solubilization a digestion; high concentration of urea will lead to carbamylation of free amino groups.

4. If necessary, adjust the pH with a very low volume of 1 M Tris–HCl, *p*H 8.0; note that the total salt concentration should not exceed 10 mM since it may interfere with the SCX chromatography.

5. To keep the salt concentration low, use pure water rather than ammonium bicarbonate buffer to dilute the sample prior to trypsin incubation.

6. During sample loading onto the SCX column it is important to monitor conductivity; under the conditions employed, conductivity higher than 4 mS/cm will lead to decreased binding of peptides – in that case the flow-through should be diluted with water and re-loaded onto the column.

7. Collection and subsequent separate analysis of the flow through is extremely important since multiply phosphorylated peptides will not bind to the SCX column; this fraction is usually the richest in the number of identified phosphorylation sites, especially in the analysis of eukaryotic phosphorylation.

8. It is recommended that the conductivity be monitored throughout the SCX run, as it is the best measure for the gradient stability; under conditions employed, at 30% of the SCX solvent "B" the conductivity typically reaches 13 mS/cm.

9. This step is optional and should be used to increase the binding specificity in the samples with very little phosphorylation (e.g., prokaryotic cell lysates); otherwise it should be avoided because it increases the probability of DHB contamination during LC-MS. Alternatively, it is possible to use lactic acid as a competitive binder instead of DHB [24].

10. C8 microcolumns are also commercially available (Proxeon Biosystems, Order Code SP121).

11. To quickly neutralize the high pH upon elution, elute the samples into a small volume (~20 µL) of the HPLC loading solvent.

12. Do not heat the samples during vacuum concentration and make sure they do not run dry.

13. The advantage of the resin used is the fact that it is active at low organic buffer content (e.g., less than 2% acetonitrile). Thereby lower amount of hydrophobic ion-pairing reagents such as heptafluorobutyric acid (HFBA), which interfere with the electrospray ionization, can be used in the HPLC solvents.

14. Note that there is no pre-column or split in this LC-MS setup.

15. Control the timing between the MS and the LC system with a standard double contact closure cable.

16. Resolution is defined at m/z = 400.

17. This gives a total scan cycle time (full scan + 3 MS 2 events) of up to 5 s.

18. This gives a total scan cycle time (full scan + 5 MS 2 events) of up to 3 s.

19. Multistage activation produces information-rich spectra, where many of the fragment ions show pronounced neutral loss of phosphoric acid (e.g., −97.97, −48.99, or −32.66 for singly, doubly or triply charged fragment ions, respectively). This information is very useful in validation of the phosphopeptide spectra.

20. Source settings have to be optimized for the emitter and nano-LC-MS setup.

REPRESENTATIVE EXAMPLES OF PHOSPHO-PEPTIDE ANALYSIS BY PROTEOMICS

Detection of Perlican phosphopeptides on MALDI

Sample: Phosphorylated (peri 276-p and peri 492-p) and dephosphorylated peptides of perlican (per 276 and 492 peptides) dissolved in water are used

Procedure:

- About 50 picomoles of peri 276-p and peri 492-p is dephosphorylated by incubation with equal volume of alkaline phosphatase diluted 1:10 times in 100mM ammonium bicarbonate for 5 min. The reaction is stopped by addition of α-cyno-4-cinnamic acid matrix (10mg/ml in 50% acetonitrile and 50% ethanol).

- Three samples (15 pmoles each), peri 276/peri 492, peri 276-p/peri 492-p and dephosphorylated peptides of peri 276-p/peri 492-p are co-crystallized with α-cyno-4-cinnamic acid matrix on the MALDI plate separately. .

- The MALDI spectrum is acquired for the three samples.

Note: The spectrums of peri 276 and dephosphorylated peri 276 should look the same. The difference of 80-90Da between both the spectrums of peri 276 and peri 276-p confirms the detection of phosphorylated state of the peptides. The same goes for peri 492 peptides.

Identification of *in vitro* E coli GST tagged phosphoprotein

Sample: The phosphoprotein is bound to beads with the GST tag. The phosphorylated (GST-protein-p) and unphosphorylated (GST-protein) forms of protein samples are used. The GST-protein is phosphorylated in vitro in presence of cold orthophosphate and purified protein kinase.

Procedure:

- About 20 µg of GST-protein-p and GST-protein are dissolved in Biorad's reagent 3 (5M urea, 2M Thiourea, 2% w/v CHAPS, 2% w/v SB-3-10, Tris 40mM, 5mM EDTA, phosphatase inhibitor cocktail, protease inhibitor cocktail and Biolyte 3-10 0.2% (v/v) and the protein isolated from the beads.

- The isolated proteins are separated on 2DE PAGE. First the proteins are separated by IEF on 3-10 IPG strips and in second dimension on 12.5% SDS PAGE. The gel is stained by silver stain.

- Since the protein of interest is known in this case, the protein spot is cut and digested in gel by 30µg of trypsin in ammonium bicarbonate.

- Alternatively the protein of interest can be detected by staining the gel with Pro-Q Diamond stain prior to digestion.
- The peptides thus obtained are treated as mentioned above in experiment 1. In short, the peptides obtained from phosphoprotein and unphosphorylated proteins are treated with alkaline phosphatase and the samples subjected to MALDI analysis. The peptides showing difference in intensities are analyzed for loss or gain of 80-90Da.
- An aliquot of the digest is subjected to IMAC column purification (microtip columns packed with POROS MC, charged with 100mM FeCl3/GaCl3, washed with 30 µl of 0.1% acetic acid and peptides eluted in 15 µl of 200 mM NaPO4, pH 8.4 for purification of phosphopeptides and subsequent LC- MS/MS analysis.

Identification of *in vivo* phosphorylation from animal tissues

Model: Normal and treated rats/mice with drug that activates AMPK.

Sample: Skeletal muscle and liver.

Procedure:

- After muscle and liver excision the tissues should be immediately frozen in liquid nitrogen and stored at -80°C.
- Frozen tissues are powdered in liquid nitrogen and dissolved in ice-cold buffer (50 mM HEPES, 150 mM NaCl, 5% glycerol, 5 mM EGTA, 2 mM $MgCl_2$, pH 7.4, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS) containing a cocktail of protease inhibitors and phosphatase inhibitors and incubated in ice for 1 hr. The liver is very soft and gets homogenized very fast. Homogenization of skeletal muscle should be done with care to ensure complete extraction of proteins.
- The sample is then centrifuged at 4000g at 4^0C for 15 min. The pellet is discarded.
- The supernatant of normal and treated samples are then processed for 2-D analysis
- Cut the spots and after trypsin digestion and processing, analyze them by MALDI/LC-MS/MS analysis.

Note: AMPK is generally activated in skeletal muscles by acute exercise. Not only AMPK but MAPK and JUNK also get phosphorylated and thus get activated by acute exercise. AMPK is activated by AMPK kinase. This attempt is to view all these protein phosphorylations in a snap shot here.

Colorimetric estimation phosphate

The phosphate from the phosphoprotein/phosphopeptides is first extracted by alkaline hydrolysis of phosphate esters of serine or threonine, the released inorganic phosphate is precipitated with calcium ions, detected by formation of an insoluble phosphomolybdate complex and then visualization of the complex with methyl green, malachite green dye. The developed color is proportional to the number of phosphate present at the phosphoprotein sites. The color is quantitated at 550 nm.

High throughput analysis for drug screening

Once the disease specific phosphoprotein that is highly regulated is identified, specific in house arrays can be designed for high throughput screening. The arrays will be specific to specific phosphoprotein.

The above methods can be applied strategically to the following applications for identification of *in vivo* phosphorylation, phosphosite detection and stoichiometric analysis of phosphates per protein.

APPLICATIONS

The abnormal phosphorylation of proteins is a cause or consequence of major diseases like cancer, diabetes and rheumatoid arthritis [25]. Between a quarter and a third of drug discovery programmes worldwide are now concentrated on targeting protein kinases and phosphatases. This drug discovery strategy has enormous potential since many proteins that will be identified as key players in pathological conditions will be up-or down-regulated, directly or indirectly by kinases or phosphatases. Thus the design and synthesis of selective, potent, cell permeable drug-like inhibitors of kinases and phosphatases holds great potential for future drug discovery programmes.

Drug Toxicity

Phosphoproteome profiling as metabolic signatures are now being applied to drug toxicity and drug efficacy in model organisms, as well as in humans and plants. Many nucleoside analog drugs, such as ribavirin and viramidine, are activated or metabolized *in vivo* through 5'-phosphorylation and dephosphorylation [26].

Metabolic Disorders

Type 2 diabetes and obesity are metabolic disease states characterized by impairments in glucose and fatty acid metabolism mediated by affected phosphorylation on key proteins like insulin tyrosine receptor kinase [27-29], AMPK [30] etc leading to a cascade of intracellular events which mediate the biological effect. Furthermore, the oral hypoglycemic agents rosiglitazone and metformin as well as hormones integral to the regulation of body adiposity, including leptin and adiponectin, are known to act in part through AMPK-mediated pathways

Apoptosis

Research in the area of programmed cell death has been an important focus of researchers since defective signaling through apoptotic pathways contributes to fatal diseases such as cancer, heart disease, Parkinson's disease [31] and AIDS. The major regulation of these pathways is through phosphorylation and dephosphorylation.

Oncology

Tyrosine kinases play a prominent role in human cancer, yet the oncogenic signaling pathways driving cell proliferation and survival have been difficult to identify, in part because of the complexity of the pathways and in part because of low cellular levels of tyrosine phosphorylation [32].

Immune disorders

Major peptides presented to immune system as antigens by foreign cells or cancer cells are phosphopeptides. Thus such peptides can be striped from the cells and then identified and studied in detail for antigenic properties by phosphopeptide profiling [33].

Conclusions and Future Directions

As described above, a variety of powerful MS methods for

detecting and characterizing phosphoproteins and phosphopeptides is available. Despite this formidable armamentarium, complete characterization of the phosphorylation state of a protein isolated in small quantities from a biological sample remains far from routine. The following is a list of analytical tools and methods that will be required to make comprehensive phosphorylation analyses routine:

1. A method for selectively enriching/purifying phosphoproteins and/or phosphopeptides that ensures recovery of all phosphorylated species and gives a low background of unphosphorylated species. Improved phospho-directed antibodies and chemistry hold much promise in this regard.
2. A method to ensure that all sites of phosphorylation are detected. Although phospho-directed chemistry and MS detection based on the lability of the phosphate moiety appear to be worthwhile avenues of pursuit, exhaustive detection of all sites of phosphorylation remains a most challenging analytical task.
3. A method for pinpointing the sites of phosphorylation. Current CID MS/MS methods are already reasonably effective, and newer methods such as electron capture dissociation hold much promise for further improvements.
4. A universal method for measuring changes in phosphorylation in vivo. Metabolic labeling and phosphorylation site labeling with stable isotopes appear to be viable approaches for attaining this goal.

In addition to the study of individual phosphoproteins, it remains critical to develop methodologies for elucidating the phosphorylation state of collections of proteins at an organelle-wide or cell-wide level. Such global techniques promise to provide a new window into the inner workings of the cell.

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