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Phosphoproteomics: Detection, Identification and Importance of Protein Phosphorylation

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1. Introduction

Reversible protein phosphorylation is one of the most important and well explored post-translational modifications. It is estimated that 30- 50% of the proteins are phosphorylated at some time point (Kalume, Molina, & Pandey, 2003). Phosphorylation is a major regulatory mechanism that controls many basic cellular processes. It may mediate a signal from the plasma membrane to the nucleus using a cascade of proteins, by which to regulate physiological and pathological processes such as cell growth, proliferation, differentiation and apoptosis (Blume-Jensen & Hunter, 2001; Hunter, 2000). Protein phosphorylation may result in alteration in protein- protein interactions, protein intracellular localization, and its activity (Blume-Jensen & Hunter, 2001; Kalume et al., 2003). Approximately 30% of drug discovery programs and R&D investment by the pharmaceutical industry target protein kinases. Knowledge of exactly when and where phosphorylation occurs and the consequences of this modification for the protein of interest can lead to an understanding of the detailed mechanism of the protein action, and ultimately to the discovery of new drug targets.

Protein phosphorylation is a fast and reversible process. It is catalyzed by kinases by attaching phosphate groups onto specific amino acids. Opposed to phosphorylation, dephosphorylation removes the phosphate groups from proteins by phosphatases. Dephosphorylation plays important role in balancing the protein phosphorylation status in signaling proteins. About 2-3% of the human genome encodes 518 distinct protein kinases (Manning et al., 2002). Four types of phosphorylation have been described based on the phosphorylation sites: (a) O-phosphorylation (serine, threonine and tyrosine), (b) N-phosphorylation (arginine, histidine and lysine), (c) S-phosphorylation (cysteine) and (d) acylphosphorylation (aspartic acid and glutamic acid) (Reinders & Sickmann, 2005). Currently, analytical methods have mainly been developed for O-phosphorylation, which is due to chemical stability of O-phosphorylation in acidic and in neutral milieu. Therefore, O-phosphorylation is the best studied among various types of phosphorylation (Reinders, 2002). In eukaryotic cells, phosphorylation occurs primarily on serine (pSer), threonine (pThr), and tyrosine (pTyr) residues, that is estimated to be in the ratio of 1800:200:1/pSer: pThr: pTyr (Kersten et al, 2006).

As aforementioned, phosphorylation is of importance for cell signaling and drug development. The lack of technologies to study all types of phosphorylation, differences in abundance and high dynamics make it difficult to have a comprehensive cover of all

phosphorylation events in cells. This chapter summarizes strategies that have been developed to characterize the phosphoproteome. These strategies include identification of phosphoproteins and phosphopeptides, localization of the exact phosphorylation sites and quantitation of phosphorylation. In addition, the applications of phosphoproteomics in life science are discussed.

2. Phosphorylation

2.1 Detection of phosphoproteins

2.1.1 Radioactive labeling of proteins with ^{32}P isotope

Radioactive labeling of proteins with ^{32}P or ^{33}P is the oldest, but still one of the most sensitive approaches for detection of phosphorylation. Under the appropriate condition, the phosphoryl groups of ^{32}P or ^{33}P are enzymatically added to the proteins. The phosphorylated proteins are then detected by autoradiography. Therefore, radioactive labeling detects all types of phosphorylation, and is not specific to only one type of phosphorylation. The proteins can be labeled with $^{32}\text{P}/^{33}\text{P}$ isotopes in vitro and in vivo. For in vitro labeling, $[\gamma\text{-}^{32}\text{P}/^{33}\text{P}]\text{-ATP}$ is used. It is a fast and convenient process, that requires (semi)purified kinase and substrate (Springer, 1991). A kinase phosphorylates its substrate in a defined mixture of the kinase, substrate, buffer, ions, ATP and $[\gamma\text{-}^{32}\text{P}/^{33}\text{P}]\text{-ATP}$. However, since the enzymatic reaction takes place in vitro, the major disadvantage is that it may not reflect the kinase activity under physiological conditions. This problem was overcome by introduction of the in vivo metabolic labeling (Wyttenbach & Tolkovsky, 2006). $^{32}\text{P}/^{33}\text{P}$ Orthophosphate is used in in vivo labeling as a source of the isotope. The radioactive orthophosphates are incorporated during metabolic processes by kinases in cells. The significant advantage of in vivo labeling is that it provides a more accurate scenario of physiological enzymatic events, and reflects cellular responses as a consequence of treatments. The drawback of in vivo labeling also exists, e.g. it has been reported that in vivo labeling with doses of radioactivity may induce DNA fragmentation, DNA repair processes, subsequently may result in the cell cycle arrest and apoptosis. Another concern is that for in vivo labeling is usually used phosphate-free medium to culture cells. This medium may differ from the medium cells are cultured. Therefore, in vivo labeling experiments are often limited in time to 4- 8 hours. The third concern of radioactive labeling (in vitro and in vivo) is that only very small amount of radioactivity will be incorporated in proteins. This requires protocols for thorough removal of non-incorporated radioactivity from phosphoproteins. The fourth concern of radioactive labeling is safety requirements. As the assays use radioactivity, corresponding safety rules have to be applied. Thus, it is very important to control quantity of the isotope and duration of labeling, take care of safety issues, and to minimize artificially-induced changes in phosphorylation.

2.1.2 Phospho-specific antibodies

In 1981, the first documented phospho-antibody was produced in rabbits immunized with benzoyl phosphonate conjugated to keyhole limpet hemocyanin (KLH) (Ignatoski, 2001). This antibody broadly recognized proteins containing phosphotyrosine. After that, there has been a rapid development in production of the phospho-antibodies. Nowadays, a large amount of phospho specific antibodies targeted to different amino acids (Ser, Thr, Tyr) at distinct sites in proteins have been produced, and widely used in the basic and clinic research (Ignatoski, 2001; Izaguirre, Aguirre, Ji, Aneskievich, & Haimovich, 1999). The

availability of phospho-specific antibodies has opened the door for the improvement of detection of phosphorylation. The advantages of phospho-antibodies consist in 4 issues. The first one is that the antibodies can be used not only with extracted proteins and peptides, but also for intact cells or tissues. The second issue is that the specificity and sensitivity may be very high if an antibody has really good quality, and antibody can detect the epitope down to femtomole range. The third issue is the antibodies can be used to enrich and purify phosphorylated proteins and peptides. The fourth issue is that there are many antibodies very useful for phosphotyrosine detection, with good specificity and minimal reactivity to either unphosphorylated tyrosine or phosphorylated serine/threonine residues. The major decisive factor for selection of antibodies is their specificity in detection of a phosphoprotein. Therefore the quality of antibodies becomes the key concern on their applications.

2.1.3 Phosphoprotein staining

Detection of phosphoproteins by staining proteins separated in the acrylamide gels with phosphor-specific dye has been widely used for almost forty years (Green et al., 1973). Historically, several phospho-staining protocols were used. The cationic carbocyanine dye "Stains-All" stains phosphoproteins, but also highly acidic proteins, DNA and RNA (Green et al., 1973). This dye is not commonly used due to its low sensitivity that is one order magnitude less sensitivity than Coomassie staining and several orders less than ^{32}P radioactivity labeling. An alternative method involves the alkaline hydrolysis of phosphate esters of serine or threonine, precipitation of the released inorganic phosphate with calcium, formation of an insoluble phosphomolybdate complex and then visualization of the complex with a dye such as malachite green, methyl green or rhodamine B (Debruyne, 1983). The detection sensitivity of the staining method is still very poor, as a protein containing roughly 100 phosphoserine residues is detectable. Besides low sensitivity, phosphotyrosine is not to be detected as it cannot be hydrolyzed. Currently, ProQ Diamond has increasingly become the first choice of phosphoprotein dye (Steinberg et al., 2003). It is a fluorescent dye, and is suitable for the detection of phosphoserine-, phosphothreonine-, and phosphotyrosine-containing proteins directly in acrylamide gels. The sensitivity of ProQ Diamond staining has been improved significantly, and is down to 1- 16 ng. However, it is still considerably less sensitive than radioactive methods. The major advantages of ProQ Diamond are constituted of 1) it can be used in combination with a total protein stain, such as SYPRO Ruby protein gel stain, allowing protein phosphorylation levels and expression levels to be monitored in the same gel, 2) it is not dependent on kinase activity, 3) greater convenience and safety of handling, and 4) the stain also seems to be specific. However, in complex protein samples with thousands of protein species resolved by 2DE some nonphosphorylated, but rather abundant proteins may also be weakly stained (Stasyk et al., 2005).

2.1.4 Mass spectrometry (MS)

Mass spectrometry is one of the most modern techniques for detection of phosphorylation. Introduction of MS has significantly advanced the research in protein phosphorylation (Peters et al., 2004). It may be applied not only for detection of phosphorylation, but also identification of phosphorylation sites. Detection of phosphorylation by MS has been based on mass spectrum generated by trypsin-digested peptides. The mass shift of m/z 79.9 or neutral loss m/z 80 or 98 compared to its theoretical peptide mass has normally been

considered as occurrence of phosphorylation. MS provides also a high speed and high sensitivity means for detection of phosphorylation. However, there are several inherent difficulties for the analysis of phospho-proteins. Firstly, signals from phosphopeptides are generally weaker as compared to non-phosphorylated peptides, as they are negatively charged and poorly ionized by MS performed in the positive mode. Secondly, it can be difficult to observe the signals from low-abundance phospho-proteins of interest in the high-background of abundant non-phosphorylated proteins. To overcome these drawbacks, enrichment of phosphoproteins or phosphopeptides before MS is necessary to apply.

2.2 Isolation and enrichment of phosphorylated proteins and peptides

2.2.1 Immunoprecipitation

Phosphospecific antibodies are an efficient tool for enrichment of phosphorylated proteins (Rush et al., 2005). Antibodies specific to phosphorylated residues are used to immunoprecipitate full-length proteins and phosphopeptides. The most notable advantage of this approach is the sensitivity provided by antibodies, as we discussed in 2.1.2. Nowadays, a variety of commercial phospho-specific antibodies with high quality are available, especially antibodies for phosphotyrosine. The lack of high quality phosphoserine/ threonine antibodies impedes the characterization of serine or threonine phosphorylations.

2.2.2 Immobilized metal affinity chromatography (IMAC)

IMAC (Andersson & Porath, 1986) is the most frequently used technique for phosphopeptide and phosphoprotein enrichment, although it was originally introduced for purification of His-tagged proteins. It employs metal chelating compounds which are covalently bound to a chromatographic support for the coordination of metal ions. Phosphorylated peptides or proteins are bound to the IMAC stationary phase by electrostatic interactions of its negatively charged phosphate group with positively charged metal ions bound to the column material via nitriloacetic acid (NTA), iminodiacetic acid (IDA), and Tris (carboxymethyl) ethylenediamine (TED) linkers. Immobilized metal ions such as Ni^{2+} , Co^{2+} , or Mn^{2+} were initially shown to bind strongly to proteins with a high density of histidines. However, immobilized metal ions of Fe^{3+} , Ga^{3+} , and Al^{3+} have been demonstrated to show better binding with phosphopeptides. On the basis of measurements of ^{32}P or ^{33}P -radioactivity in whole cell extracts and in phosphoprotein samples after enrichment, IMAC-based techniques have been reported to recover up to 70–90% of total phosphoproteins (Dubrovskaya & Souchelnytskyi, 2005). IMAC procedures have become very popular rapidly due to its good compatibility with subsequent separation and detection techniques such as LC-ESI-MS/MS and MALDI MS. One of the major drawbacks of IMAC-based strategies is the nonspecific binding of peptides containing acidic amino acids, that is Glu and Asp, and the strong binding of multiply phosphorylated peptides. Nonspecific binding of acidic peptides can be diminished by esterification of carboxylic acids to methyl esters using HCl-saturated dried methanol (Ficarro et al., 2002). Reaction conditions have to be chosen carefully to avoid both incomplete esterification and side reactions because they increase sample complexity. Another disadvantage is that despite following a common binding-washing-eluting procedure, IMAC experimental conditions are very variable and care should be taken, as small variations in the experimental conditions (for example, pH, ionic strength, or organic composition of the solvents) could drastically affect the selectivity of the IMAC stationary phase.

2.2.3 Strong cation exchange chromatography (SCX)

Strong cation exchange chromatography has been used in the enrichment of phosphorylated peptides (Peng et al., 2003). This procedure is based on the fact that under acidic conditions (pH 2.7) phosphorylated peptides are single positively charged and amenable to further separation from nonphosphorylated peptides that usually have a net charge of 2+ at low pH. One of the main advantages of this method is that complex peptide mixtures can be analyzed directly, since it can be connected directly to LC-MS/MS for identification or sequencing (Villen & Gygi, 2008). However, this strategy does not have high specificity and the fractions enriched in phosphopeptides also contain a high percentage of contaminants. Therefore, it's very common to combine SCX with other enrichment methods, i.e IMAC and TiO₂.

2.2.4 Titanium dioxide (TiO₂)

A promising alternative to the use of IMAC for the enrichment of phosphorylated peptides was first described by Pinkse et al (Pinkse et al., 2004). The approach is based on the selective interaction of water-soluble phosphates with porous titanium dioxide microspheres via binding at the TiO₂ surface. Phosphopeptides are trapped in a TiO₂ precolumn under acidic conditions and desorbed under alkaline conditions. An increased specificity for phosphopeptides has been reported. Another advantage of this approach is that it can be easily coupled with a LC-MS/MS workflow (Ishihama et al., 2007; Marcantonio et al., 2008). Nevertheless, TiO₂-based columns may retain nonphosphorylated acidic peptides. Peptide loading in 2, 5-dihydroxybenzoic acid (DHB) has been described to efficiently reduce the binding of nonphosphorylated peptides to TiO₂ while retaining high binding affinity for phosphorylated peptides. This improved TiO₂ procedure was found to be more selective than IMAC.

2.2.5 Chemical modification

Biotin tagging by β -elimination and Michael addition

A number of chemical modification strategies were developed in which the phosphate group has been replaced with a moiety that is chemically more stable than phosphate. One such method employs β -elimination of the phosphate from phosphothreonine or phosphoserine and results in the formation of dehydroaminobutyric acid or dehydroalanine, respectively. This product can be detected directly using tandem MS (Thompson et al., 2003). Alternatively, Michael addition is used to add a reactive thiol to dehydroaminobutyric acid or dehydroalanine to allow attachment of an affinity tag. Biotin is a widely used affinity tag and it permits purification of the chemically modified (previously phosphorylated) peptides (Meyer et al., 1991). This chemical modification is not applicable to phosphotyrosine residues and suffers from side reactions in which nonphosphorylated serine can be tagged.

2.3 Identification of phosphorylation sites

2.3.1 Two-dimensional (2D) phosphopeptide mapping

2D phosphopeptide mapping is a traditional biochemical method for identification of protein phosphorylation sites (Blaukat, 2004). After metabolically labeling cells with radioactive phosphate, the protein of interest is isolated by immunoprecipitation, subsequently subject to enzymatic digestion. The digested phosphopeptide is visualized by

2D phosphopeptide mapping (electrophoresis and thin-layer chromatography). To determine a phosphorylation site, labeled spots from the 2D phosphopeptide map are excised, and a combination of phosphoamino acid analysis and Edman sequencing is performed by monitoring the loss of radioactivity in each cycle. It should be noted that phosphorylation sites identified by 2D mapping need further validation (Nagahara et al., 1999). The most common way for confirmation is to mutate the phosphorylation sites and compare the phosphopeptide maps for the wild-type with those from mutant proteins. Although this method for phosphorylation identification is very useful, it still contains some limitations. It is time consuming process; care must be taken when label the cells with radioactivity; it only studies single protein, and can not apply to large scale identification of phosphorylation sites. Attempts were also made to combine 2D phosphopeptide mapping and MS analysis of recovered phosphopeptides by using 2D phosphopeptide mapping and HPLC purification before MS (Figure 1).

Fig. 1. An overview of techniques for enrichment and analysis of phosphorylated proteins or peptides using MS-based detection methods.

2.3.2 MS fragmentation

2.3.2.1 Collision-induced dissociation (CID)

PTMs on proteins often show greater susceptibility to cleavage by collision-induced fragmentation in the mass spectrometer than the peptide backbone. This characteristic may be used in different analytical strategies: 1) detection of the low mass 'signature' or 'marker' ions generated from the modification itself, 2) detection of the loss of the modification from the peptide precursor. Such targeted MS/MS analysis can enhance the specificity and sensitivity of phosphopeptide analysis, particularly for complex samples consisting of mixtures of phosphorylated and nonphosphorylated peptides. There are two most common precursor ion-scanning modes implemented on triple quadrupole mass spectrometers

(Figure 1). When phosphopeptides are fragmented by CID in the negative ion mode, a characteristic product ion (PO3) is generated giving rise to a peak at m/z 79 in the product spectrum (Collins et al., 2005). The detection of this marker ion has been used in various analytical setups. For example, a list of putative phosphopeptide ions can be generated by precursor ion scanning, a follow up analysis in positive ion mode is then performed to sequence these candidates by MS/MS using DDA (data-dependent acquisition) mode. Alternatively, detection of the precursor ion can be performed in positive ion mode, conduct MS/MS sequencing directly. Phosphotyrosine-containing peptides yield a characteristic immonium ion at m/z 216.043 from the loss of phosphotyrosine in the positive ion mode CID. Therefore, targeted monitoring of the precursor ion of 216.043 is useful for the detection of phosphotyrosine-containing peptides. This method was reported with good sensitivity, enabling the detection phosphotyrosine peptides from subpicomole amounts of gel-separated proteins (Steen et al., 2001).

2.3.2.2 Electron capture dissociation (ECD) and Electron transfer dissociation (ETD)

In tandem mass spectra of phosphopeptides generated by CID, limited or weak fragment ions spectra produce many false-negative as well as false-positive identifications especially for large, multiply charged and/or multiply phosphorylated peptides. Emerging alternative fragmentation techniques such as electron capture dissociation (ECD) and electron transfer dissociation (ETD) provide complementary sequence information for protein and peptide characterization, and are also applicable to the analysis of post-translational modifications (PTMs). These approaches induce more extensive cleavage along the peptide backbone and therefore provide excellent sequence tags, which retain labile PTMs (such as phosphorylation, glycosylation, acylation, ubiquitination and sumoylation) on backbone fragments. This feature enables direct and unambiguous assignment of the sites of modification. A further benefit is that these approaches are better suited for the analysis of large peptides, permitting the detection of multiple PTMs.

In ECD, multiply protonated ions capture low energy electrons and upon the following charge neutralisation, the resulting radical cations dissociate along the peptide backbone to produce a series of c and z type fragment ions while retaining the labile PTM group (Zubarev et al., 1998). Since the electron capture process requires low energy electrons (<10 eV) and long interaction times, the application of ECD was traditionally confined to instruments that employ static electromagnetic fields that avoid energizing or heating electrons, such as Fourier transform ion cyclotron resonance (FT-ICR) MS. Recently however, the addition of magnetic fields to ion traps have allowed for ECD in such electrodynamic trapping instruments (Baba et al., 2004) and the use of ECD in a digital ion trap mass spectrometer has also been reported (Ding & Brancia, 2006).

Electron transfer dissociation (ETD) is similar to ECD in that it also induces relatively non-selective cleavage of the N-C α bond on a peptide's backbone producing c- and z-product ions, while maintaining phosphate groups and other potentially labile modifications (Syka et al., 2004). However, rather than involving the direct capture of an electron, ETD involves transfer of an electron to the multiply protonated precursor ion from a singly charged radical anion. The use of electron donors makes ETD amenable for use in quadrupole ion trap mass spectrometers which utilize rf fields for simultaneous storage and reaction of ions with positive and negative polarities. ETD fragmentation of phosphopeptides results in retention of phosphate groups in the sequence, allowing easier assignment of the exact site of modification. Moreover, these fragment ions are generated with good efficiency, making this a very promising approach for the analysis of phosphopeptides (Chi et al., 2007).

2.3.2.3 Photodissociation (PD)

Furthermore, ions in the gas-phase may be excited and subsequently dissociated by absorption of the photons. Photodissociation uses a laser that is directed through a window to irradiate the interior of the analyser. The mechanism of fragmentation by photodissociation involves the absorption by one or more photons. As each photon is absorbed, the ion increases its internal energy. The energy accumulates and finally it is sufficient to provoke dissociation resulting in gas-phase fragmentation of the ion. Ion activation may be achieved using infrared lasers (Brodbeck & Wilson, 2009). Due to its relatively low energy (~ 0.1 eV/photon), the absorption of multiple IR photons (tens to hundreds) are required for ion dissociation. Like CID, IRMPD is a “slow heating” method and allows for intramolecular energy redistribution over all of the vibrational degrees of freedom prior to the next photon absorption event (McLuckey & Goeringer, 1997). As a result, ergodic dissociation of low-energy pathways predominates and the resulting spectra are generally comparable with those obtained by CID. Photodissociation in the UV range has targeted common chromophores such as the amide bonds of a peptide using 193 and 157 nm light, as well as residue-specific chromophores such as aromatic amino acids using 220, 266, and 280 nm light (Reilly, 2009). Photodissociation has some advantages over the aforementioned methods. It is relatively selective, as only ions absorb the wavelength of the light used are activated. These techniques are most often used with ion trapping mass spectrometers.⁷

2.3.3 Site-specific microarrays

Site-specific microarray use oriented peptide libraries to map target specificity of kinases. This approach is based on kinase consensus sequences and phosphorylation prediction algorithm. It is thought that many phosphorylation sites tend to occur in accessible and flexible regions in three dimensional protein structures, suggesting that phosphorylation of linear peptide sequences *in vitro* should be similar to phosphorylation of the intact protein for the majority of sites. Data derived from peptide array experiments is consistent with known kinase consensus sequences, and is therefore a useful tool for studying phosphorylation. Peptide microarrays consist of synthetic peptide sequences deposited on to glass slides or attached to a derivatised surface, usually in triplicate, with phosphorylation site substitutions as controls. The peptides could map the entire sequence of a protein or correspond to a dataset of peptides that for example may have been identified from an *in vivo* sample, by MS. The *in vitro* phosphorylation reaction is performed in the presence of radiolabelled ATP, the array exposed to film and the image captured. Once the set of peptides have been synthesized, a large number of these microarrays can be made to screen many kinases relatively quickly (Kemp et al., 1975; Zetterqvist et al., 1976). The limitation of site-specific microarray is that, *in vitro* data is not sufficient on its own to definitively prove that a kinase may phosphorylate a given site *in vivo*. It is reasonable to use this peptide array technology as a first approach to screen for possible substrates (Diks et al., 2004; MacBeath & Schreiber, 2000), but further validation is required.

2.4 Quantification of protein phosphorylation

The field of phosphorylation quantitation by proteomics has made important advances over the last few years (Nita-Lazar et al., 2008). In general, quantitative phosphoproteomics can

be performed as gel based and non-gel based (shotgun), e.g LC-MS/MS. Two-dimensional gel electrophoresis is a classical and powerful analytical method in proteomics that can separate complex mixtures of proteins based on charge (by isoelectric focusing) and apparent molecular mass (by sodium dodecyl sulfate polyacrylamide gel electrophoresis). In contrast to LC-MS/MS that analyzes digested peptides, 2DE delivers a map of intact proteins, which reflects changes in protein expression, isoforms or post-translational modifications. These changes can be confirmed by 1D or 2D Western blot analysis. Some forms of post-translational modification such as phosphorylation, glycosylation or limited proteolysis are easily located in 2DE gels as they appear as distinct spot trains along the horizontal and/or vertical axis. In 2DE, stoichiometry of phosphorylation can be readily determined by quantifying the spot intensity of each phosphorylated form. Furthermore, antibody-based approaches, using phosphorylation site-specific antibodies/Western blot analysis, ProQ Diamond staining and ^{32}P radioactive labeling are most frequently used approaches for gel-based phosphoprotein quantification (Agrawal & Thelen, 2006; Gorg et al., 2004).

Shotgun proteomics, where a peptide mixture from a sample is analyzed by LC-MS/MS without separating proteins on gels prior to the analysis, is a robust and high-throughput method that enables identification of thousands of proteins in a single analysis. There are many quantification methods in LC-MS/MS analysis, as summarized in the figure 2. Which method should be selected depends on the accuracy required, the sample source (from cultured cells or tissues) and the number of samples to be compared.

The easiest way is a label-free method based on the spectral counts of identified peptides. An abundant peptide is represented by a large LC peak eluted for a long time and has more chance of being analyzed by MS/MS. Thus, the number of observed spectra assigned to a particular peptide is a semi-quantitative measure of the abundance of the peptide. Although the accuracy of quantification using spectral counts is not high, it is convenient for analyzing large quantitative differences between samples. Another label-free method measures the intensity of MS chromatograms. A number of methods have been developed to quantify peptides/proteins from peak heights in shotgun proteomics using an internal control. Using high-resolution MS instruments, a peptide ion can be analyzed accurately in the low parts per million mass unit range, and it facilitates the peptide signal mapping across a few or multiple LC-MS measurements, using their mass to charge and retention time dimension. Thus, this method depends on the mass resolution, the mass precision and the consistency of the retention time to match the same peptides among different LC-MS analyses. It is essential to use a high-resolution MS, as well as a sensitive and reproducible nano-LC where the retention time of a particular peptide in crude extract behaves exactly the same.

Relative quantification based on differential stable isotope labeling is frequently used for quantitative phosphoproteomic analyses by MS. Although many techniques have been developed, only a few methods have been used in multiple laboratories. These include isotope-coded affinity tags, stable isotope labeling by amino acids in cell culture (SILAC) and the recently introduced chemical labeling by tandem mass tags, such as isobaric tag for relative and absolute quantitation (iTRAQ). SILAC and iTRAQ are currently the most frequently used techniques in quantitative MS-based phosphoproteomics. In SILAC, cell cultures to be compared are differentially labeled with amino acids containing stable isotopes, usually $^{13}\text{C}_6$ -Lys and/or $^{13}\text{C}_6$ -Arg, and normal amino acids. Lysates from differentially labeled cells are then mixed, digested with protease and analyzed by LC-MS/MS. As a result, differentially labeled peptides (light and heavy) with the same amino

Fig. 2. Quantification methods for liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis. There are two major methods for quantification: label-free, labeling with stable isotopes. The two main non-labeling methods are based on the intensity of MS chromatograms and the spectral counts of identified peptides. Labeling methods are classified into two major groups: metabolic labeling and in vitro labeling. The representative of the metabolic labeling is SILAC. In SILAC, two cell cultures to be compared are differentially labeled with heavy amino acids containing stable isotopes (heavy) and normal amino acids (light). Lysates from differentially labeled cells are mixed, digested with protease and analyzed by LC-MS/MS. Differentially labeled peptides having the same amino acid sequence are detected in MS spectrum, and the relative abundance of the peptides can be compared by calculating their ratio. The representative of the in vitro labeling is performed using isobaric amine-specific tandem mass tags, such as iTRAQ. The iTRAQ reagent consists of reporter regions with 1 Da difference (molecular weight: 114, 115, 116...) and balance regions that adjust the molecular weight of the labeled parent ions (molecular weight: 31, 30, 29...). Each tag generates a unique reporter ion in the MS/MS spectra, and the relative abundance of the peptides can be compared by calculating their ratio.

acid sequence are detected in the MS spectrum, and the relative abundance of the peptides derived from different samples can be compared by calculating their ratio. Isobaric tagging for relative and absolute quantitation/tandem mass tags is a recently developed protein quantification method that uses isobaric amine-specific tandem mass tags and quantification in MS/MS instead of MS spectra. In MS spectra, the differentially labeled peptides possess the same mass by using the balance region in the tag and are represented in a combined single peak (Figure 2). However, each tag generates a unique reporter ion, and the intensities of the reporter ions in the MS/MS spectra are compared for protein quantification. iTRAQ can comparatively analyze four or eight different conditions in one

experiment. This chemical labeling method is suitable for the phosphoproteomic analysis of tissue and clinical samples.

2.5 Application of phosphoproteomics

2.5.1 MS based applications

As described before, MS based phosphoproteomics can be performed at either gel or gel-free based level. Gel based phosphoproteome profiling indicates that intact phosphoproteins were isolated and enriched from samples, and subsequently subject to 1DE or 2DE. To explore changes in protein phosphorylation in MCF-7 cells overexpressing Samd2/3 in response to TGF β 1 stimulation, Stasyk et al (Stasyk et al., 2005) generated 2DE gels using ^{32}P labeled proteins, 32 proteins were identified with high confidence. One of the identified targets, transcription factor-II-I (TFII-I), was found in three phosphoprotein spots of similar molecular mass, suggesting at least three sites of phosphorylation of TFII-I. 2D phosphopeptide mapping of TFII-I identified Ser371 and Ser743 as two phosphorylation sites. Mutation of Ser 371 and Ser743 led to the abrogation of TGF β 1-dependent regulation of Cyclin D2, Cyclin D3, and E2F2 gene expression. Our lab previously developed a quantitative phosphoproteomics approach using phosphoprotein enrichment by Fe-IMAC followed by 2DE, which allows recovery of up to 90% of phosphoproteins, and can be applied to cultured cells and tissues (Dubrovska & Souchelnytskyi, 2005). We applied this approach to investigate the crosstalk of EGF and TGF β signaling pathway in MCF-7 cell, and identified 47 convergent components of these two pathways. Systemic analysis identified MEK1 and CK1 as the primary common components of EGF and TGF β signaling pathway in regulation of cell proliferation. Cell proliferation analysis showed that inhibition of MEK1 and CK1 can affect cell proliferation in the context of EGF and TGF β treatment. And our experimental data also suggested that cross-talk between EGF and TGF β may affect the responsiveness to Iressa in MCF-7 cell. Huber et al. performed two-dimensional differential gel electrophoresis (2D-DIGE) after purification of endosomes from EGF-treated mouse epithelial cells and identified 23 endosomal targets of EGF receptor signaling, such as R-Ras (Stasyk et al., 2007). Tang et al. performed 2D-DIGE analysis of phosphoprotein and plasma membrane fractions from brassinosteroid-treated Arabidopsis (Tang et al., 2008) and identified homologous protein kinases as key transducers of this steroid hormone signaling in plants (Tang et al., 2008). Thus, the combination of enrichment of phosphoproteins and 2D is a powerful proteomics approach for unraveling protein kinase-mediated signaling networks.

Gel-free (shotgun) proteomics, e.g. LC-MS/MS analysis have been developed and successfully applied to quantify phosphopeptides from various cells and tissues. Ineffective erythropoiesis in human hematopoietic stem cells has been implicated in Hemoglobin E/beta-thalassemia. Ponnikorn et al. compared the phosphoproteomics of human hematopoietic cells between healthy donors and Hemoglobin E/beta-thalassemia patients (Ponnikorn et al., 2011). They enriched the phosphoproteins by IMAC, followed by LC-MS/MS for identification, and found 229 differentially phosphorylated proteins. To investigate the mechanisms of resistance to Her2 tyrosine kinase inhibitor lapatinib, Arteaga's group profiled the tyrosine phosphoproteome of sensitive and resistant cells using an immunoaffinity enrichment and mass spectrometry method (Rexer et al., 2011). Peptides containing phosphotyrosine were isolated directly from protease-digested cellular protein extracts with a phosphotyrosine-specific antibody and were identified by tandem mass spectrometry. They found increased phosphorylation of Src family kinases (SFKs) and

putative Src substrates in several resistant cell lines. Treatment of these resistant cells with Src kinase inhibitors partially blocked PI3K-Akt signaling and restored lapatinib sensitivity. Further, SFK mRNA expression was upregulated in primary HER2+ tumors treated with lapatinib. Finally, they observed that the combination of lapatinib and the Src inhibitor AZD0530 was more effective than lapatinib alone at inhibiting pAkt and growth of established HER2-positive BT-474 xenografts in athymic mice. Ståhl et al. profiled phosphoproteome of ephrin and Eph signaling circuit. They combined SCX chromatography and TiO₂ for enrichment of phosphopeptides followed by nano-LC and MS analysis (Stahl et al., 2011), and identified 1083 unique phosphorylated proteins. Out of these, 150 proteins were found only when ephrin B3 is expressed, whereas 66 proteins were found exclusively in U-1810 cells with silenced ephrin B3. Cantrell's group reported an unbiased analysis of the cytotoxic T lymphocyte (CTL) serine-threonine phosphoproteome by high-resolution mass spectrometry (Navarro et al., 2011). They used SILAC and IMAC based phosphopeptide enrichment, and identified approximately 2,000 phosphorylations in CTLs, of which approximately 450 were controlled by T cell antigen receptor (TCR) signaling. SILAC-based method also applied to study phosphorylation changes in EGF-stimulated HeLa cells (Olsen et al., 2006). After enrichment of phosphopeptides with SCX and TiO₂, temporal profiles of 6600 unique phosphorylation sites on 2244 proteins were determined, including many known members of the EGF receptor signaling pathway. More recently, the cell cycle profiles of 20,443 phosphorylation sites in 6027 proteins have been determined and the site-specific stoichiometry of more than 5000 sites has been achieved by combining the results from corresponding non-phosphorylated peptides (Olsen et al., 2011). Comparative studies have revealed that different proteomic strategies are complementary to each other. For example, different phosphopeptide enrichment methods show distinct and partially overlapping preferences in phosphopeptide recovery. Bodenmiller and colleagues compared three different phosphopeptide enrichment approaches, phosphoramidate chemistry (PAC), IMAC and TiO₂. They observed that among repeat isolates for each method pattern, overlap was ranging from an average of 80% for PAC (average of 6,643 features per run (FPR)), 76% for TiO₂ (8,459 FPR), 74% for IMAC (9,312 FPR) (Bodenmiller et al., 2007). This suggested that no single method is sufficient for a comprehensive phosphoproteome analysis, and combination of different approaches for enrichment can improve the comprehensiveness of phosphoproteins. Furthermore, phosphoproteomic profiling of the ERK pathway using 2D-DIGE, label-free precursor ion scanning and SILAC identified surprisingly different subsets of ERK targets (Kosako & Nagano, 2011). Thus, a combination of various phosphoproteomic strategies, such as LC-MS/MS, 2DE and peptide (protein) microarrays, can increase the reliability and comprehensiveness of the data obtained.

2.5.2 Arrays-based application

Protein microarray technology offers the potential for profiling the proteome without employing separation techniques and evaluating protein biochemistry in a high-throughput and systematic manner. Synder's group have done large amount of work in protein microarray, including application in phosphoproteomics (Kafadar et al., 2003; Ptacek et al., 2005; Zhu et al., 2000). In 2000 his group screened 119 of the 122 yeast kinases with 17 different substrates (including the kinases themselves for monitoring autophosphorylation) on a prototype of protein microarray (Zhu et al., 2000). The substrates were immobilized onto nanowell protein chips and phosphorylation events were identified by adding ³³P-γ-

ATP and a specific yeast kinase and exposing the chip to a phosphoimager. They discovered that more than 60% of the kinases autophosphorylated themselves, and 94% of the tested kinases had at least one substrate in vitro, with 32 of them specifically phosphorylating one or two substrates. Twenty-seven kinases were found to phosphorylate poly (Tyr-Glu), which quadrupled the number of identified tyrosine kinases (seven) reported at that time. Moreover, these tyrosine kinases preferentially contain 3 conserved lysines and one conserved methionine near the catalytic region, indicating their potential roles in substrate selection. The same method was later used to identify Hrr25p as a kinase for the zinc-finger transcription factor Crz1, which turned out to negatively regulate Crz1 activity and nuclear localization by phosphorylation in vivo (Kafadar et al., 2003). His group later expanded the study to search for the substrates of 87 different *S. cerevisiae* kinases in a large set of more than 4400 full-length, functional yeast proteins with a yeast protein microarray containing 4400 yeast proteins (Ptacek et al., 2005). In this study they discovered about 4200 phosphorylation events affecting 1325 proteins and generated the first version of the phosphorylation network in yeast. In contrast to previous protein arrays that immobilize the probe, Paweletz et al developed reverse phase protein array, which immobilizes the whole repertoire of patient proteins that represent the state of individual tissue cell populations undergoing disease transitions (Paweletz et al., 2001). A high degree of sensitivity, precision and linearity was achieved, making it possible to quantify the phosphorylated status of signal proteins in human tissue cell subpopulations. Using this novel protein microarray they have analyzed the state of pro-survival checkpoint proteins at the transition stage from patient matched histologically normal prostate epithelium to prostate intraepithelial neoplasia (PIN) and then to invasive prostate cancer. Cancer progression was associated with increased phosphorylation of Akt ($P < 0.04$), suppression of apoptosis pathways ($P < 0.03$), as well as decreased phosphorylation of ERK ($P < 0.01$). c-Src tyrosine kinase plays a critical role in signal transduction downstream of growth factor receptors, integrins and G protein-coupled receptors.

Amanchy et al. employed peptide microarrays approach and identified tyrosine phosphorylation sites in c-Src substrates (Amanchy et al., 2008). They designed custom peptide microarrays containing all possible tyrosine-containing peptides and their mutant counterparts containing a Tyr → Phe substitution from the identified substrates. In all, 624 WT or mutant (312 WT and 312 MUT) peptides from 14 proteins were spotted with each sequence being represented in triplicate, on to the glass slides. c-Src kinase assays were performed on the peptide microarrays and the arrays subsequently exposed to phosphorimager screen. From this analysis, 12 out of 14 proteins phosphorylation sites were identified.

3. Challenges, limitations, future directions and potential

The term 'phosphoproteomics' describes a subdiscipline of proteomics that is focused on deriving a comprehensive view of the extent and dynamics of protein phosphorylation. Phosphoproteomics greatly expands knowledge about the numbers and types of phosphoproteins, and promotes rapidly the analysis of entire phosphorylation-based signaling networks. The combination of quantitative methods and phosphoproteomics has generated powerful technologies for studying cellular signaling. However, there are still many challenges to the approach itself. Firstly, further improvements of the comprehensiveness are necessary. Ideally one could identify every single phosphorylation,

independent of its concentration. However, currently most of the time people only look at most abundant ones. Lack of comprehensiveness impacts reproducibility. Data-dependent acquisition in MS is inherently irreproducible, so alternative ways of choosing ions for further fragmentation are needed. Additional complementary techniques are also needed, such as starting with proteases other than trypsin. Secondly, optimization of enrichment techniques for phosphopeptides and phosphoproteins also pose a significant challenge. As we discussed in 2.5.1, currently no single enrichment method can fully recovery of phosphoproteins and phosphopeptides. Combination of different enrichment methods will be an efficient way to this problem. Thirdly, the interpretation of quantitative phosphoproteomics studies is complicated because each differential phosphorylation event integrates both changes in protein expression and phosphorylation. Studies have been performed by parallel comparisons of protein expression and phosphorylation in *S. cerevisiae*, and it has been found that 25% of seemingly differential phosphopeptides now attributed to changes in protein expression (Wu et al., 2011). Hence, correct interpretation of comprehensive phosphorylation dynamics requires normalization by protein expression changes. In addition, despite the vast amount of quantitative phosphoproteomic data generated in recent studies, validation of these data has been quite limited. Furthermore, although large amount phosphorylation sites identified, most of these studies did not in-depth investigate the biological functions of the phosphorylation sites in signaling transduction.

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Integrative Proteomics

Edited by Dr. Hon-Chiu Leung

ISBN 978-953-51-0070-6

Hard cover, 442 pages

Publisher InTech

Published online 24, February, 2012

Published in print edition February, 2012

Proteomics was thought to be a natural extension after the field of genomics has deposited significant amount of data. However, simply taking a straight verbatim approach to catalog all proteins in all tissues of different organisms is not viable. Researchers may need to focus on the perspectives of proteomics that are essential to the functional outcome of the cells. In Integrative Proteomics, expert researchers contribute both historical perspectives, new developments in sample preparation, gel-based and non-gel-based protein separation and identification using mass spectrometry. Substantial chapters are describing studies of the sub-proteomes such as phosphoproteome or glycoproteomes which are directly related to functional outcomes of the cells. Structural proteomics related to pharmaceuticals development is also a perspective of the essence. Bioinformatics tools that can mine proteomics data and lead to pathway analyses become an integral part of proteomics. Integrative proteomics covers both look-backs and look-outs of proteomics. It is an ideal reference for students, new researchers, and experienced scientists who want to get an overview or insights into new development of the proteomics field.

How to reference

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Min Jia, Kah Wai Lin and Serhiy Souchelnytskyi (2012). Phosphoproteomics: Detection, Identification and Importance of Protein Phosphorylation, Integrative Proteomics, Dr. Hon-Chiu Leung (Ed.), ISBN: 978-953-51-0070-6, InTech, Available from: <http://www.intechopen.com/books/integrative-proteomics/phosphoproteomics-detection-identification-and-importance-of-protein-phosphorylation>

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